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3,13-DIMETHYLHEPTADECANE: MAJOR SEX PHEROMONE COMPONENT OF THE WESTERN FALSE HEMLOCK LOOPER, *Nepytia freemani* MUNROE (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—3,13-Dimethylheptadecane (3,13-dime-17Hy) is the major sex pheromone component of the western false hemlock looper (WFHL), *Nepytia freemani* Munroe. It was identified in extracts of female pheromone glands by coupled gas chromatographic–electroantennographic detection (GC-EAD) and coupled GC–mass spectroscopy (GC-MS). Traps baited with 100 μg of 3,13-dime-17Hy attracted large numbers of male WFHL. Of five additional candidate pheromone dimethylated hydrocarbons, only 3,13-dimethylhexadecane attracted male WFHL. However, neither 3,13-dime-16Hy nor the other four compounds enhanced attraction to 3,13-dime-17Hy when tested in binary or ternary combination at respective ratios of 100:10, 100:1, or 100:1:1. Identification of the complete WFHL sex pheromone requires structural elucidation of all 12 EAD-active components in gland extracts, determination of their chirality, and field testing of antennally active isomers in appropriate combinations and ratios. Stereoisomeric 3,13-dime-17Hy as trap bait may already be used to monitor WFHL populations.

Key Words—Lepidoptera, Geometridae, sex pheromone, 3,13-dimethylhep-

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tadecane, 3,13-dimethylhexadecane, 3,13-dimethyloctadecane, 5,13-dimethylheptadecane, 3,11-dimethylhexadecane, 3,11-dimethylpentadecane.

INTRODUCTION

The western false hemlock looper (WFHL), *Nepytia freemani* Munroe, occurs in the northwestern United States and southwestern Alberta and British Columbia (Ferris and Woensdregt, 1983), feeding on a variety of coniferous trees including Douglas fir, *Pseudotsuga menziesii* (Mirb) Franco; white fir, *Abies concolor* (Gord. & Glend.) Lindl.; western larch, *Larix occidentalis* Nutt.; western hemlock, *Tsuga heterophylla* (Raf.) Sarg.; and Engelmann spruce, *Picea engelmannii* Parry (Furniss and Carolin, 1977). In British Columbia, populations of the WFHL increased to very high levels four times between 1947 and 1984 (Harris et al., 1985), but epidemics occurred only in the drier part of the Douglas fir range (Shepherd, 1977). Repeated defoliation of Douglas fir from 1947 to 1949 in the Windermere Valley resulted in top killing and scattered tree mortality over several thousand hectares and prompted an application of DDT over 4400 ha to protect the threatened Christmas tree industry (Ferris and Woensdregt, 1983). Annual appraisal of WFHL larval populations by Forestry Canada's Forest Insect and Disease Survey (FIDS) allows prediction of population level and potential damage in the following year, but the use of synthetic sex pheromone in survey traps could greatly facilitate and improve the monitoring of WFHL populations. The presence of a sex pheromone has been demonstrated in the WFHL (Shepherd, 1979). We report the identification and field testing of sex pheromone components of the WFHL.

METHODS AND MATERIALS

Laboratory Analyses

Fourth- and fifth-instar WFHL larvae were field collected near Kamloops, British Columbia, in June 1991 and reared to the adult stage in the laboratory at a photoperiod of 14:10 (L:D). Four to 5 hr into the scotophase (Shepherd, 1979), pheromone glands of 2- to 3-day-old virgin females were removed and extracted for 5 min in hexane. Extracts were subjected to gas chromatographic–electroantennographic analysis (GC-EAD) (Arn et al., 1975) on two capillary columns (Hewlett Packard 5890A; DB-210, DB-1, each 30 m × 0.25 mm ID). Coupled GC–mass spectroscopy (GC-MS) (Hewlett Packard 5985B) in full-scan and selected ion monitoring mode (SIM) was conducted to identify pheromone components or confirm their presence in gland extracts. For GC-MS-SIM, full-scan electron impact spectra of synthetic candidate compounds were obtained to select diagnostic ions. In sequence, 200 pg of synthetic compounds, hexane,

and a concentrated pheromone gland extract were then chromatographed, each time scanning for the diagnostic ions. Synthetic candidate pheromone components were further subjected to GC-EAD analyses to compare their EAD activity with those of female-produced compounds.

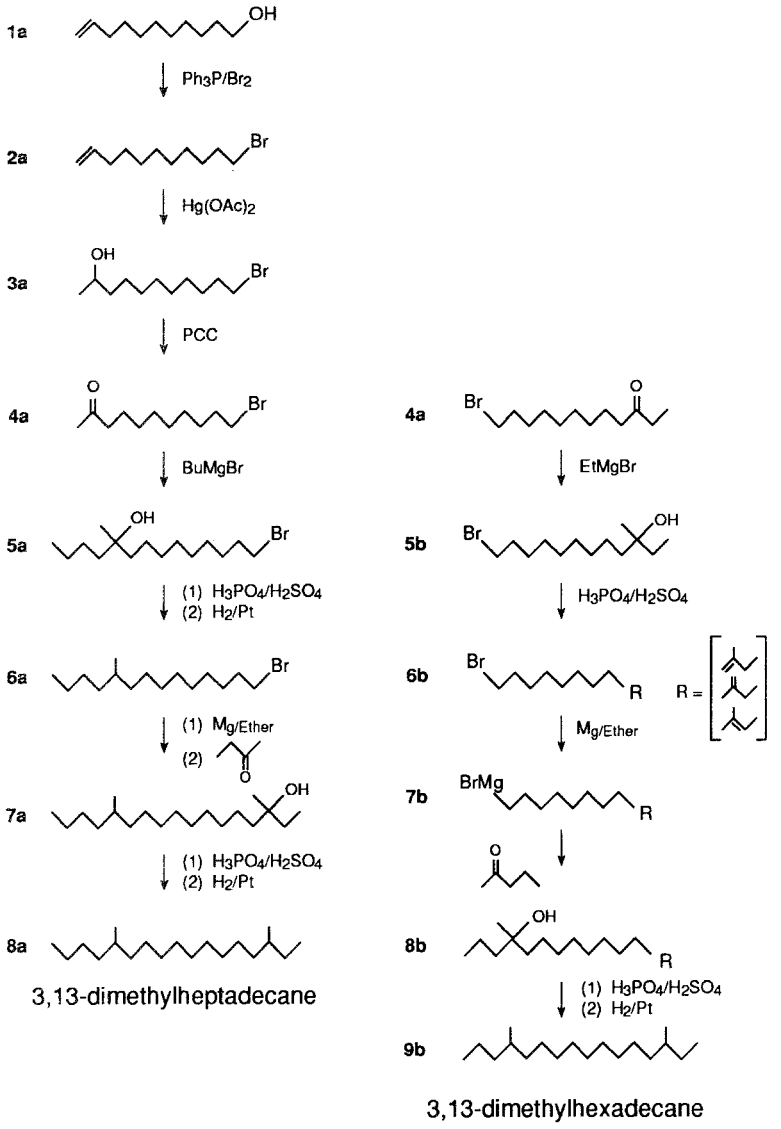
Synthesis of Dimethylated Hydrocarbons

3,13-Dimethylheptadecane (3,13-Dime-17H:H). 10-Undecen-1-ol (Scheme 1, **1a**)) (Aldrich, Milwaukee, Wisconsin) was treated with triphenylphosphine dibromide in dichloromethane containing 2 equivalents of pyridine. The resulting bromoundecene **2a** was reacted with mercuric acetate in aqueous THF for 0.5 hr followed by aqueous sodium borohydride to yield 11-bromo-2-undecanol **3a.** Treatment of **3a** with pyridinium chlorochromate (PCC) produced the corresponding ketone **4a,** which was reacted with butylmagnesium bromide to yield 14-bromo-5-methyl-5-tetradecanol **5a.** Dehydration at 25°C with a mixture of phosphoric and sulfuric acids (10:1) resulted in bromoalkenes. Hydrogenation over 5% platinum on asbestos resulted in **6a,** which was converted in diethyl ether (Et_2O) to the Grignard reagent and reacted with 2-butanone to yield **7a.** Dehydration of **7a,** followed by hydrogenation as above, produced **8a** with 5% overall yield. EI mass spectrum *m/z*: 268 (M^+) 1%, 239 ($M-C_2H_5^+$) 5%, 211 ($M-C_4H_9^+$) 11%, 85 ($C_6H_{13}^+$) 100%, 84 ($C_6H_{12}^+$) 54%, 57 ($C_4H_9^+$) 96%, 56 ($C_4H_8^+$) 39%, 55 ($C_4H_7^+$) 42%.

3,13-Dimethylhexadecane (3,13-Dime-16:H). The previously prepared ketone **4a** was reacted with excess ethylmagnesium bromide to give 12-bromo-3-methyl-3-dodecanol **5b.** Dehydration of **5b** with a mixture of phosphoric and sulfuric acids (10:1) resulted in bromoalkenes **6b,** which were converted to Grignard reagent **7b** in Et_2O, and reacted with 2-pentanone to yield the dimethylated unsaturated alcohols **8b.** Dehydration and hydrogenation of **8b** yielded **9b** with 9% overall yield. EI spectrum *m/z* of **9b:** 254 (M^+) 1%, 225 ($M-C_2H_5^+$) 4%, 211 ($M-C_3H_7^+$) 4%, 71 ($C_5H_{11}^+$) 77%, 70 ($C_5H_{10}^+$) 42%, 57 ($C_4H_9^+$) 100%, 56 ($C_4H_{10}^+$) 25%, 43 ($C_3H_7^+$) 88%.

Field Bioassay

In August–September 1991, field experiments were conducted 30 km west of Kamloops, British Columbia, in a widely spaced mature Douglas fir stand with noticeable, recent defoliation. Experiments were set up in randomized complete blocks, with traps and blocks at approximately 40-m intervals. Traps were suspended 1.5–2 m above the ground and baited with rubber septa (Thomas Scientific, Swedesboro, New Jersey) impregnated with candidate pheromone components in HPLC grade hexane. "Guard" traps baited with 100 μg of 3,13-dime-17:H were placed at the end of trap lines to avoid bias in catches of experimental traps located at the upwind end of each trapping line.



SCHEME 1.

Delta 2-liter milk cartons served as traps. The inner 855 cm^2 surface was covered with adhesive Tangle-Trap (Tanglefoot Company, Grand Rapids, Michigan) to retain moths approaching the chemical-impregnated septum pinned to the middle of the trap. Traps were recorded and advanced one position daily, and traps containing more than 20 moths were replaced, reusing the same lure.

The first experiment tested the following six candidate pheromone components alone at 100 μg each: 3,11-dimethylpentadecane (3,11-dime-15Hy), 3,11-dimethylhexadecane (3,13-dime-16Hy), 3,13-dimethylhexadecane (3,13-dime-16Hy), 5,13-dimethylheptadecane (5,13-dime-17Hy), 3,13-dimethylheptadecane (3,13-dime-17Hy) and 3,13-dimethyloctadecane (3,13-dime-18Hy). The second and third experiment tested the major sex pheromone component, 3,13-dime-17Hy, alone (100 μg) and in binary combinations with the other five candidate components at ratios of 100:10 and 100:1. A final experiment employed Unitraps (Phero Tech Inc., Delta, British Columbia V4G 1E9) and tested 3,13-dime-17Hy alone and in binary and ternary combination with the other candidate pheromone components at respective ratios of 100:1 and 100:1:1.

RESULTS

Laboratory Analyses

GC-EAD analyses of female gland extracts revealed 12 EAD-active compounds (Figure 1) with retention indices similar to nona-, octa-, hepta-, and hexadecane. The structure of the major EAD-active and FID-detectable compound (X in Figure 1) was derived from a mass spectrum containing 54 female equivalents (FEQ). The fragmentation pattern indicated 3,13-dime-17Hy (Figure 2). Identical mass spectral and retention characteristics of authentic 3,13-dime-17Hy with those of the female-produced compound confirmed this structural assignment.

GC-EAD analysis of pheromone gland extracts on DB-210 and DB-1 columns revealed a similar antennal response pattern, but GC-EAD response VIII disappeared upon hydrogenation, suggesting that all compounds but VIII are saturated hydrocarbons. Based upon previous experience with mono- and dimethylated hydrocarbons (Gries et al., 1991a) and with model compounds, which indicated how retention indices are affected by methyl branches in relation to both their position in and the chain length of the molecule, candidate pheromone components were synthesized. 3,11-Dime-15Hy, 3,11-dime-16Hy, 3,13-dime-16Hy, 5,13-dime-17Hy, and 3,13-dime-18Hy coincided with GC-EAD responses I, III, IV, VII, and XII (Figure 1), respectively. All synthetic compounds elicited good antennal responses. EAD activity of 5,13-dime-17Hy was as strong as that of 3,13-dime-17Hy, the major sex pheromone component.

GC-MS analyses of authentic standards and 200 FEQ of gland extract and scanning for ions diagnostic of methyl branch positions resulted in exact retention time and excellent ion ratio matches of synthetic 3,13-dime-16Hy and 3,13-dime-18Hy versus female-produced compounds: synthetic 3,13-dime-16Hy: *m/z* 70 (67), 211 (33), gland extract: *m/z* 70 (64), 211 (36); synthetic 3,13-dime-

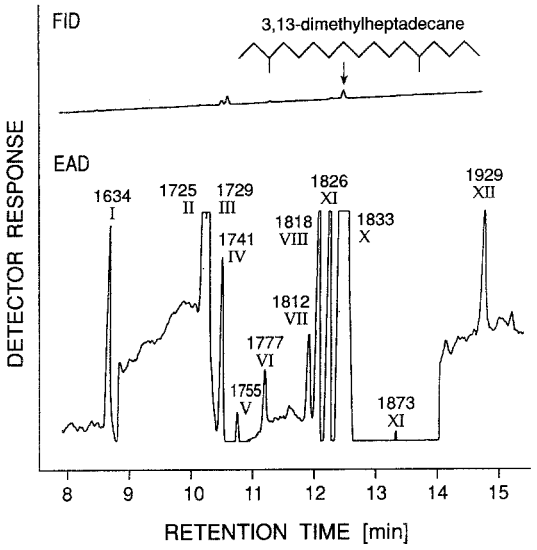


FIG. 1. Detector responses to one female equivalent of pheromone extract. Chromatography: DB-210 column, 1 min at 70°C, 20°C/min to 130°C, 2°C to 220°C. The antennal recording was carried out with a male *N. freemani* antenna. Antennal responses are superscripted by their retention indexes. I: 3,11-dime-15Hy, IV: 3,13-dime-16Hy, X: 3,13-dime-17Hy, XII: 3,13-dime-18Hy; III and VII were tentatively identified as 3,11-dime-16Hy and 5,13-dime-17Hy, respectively. The structural identity of EAD-active compounds II, III, IV, VII, IX, XI is not yet known.

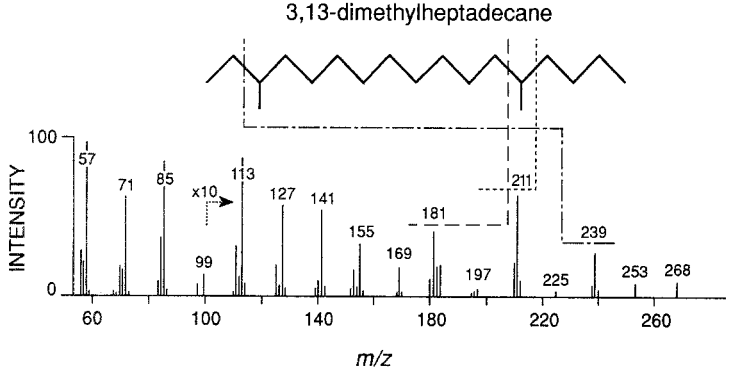


FIG. 2. Mass spectrum of 3,13-dimethylheptadecane present in female pheromone gland extracts.

18Hy: *m/z* 91(81), 211 (19), gland extract: *m/z* 91 (81), 211 (18). 3,11-Dime-15Hy was barely detectable in gland extracts; synthetic 3,11-dime-15Hy: *m/z* 183 (63), 211 (36), gland extract: *m/z* 183 (58), 211 (41). 3,11-Dime-16Hy and 5,13-dime-17Hy were not detectable in female extracts by GC-MS in SIM mode.

Field Trapping

Of the six candidate pheromone components tested alone, only 3,13-dime-17Hy and 3,13-dime-16Hy attracted male moths (Figure 3). 3,13-Dime-17Hy was significantly most attractive and is the major sex pheromone component in WFHL. All six candidate pheromone components combined at 100 μg each completely inhibited response (Figure 3). Binary and ternary combinations of 3,13-dime-17Hy with the other components at respective ratios of 100:10, 100:1 and 100:1:1 did not enhance attraction to 3,13-dime-17Hy alone.

DISCUSSION

Dimethylated hydrocarbons as lepidopteran sex pheromone components have only been recently identified. 5,9-Dimethylheptadecane (Francke, 1987; Francke et al., 1987) and 5,9-dimethyloctadecane (Riba et al., 1990) are major

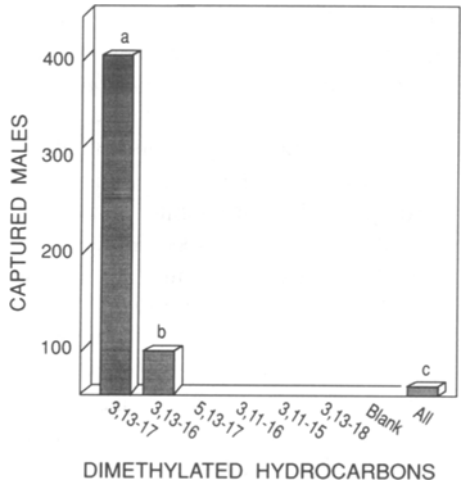


FIG. 3. Total catches of male *Nepytia freemani* in sticky traps baited with candidate pheromone components alone (100 μg each) and in a mixture of six components at 100 μg each, August 24–27, 1991, Savona, British Columbia. $N = 28$. Bars superscripted by the same letter are not significantly different (ANOVA followed by Duncan's multiple range test, $P < 0.05$; treatments that did not attract any moths were excluded from the analysis).

and minor sex pheromone components, respectively, of the mountain-ash bentwing, *Leucoptera malifoliella* (Costa), formerly *L. scitella* (Zeller) (Lepidoptera: Lyonetiidae). 5,9-Dimethylpentadecane is a very attractive sex pheromone component of the coffee leaf miner, *Leucoptera coffeella* (Francke et al., 1988), and 5,11-dimethylheptadecane and 2,5-dimethylheptadecane comprise the sex pheromone of the eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Guen.) (Gries et al., 1991a,b). The same two dimethylheptadecanes and 7-methylheptadecane are sex pheromone components of the western hemlock looper, *L. f. lugubrosa* (Hulst) (Gries et al., 1993). In this paper we report 3,13-dime-17Hy as major sex pheromone component of the WFHL. It is a new dimethylated hydrocarbon to be identified as a sex pheromone component in the Lepidoptera.

Accumulation and concentration of 54 female equivalents of pheromone gland extracts was sufficient to obtain a mass spectrum of the major EAD-active compound (Figure 2). As mass spectra of mono- and dimethylated hydrocarbons have diagnostic ions indicative of methyl branch positions (Pomonis et al., 1980), the compound was readily identified as 3,13-dime-17Hy. In field trapping experiments, 3,13-dime-17Hy alone attracted a large number of male looper (Figure 3). 3,13-Dime-16Hy by itself was also attractive, but in binary combination with 3,13-dime-17Hy did not enhance attraction. With its homologous molecule structure, it may have only mimicked behavioral activity of the major sex pheromone component. Field attraction to pheromone homologs has also been reported in the gypsy moth, *Lymantria dispar* (L.), (Sarmiento et al., 1972) and in the geometrid moth *Semiothisa signaria dispunctata* (Walker) (Millar et al., 1987).

None of the other candidate pheromone components was attractive by itself or in combination with 3,13-dime-17Hy at the two ratios tested. All six components combined at 100 μg each even inhibited response by WFHL males. As 3,13-dime-17Hy in gland extracts was exceedingly more abundant than other candidate pheromone components, equal amounts of compounds in the six-component blend were likely unnatural and possibly also inhibitory.

Lack of synergism between pheromone components and inhibition of response to the 6-component blend may have also been caused by the presence of unnatural stereoisomers in synthetic compounds. Each of the field tested dimethylated hydrocarbons occurred as a mixture of all possible stereoisomers. As shown in *Leucoptera scitella* (Zeller) (Tóth et al., 1989) and *Lambdina fiscellaria* (Li et al., 1993), male WFHL are likely to be attracted to only one stereoisomer of the respective dimethylated hydrocarbons in pheromone gland extracts. Although the presence of synthetic unnatural stereoisomers did not interfere with attraction of male *L. scitella* and *L. fiscellaria*, unnatural stereoisomers may have inhibited response by male WFHL.

Identification of the complete WFHL sex pheromone requires structural

elucidation of all 12 EAD-active components in gland extracts, determination of their chirality (if optically active), and field testing of antennally active isomers in appropriate combinations and ratios. Stereoisomeric 3,13-dime-17Hy as trap bait may be used to monitor WFHL populations.

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NMR SPECTRAL ASSIGNMENT OF LACTONE PHEROMONE COMPONENTS EMITTED BY CARIBBEAN AND MEXICAN FRUIT FLIES[1]

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Abstract—By utilizing one- and two-dimensional nuclear magnetic resonance techniques, the complete assignments of the proton and carbon spectra of the lactones anastrephin, epianastrephin, and suspensolide have been accomplished. These compounds are pheromone components for both the Caribbean and Mexican fruit flies. The relative stereochemistries of anastrephin and epianastrephin were demonstrated by nuclear Overhauser difference spectroscopy. With complete spectral assignments now available, biosynthesis of these molecules may be studied by feeding specific isotopically labeled nutrients to flies and subsequently analyzing the volatiles produced for the presence and position of the labelling isotope.

Key Words—Caribbean fruit fly, *Anastrepha suspensa*, Mexican fruit fly, *Anastrepha ludens*, anastrephin, epianastrephin, suspensolide, NMR, NOE, HETCOR.

INTRODUCTION

The pheromone chemistry of Caribbean (*Anastrepha suspensa* Loew) and Mexican (*Anastrepha ludens* Loew) fruit flies, which are both major crop pests, has been of interest for many years. These flies share several pheromone components (Rocca et al., 1992), including the unusual lactone compounds anastrephin and epianastrephin [4-ethenyl-4,7a-dimethyl-2(3H)-hexahydrobenzofuranone] (Bat-

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tiste et al., 1983; Stokes et al., 1983), and suspensolide [(3E,8E)-4,8-dimethyldeca-3,8-dien-10-olide] (see Figures 1 and 2 below) (Chuman et al., 1988; Rocca et al., 1992). It has recently been suggested that the anastrephin compounds and suspensolide may be metabolically related through interconversion or through a common precursor (Rocca et al., 1992). One method by which this hypothesis can be explored is to use isotopically labeled precursors in diets supplied to the flies and to examine the spectra of the resulting components emitted by the flies. The utilization of ^{14}C labeling to study biosynthesis is common. For example, nutrients labeled with ^{14}C have been used to study the biosynthesis of the pheromone of *Dacus oleae* (Gmelin) (Tsitsipis, 1975) with incorporation rates of 3–22% depending on the nutrient studied. The incorporation of ^{14}C, however, is usually utilized to determine the presence or absence of the label in a chemical compound. Although the level of incorporation gives some clues about biosynthesis, the ability to determine the exact location of the label in an isolated compound would be of greater utility. To this end the ^{13}C and ^{1}H NMR spectra have been completely assigned for the aforementioned lactones.

For fruit flies, it is common to be able to isolate 5- to 50-μg quantities of pure compounds for NMR analysis. If more material is needed, multiple collections can be performed and combined. This quantity of material is more than adequate for standard proton spectra. The natural abundance of ^{13}C in nature is 1.1%. The presence of ^{13}C in ^{1}H spectra is apparent as satellites with intensities 1.1% of the primary proton resonances. An increase in the abundance of ^{13}C in a molecule results in a proportional increase in satellite intensity. It is therefore possible to determine the positions of ^{13}C in labeled molecules by the observation of these satellites in the proton spectrum even if the level of incorporation is less than 10% and well below the concentration necessary for the direct observation of ^{13}C. The use of inverse detection for heteronuclear correlation experiments can even allow a two-dimensional NMR experiment to be performed (Martin and Crouch, 1991). In addition, the availability of complete NMR assignments of the pheromone lactones will facilitate the identification of lactones in other related *Anastrepha* species.

Carbon and proton NMR spectra were used previously to infer the structures of the lactone volatiles, but the structures were confirmed only through synthesis (Battiste et al., 1983, 1988). In order to be precise in the interpretation of the spectra of isotopically enriched compounds that have been isolated from insects, complete spectral assignments would be of great utility. A series of both one- and two-dimensional NMR techniques have been used to accomplish this goal. A modification of the standard heteronuclear correlation experiment (HETCOR) (Maudsley and Ernst, 1977; Bax and Morris, 1981), which allows the direct observation of secondary coupling between protons and carbons, enabled the direct assignment of the tertiary carbons with isolated fragments within the

molecules. Nuclear Overhauser effect (NOE) difference spectroscopy (Anet and Bourn, 1965; Derome, 1987) confirmed the relative stereochemistries of anastrephin and epianastrephin.

The temperature dependency of the spectrum of suspensolide was also explored. At low temperatures suspensolide resolves into two different conformations. At elevated temperatures the spectra indicated a single average conformation on the NMR time scale. Degradation occurred above 325 K and isolated vinyl groups were observed in the degradation products. Since a study of the thermal degradation of suspensolide was not the object of this work, no attempt was made to characterize the degradation products.

METHODS AND MATERIALS

Materials. Optically pure synthetic samples of anastrephin, epianastrephin, and suspensolide were obtained from Nitto Denko Co., Osaka, Japan.

NMR Spectra. NMR spectra were obtained on a Bruker AC-300 spectrometer equipped with an Aspect 3000 computer and a 5-mm dual $^{1}H/^{13}C$ probe. The operating frequencies were 300.135 MHz for ^{1}H and 75.469 MHz for ^{13}C. Standard Bruker automated acquisition programs were used for all experiments. All one-dimensional spectra were from 64K data points. The spectral width for ^{1}H was set at 6024 Hz and for ^{13}C at 18519 Hz with acquisition times of 5.439 and 1.769 sec, respectively. The resolution of the spectra was 0.565 Hz per point for ^{13}C and 0.184 Hz/pt. for ^{1}H. The numbers of scans for ^{1}H and ^{13}C were 16 and 128, respectively.

The spectra and assignments were obtained with $CDCl_3$ solutions of 50 mg of sample (50 μl for suspensolide) dissolved in 0.35 ml of $CDCl_3$ with TMS as internal reference. Partial spectral assignments were based on ^{1}H and ^{13}C (^{1}H decoupled) chemical shifts, ^{1}H–^{1}H coupling constants, proton splitting patterns, and integrated proton intensities. Standard ^{13}C DEPT (Doddrell et al., 1982; Derome, 1987), ^{1}H COSY (Aue et al., 1976; Nagayama et al., 1980), and ^{13}C–^{1}H HETCOR experiments were performed to complete the spectral assignments.

Nuclear Overhauser effect difference spectroscopy experiments were performed on epianastrephin and anastrephin to confirm their relative stereochemistries. Twenty pairs of resonance/off-resonance spectra of 16 transients each with the difference taken directly on the FID signal were performed. The relaxation delay and presaturation time were set at 2 sec each. The samples for the NOE experiments were degassed by a minimum of five freeze–pump–thaw cycles (Derome, 1987) directly in the NMR tube utilizing a liquid nitrogen bath. For elevated temperature experiments, one-dimensional ^{1}H and ^{13}C spectra were taken on 50 μl of suspensolide dissolved in 0.4 ml of DMSO-d_6 at temperatures up to 380 K. Reduced temperature spectra were performed on suspensolide in $CDCl_3$ to 220 K. Chemical shifts were assigned relative to TMS.

Anastrephin was used to optimize the time delay in the standard HETCOR experiment to enhance the secondary couplings (Croasmun and Carlson, 1987) so that connectivity information could be obtained without having to perform time-consuming experiments such as INADEQUATE (Ernst et al., 1987). Standard HETCOR experiments were used to determine the correlation of the proton spectrum with the carbon spectrum, and carbon DEPT experiments were used to assign the number of protons attached to each carbon and determine if the individual protons attached to any particular carbon were in a chemically unique environment. The optimum value of the coupling constant used to modify the HETCOR experiment for secondary $^{1}H-^{13}C$ coupling was determined to be 10 Hz. Normal primary couplings range from 120 to 180 Hz.

RESULTS

Anastrephin and Epianastrephin. The proton spectra of anastrephin and epianastrephin are very similar. Two isolated methyl groups and an isolated vinyl group are immediately discernible. The remainder of the proton spectra for these molecules is complicated by multiple- and second-order couplings. The rigid structure of the molecules contributes to protons on disubstituted carbons having different magnetic environments and thus distinct chemical shifts, making the complete assignment of the spectra difficult.

Table 1 shows the results from the carbon DEPT and standard HETCOR experiments. From these data one can immediately assign the resonances at carbons 1–6 based on chemical shift and DEPT analysis. The remaining resonances were ambiguous in assignment.

By utilizing the secondary coupling HETCOR experiment, carbons 2, 5, 6, and 8 indicated a strong secondary coupling to the protons on carbon 9. This allowed the assignment of carbons 8 and 9 and confirmed 6. Carbons 4, 5, and 7 indicated strong secondary couplings to the protons on carbon 12, which allowed the assignment of carbons 7 and 12 and confirmed the assignment of carbon 4. Carbon 5 indicated secondary coupling to protons on carbon 10 as did carbon 1. The COSY spectrum indicated coupling between protons on carbons 7, 8, and 11. With this observation, all carbon resonances were assigned for anastrephin and epianastrephin.

The relative stereochemistries of carbons 4, 5, and 6 can be inferred by the NOE difference experiment. By irradiating the resonance of protons on carbon 9 in both anastrephin and epianastrephin and observing the positive NOE difference spectra, only those protons that are in the immediate vicinity of the protons on carbon 9 will be enhanced. Irradiating the protons on carbon 9 resulted in enhancement of the protons at carbons 2, 3, 5, and 10 for anastrephin, and at 2, 3, 10, and 12 for epianastrephin. Enhancement of the protons attached

TABLE 1. NMR ASSIGNMENTS FOR ANASTREPHIN AND EPIANASTREPHIN

	Anastrephin			Epianastrephin		
#[a] DEPT	^{13}C ppm	^{1}H ppm: J (Hz)	NOE[b]	^{13}C ppm	^{1}H ppm: J (Hz)	NOE
1(0)	176.1			176.1		
2(1)	140.0	5.89: 11.2, 17.2	+++	147.8	5.69: 10.6, 17.6	+++
3(2)	112.9	(5.11, 5.06): 11.2, 17.2	+++	111.6	(5.00, 4.95) 10.6, 17.6	+++
4(0)	86.3	<1.0		86.0	0.74	
5(1)	55.5	2.13: 6.8, 14.7	+++	53.5	2.14: 6.4, 14.8	
6(0)	38.6			38.5		
7(2)	37.2	1.94 1.64		37.0	2.04 1.98	
8(2)	36.0	2.08 2.01		37.9	1.54 1.46	
9(3)	30.3	1.03	***	16.4	1.07	***
10(2)	29.0	2.51: 14.7, 16.4 2.40: 6.8, 16.4	+++	29.5	2.40: 14.8, 16.4 2.25: 6.4, 16.4	+++
11(2)	20.4	1.83 1.76		20.4	1.84	
12(3)	20.2	1.25		20.9	1.39	+++

[a]Numbering scheme refers to Figure 1 and the order is that of the ^{13}C chemical shifts for anastrephin. The DEPT experiment determined the number of attached protons.
[b]NOE difference spectroscopy (*) irradiated resonance, (+) indicates positive NOE.

to carbon 8 was not observed. The proton resonances associated with carbon 8, as assigned by the HETCOR experiment, are broadened by complex coupling due to the rigidity of the molecule. This fact would make observation of direct NOE enhancement more difficult than the enhancement of the sharp resonances that were observed. The enhancement of the 2.4 ppm proton resonance attached to carbon 10 in epianastrephin was greater than for the 2.25 ppm resonance. One would expect enhancement for the protons at carbons 2 and 3, and possibly at one of the two protons at carbon 10 for each molecule. The perturbation at carbon 5 in anastrephin confirmed that carbon 9 and the proton on carbon 5 are on the same side of the ring and on the opposite face of the ring from carbon 12. For epianastrephin carbons 9 and 12 were on the same side of the ring and

opposite the proton on carbon 5. The completed assignments are shown in Figure 1.

Suspensolide. The results for DEPT and standard HETCOR experiments are shown in Table 2. The spectra indicated two isolated methyl groups and, according to the carbon chemical shifts, two trisubstituted *trans* double bonds.

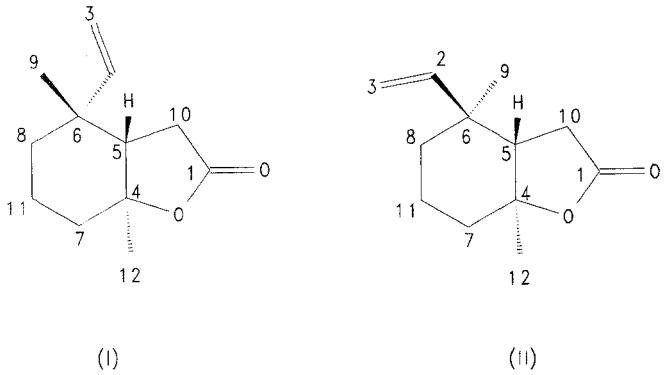

FIG. 1. (I) Anastrephin, (II) epianastrephin. Numbering scheme refers to the order of carbon chemical shifts of anastrephin.

TABLE 2. NMR ASSIGNMENTS FOR SUSPENSOLIDE

#[a] DEPT	^{13}C ppm	^{1}H ppm: J (Hz)	^{13}C (A)[b] ppm	^{13}C (B)[b] ppm
1(0)	170.4		171.8	169.4
2(0)	144.7		145.9	144.1
3(0)	142.7		143.8	143.1
4(1)	120.0	5.11:8.0	119.2	119.7
5(1)	115.7	4.85:8.1	113.6	116.3
6(2)	61.7	4.65	61.5	61.9
7(2), 8(2)	41.8	2.20	42.3, 41.7, 41.5, 41.1	
9(2)	36.1	2.90	36.9	35.2
10(2)	25.9	1.87	25.8	
11(3)	16.0	1.66	15.5, 15.2	
12(3)	15.5	1.60		

[a] Numbering scheme refers to suspensolide ^{13}C chemical shift. The DEPT experiment determined the number of attached protons.

[b] The symbols (A) and (B) refer to the two conformations observed at 220 K.

The proton resonances on the double bonds were triplets, indicating that the protons were on carbons adjacent to CH_2 groups. The remaining proton resonances were broad and featureless at room temperature, which did not allow an analysis of coupling. The lack of well-defined multiplets suggested that the molecule was in equilibrium between different conformations at room temperature. The modified HETCOR experiment indicated that carbons 2 and 4 were coupled with protons on carbon 11 and that carbon 3 was coupled with protons on carbon 12. The secondary coupling of carbon 5 with protons on carbon 12 were not observed. Carbons 2, 4, and 11 form a trisubstituted double bond fragment with attached methyl group of carbon 11, and 3, 5, and 12 another. COSY spectroscopy indicated coupling between the protons on carbon 4 with protons on carbons 6 and 11, and 5 with protons on carbons 9 and 12. Carbons 1 and 6 can be assigned on the basis of chemical shift. Only by elimination and inference can the resonances associated with carbons 7, 8, and 10 be assigned since no direct coupling could be deconvoluted at room temperature. Carbons 7 and 8, and their attached protons, have the same chemical shifts in both the carbon and proton spectra. The integrated proton intensity at 2.2 ppm associated with carbons 7 and 8 indicated four protons. The DEPT analysis indicated the carbon resonance represented a CH_2 group; thus there must be two coincidently equivalent carbons. The chemical shift for carbon 10 is indicative of a aliphatic environment that completes the spectral assignment. The assignments are shown in Figure 2.

In order to explore the possibility that suspensolide exists in multiple conformations, a series of reduced temperature experiments were performed. The carbon spectrum was monitored as the probe temperature was reduced from room temperature to 220 K in 10°C increments. At 250 K, the resonances for carbons 1, 2, 5, and 9 had lost almost all intensity. At 230 K all resonances except 10, 11, and 12 were showing some signs of resolution. At 220 K each carbon, except 10–12, was split into two resonances. Carbons 7 and 8 were

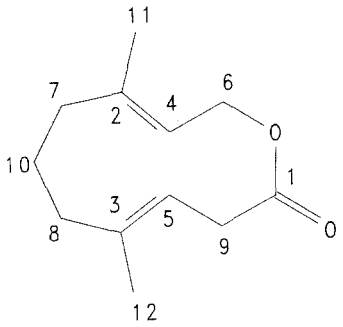

FIG. 2. Suspensolide. Numbering scheme refers to the order of carbon chemical shifts.

now represented by two distinct resonances each. Through the use of the normal and modified HETCOR experiments, the resonances for the separate conformational isomers could be partially assigned. These results are also tabulated in Table 2 and labeled as conformers A and B.

Suspensolide in DMSO-d_6 was exposed to sustained elevated temperatures and NMR spectra were recorded. At 305 K the proton resonance associated with carbon 9 was a doublet. At 315 K the same protons were resolved into a clean doublet and some multiplet structures appeared at the proton resonances associated with carbons 7 and 8. The methyl groups were split into doublets, and the triplets associated with the protons on carbons 4 and 5 became a triplet of quartets as expected. At 325 K the proton resonance associated with carbon 6 became a doublet. At 340 K a multiplet structure appeared at the resonance associated with carbon 10. Hints of resonances not associated with suspensolide begin to appear at 325 K and were apparent as complex multiplets at 340 K in the proton spectra. No evidence for any resonances other than those associated with suspensolide were indicated in the carbon spectra at 340 K. At 360 K new carbon resonances started to appear. At 380 K major degradation had taken place and the spectra were too complex to be deconvoluted. There were proton multiplet patterns present that were characteristic of terminal vinyl groups.

DISCUSSION

Through the utilization of readily available NMR pulse sequences, the spectral assignments of anastrephin, epianastrephin, and suspensolide have been accomplished. Complete carbon spectral assignments were possible from the spectra alone, without utilizing synthetic methods. The utility of the simple modification to the HETCOR experiment described here for elucidating connectivity was demonstrated. The method allows the elucidation of carbon connectivities with acquisition times of 2 hr when utilizing sample sizes adequate for the rapid acquisition of natural abundance one-dimensional ^{13}C spectra. Other experiments that give carbon connectivities directly, such as INADEQUATE, may require days of acquisition time with natural abundance samples, rendering the experiment too expensive where instrument time is in high demand. For the anastrephin molecules, the NOE difference spectrum was capable of determining the relative stereochemistries of the two compounds directly. The original structure proof required comparison of the spectra of all possible isomers (Battiste et al., 1983) with the natural material. The information provided here will facilitate the elucidation of the biosynthesis of these compounds by rearing flies with nutrients that have been specifically labeled with isotopes, such as ^{13}C, followed by performing standard one-dimensional ^{1}H NMR experiments with volatiles collected from flies. Since the presence of incorporated ^{13}C in the

collected volatiles would be observable as satellites in the ^{1}H spectrum, standard collection and purification procedures can be utilized for analysis without needing enough material for a direct ^{13}C spectral analysis.

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PHENOLICS IN ECOLOGICAL INTERACTIONS: THE IMPORTANCE OF OXIDATION

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Abstract—The ecological activities of plant phenolics are diverse and highly variable. Although some variation is attributable to differences in concentration, structure, and evolutionary history of association with target organisms, much of it is unexplained, making it difficult to predict when and where phenolics will be active. I suggest that our understanding is limited by a failure to appreciate the importance of oxidative activation and the conditions that influence it. I summarize examples of oxidative activation of phenolics in ecological interactions, and argue that physicochemical conditions of the environment that control phenolic oxidation generate variation in ecological activity. Finally, I suggest that measurements of oxidative conditions can improve our predictions of phenolic activity and that experiments must be designed with conditions appropriate to the biochemical mode of phenolic action.

Key Words—Phenolics, tannins, plant defense, plant-animal interactions, plant-herbivore interactions, plant-microbe interactions, phenol oxidase, peroxidase.

INTRODUCTION

Phenolics are the only putative defensive molecules ubiquitous in plants. They have played a central role in theories of plant–herbivore interactions, including apparency theory (Feeny, 1976; Rhoades and Cates, 1976) and the resource availability hypothesis (Coley et al., 1985). Despite over 30 years of research on their ecological activity, however, there is significant controversy over their importance (Bernays, 1981; Zucker, 1983; Mole and Waterman, 1987; Martin et al., 1987; Bernays et al., 1989). This controversy is a result of dramatic incongruities between the proposed and realized effects of phenolics on organisms and ecosystems. Proposed initially to have ecological activity as plant

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defenses against pathogens and herbivores (Kosuge, 1969; Feeny, 1969), their realized effects are much more diverse. They may influence detritivores as well as herbivores and pathogens and affect processes occurring at the ecosystem as well as organismal levels. Their effects may be positive as well as negative, and include feeding deterrence, feeding stimulation, digestion inhibition, digestion stimulation, toxicity, toxicity amelioration, disease resistance, signal inhibition, signal transduction, and nutrient cycle regulation (Schultz, 1989; Bernays et al., 1989; Friend, 1981; Peters and Verma, 1990; Schlesinger, 1991; Schultz et al., 1992). Furthermore, the type and magnitude of their effects vary with the situation and the organism, such that it has been very difficult to predict when and where phenolics will be active. In this paper, I argue that the activity of plant phenolics is more predictable on the basis of their mode of action.

I suggest that phenolics require oxidation for most of their ecological activity; thus variation in phenolic activity often results from variation in oxidative conditions. Oxidative activation of phenolics has not received much attention, primarily because of the early assumption that ecological activity depends on hydrogen bonding (Feeny, 1968, 1969). However, there are many examples of ecological activity of oxidatively activated phenolics and, frequently, a high probability of phenolic oxidation in the environment. As a consequence, ecological activity can vary with the activity of enzymes and oxidants, even when the concentration and composition of phenolics are constant. Thus, oxidative activation may explain much of the variation observed in individual and ecosystem-level responses to phenolics. Furthermore, we may be able to improve our predictions of the consequences of additions of phenolics to ecological systems by measuring the oxidative strength of the environment.

In this paper, I discuss sources of variation in phenolic activity, potential biochemical modes of action, and documented modes of action. I argue that (1) phenolics have several modes of action, but oxidative activation is the most common; (2) physicochemical conditions of the environment control phenolic oxidation and thus generate variation in ecological activity; (3) oxidative conditions can be used to predict the activity of phenolics; and (4) experiments must be designed with conditions appropriate to the mode of phenolic action.

SOURCES OF VARIATION IN PHENOLIC ACTIVITY

Variation in the activity of phenolics arises from three major sources: (1) evolutionary differences in tolerance among organisms, (2) variation in phenolic structure and concentration, and (3) variation in conditions influencing the mode of phenolic action, especially oxidative activation. Although I emphasize the importance of mode of action throughout this paper, differences in evolutionary history and phenolic structure and concentration account for some of the vari-

ation in the activity of phenolics in ecological systems, and are discussed briefly here.

Evolutionary differences among organisms can explain some of the interspecific variation in phenolic activity. In general, organisms with an evolutionary history of association with phenolics are less sensitive to their deleterious effects, and in some cases even benefit from them (e.g., Austin et al., 1989; Berenbaum, 1984; Bernays et al., 1980; Lindroth et al., 1988; Karowe, 1989; Robbins et al., 1991; Steinberg and Van Altena, 1992; Steinberg et al., 1991). The mechanistic basis of this tolerance/benefit is largely unknown (Appel and Schultz, 1992; McArthur et al., 1991).

Temporal and spatial variation in structure can also explain some of the variation in phenolic activity (Zucker, 1983). Phenolics are an extremely diverse group of compounds with different modes of action, and, as a result, different ecological activities. Major structural classes of polymeric phenolics (e.g., hydrolyzable vs. condensed tannins) and monomers often differ in ecological activity, as do differently substituted compounds (Hagerman and Butler, 1991; Nichols-Orians, 1991; Keating et al., 1988; Harborne, 1988; Jones and Klocke, 1987; Elliger et al., 1981). Even stereoisomeric differences can influence phenolic activity (Clausen et al., 1990).

Variation in phenolic concentration can also influence activity, and studies must be done at ecologically appropriate concentrations. While this may seem obvious, there is a surprising number of studies in which single and/or ecologically unrealistic concentrations are used. Ecological responses are invariably threshold phenomena, with ranges of sensitivity and tolerance. Even for organisms that can tolerate high levels of dietary phenolics, there are thresholds beyond which negative effects are observed. Levels of plant phenolics are influenced by phenology (maturation of foliage and seasonal patterns of litter deposition), stress (e.g., nutrient, light, osmosis, acid rain, elevated CO_2), disturbance (defoliation, deforestation, and fire), and genotype, all of which have been shown to influence herbivore performance (Waterman and Mole, 1989; Reimer and Whittaker, 1989; Bryant et al., 1991; Tallemy and Raupp, 1991; Cooper et al., 1988; Rossiter et al., 1988; Lindroth et al., 1992; Cole et al., 1988).

A potentially common and important source of variation in the ecological activity of phenolics, however, is variation in phenolic oxidation state. Activity often requires oxidation, which is influenced by many biotic and abiotic factors. Phenolics are readily oxidized by enzymes and oxidants found in leaves, detritus, soil, water, and the digestive tracts of herbivores and detritivores (Friend, 1979, 1981; Vaughn and Duke, 1984; Duffey and Felton, 1989; Felton and Duffey, 1991a; Larson, 1988; Tate, 1987; Stevenson, 1982). Available data summarized in this paper indicate temporal and spatial variation in the amount and/or activity of these enzymes and oxidants. As a consequence, phenolic

activity may also exhibit temporal and spatial variation simply due to variation in phenolic oxidation state.

POTENTIAL BIOCHEMICAL MODES OF PHENOLIC ACTION

The ecological activity of phenolics depends on the physicochemical conditions in which phenolics occur, because these determine chemical modification(s) and modes of action. Here I provide a brief introduction to phenolic chemistry, modes of action, and modification by physicochemical conditions.

Phenolics are a chemically diverse and widespread group of compounds. Although ubiquitous and diverse in plants, all organisms produce simple phenolics denovo or from aromatic amino acids. A phenolic is characterized only by the presence of an aromatic ring bearing one or more hydroxyls. Phenolics are found in chemical classes as diverse as alkaloids, flavonoids, terpenoids, and glycosides as products of a large number of metabolic pathways. There are excellent reviews of several of these structural groups (Hagerman and Butler, 1991; Harborne, 1991a,b).

The broad chemical reactivity of phenolics arises from the transformations they undergo in the presence of enzymes and under different conditions of pH and E_h. Phenolics may be oxidized to quinones, or ionized to phenolate ions (Figure 1). All three forms may participate in reactions with other molecules. Phenolics can act as reducing agents (electron donors), and quinones as oxidizing agents (electron acceptors). In fact, phenolics such as tocopherol and ascorbic acid are used extensively as electron donors (antioxidants) in food and manufacturing (Schuler 1990).

Phenolics occur in both monomeric and polymeric forms. The polymeric form, or "tannin" was initially ascribed the greater ecological importance because of its ability to bind proteins in vitro (Feeny, 1976; Rhoades and Cates, 1976; Swain, 1979). However, monomeric phenolics also have ecological activity (Harborne, 1991a; Jones and Klocke, 1987; Isman and Duffey, 1982; Reese, 1978), and, in some cases may be more active than polymers, particularly towards pathogenic microbes (Schultz et al., 1992).

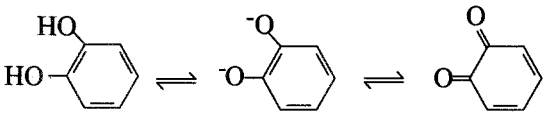


FIG. 1. The oxidation states of phenolics.

The toxicity of phenolics results from several different modes of action, including binding and oxygen radical formation. Phenolic binding has received the bulk of experimental attention and occurs readily in vitro with many types of molecules, including proteins, lipids, metals, and carbohydrates (Takechi and Tanaka, 1987). When these molecules are nutrients, enzymes, morphological features, or pathogens, binding can have a dramatic impact on digestion and disease resistance.

Phenolics can participate in four major types of bonds; hydrophobic, hydrogen, ionic, and covalent, in order of increasing strength (Table 1 and Figure 2) (Ebbing, 1987). Hydrophobic bonds are formed by attraction between the aromatic ring of the phenolic and hydrophobic regions of other compounds, like aliphatic and aromatic side chains of amino acids and aliphatic side chains of fatty acids (Oh et al., 1980). Unlike other types of bonds, hydrophobic bonds result from changes in entropy rather than enthalpy, and the entropic factors are determined primarily by the solvent, rather than the solute. Hydrophobic bonds are reversible, and independent of pH.

TABLE 1. CHARACTERISTICS OF BONDS IN WHICH PHENOLICS PARTICIPATE

	Type of bond			
Characteristic	Hydrophobic	Hydrogen	Ionic	Covalent
Strength (kJ/mol)	<4	10–40	100–1000	100–1000
Reversibility	reversible	reversible	reversible	irrevers.
Oxidation state	phenolic	phenolic	phenolate ion	phenolic or quinone
pH of formation	any	<8	>8	>8

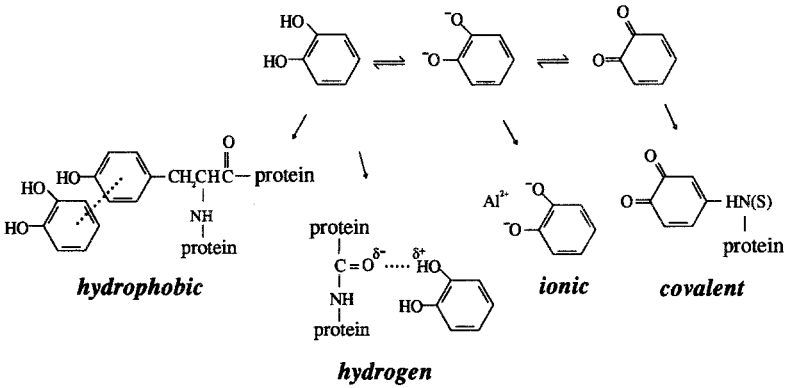


FIG. 2. Potential modes of phenolic binding.

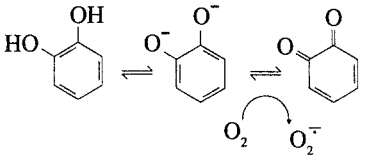
Hydrogen bonds are formed by sharing of the phenolic proton with other compounds, like the numerous amide carbonyl groups of proteins. Hydrogen bonds are reversible, and occur only when the phenoxyl hydrogen is present, i.e., at pHs below its pK_a of approximately 9.

Ionic bonds are formed only when the phenolic is ionized to a phenolate ion, i.e., when the hydrogen ion is removed from the phenoxyl group, leaving a net negative charge. Phenolate ions are highly reactive because they are readily oxidized. Ionic bonds are formed by attraction between the phenolate ion and cationic portions of other compounds. Ionic bonds are reversible and formed nonenzymatically at alkaline pHs, i.e., above the hydroxyl pK_a of approximately 9.

Covalent bonds are formed between a ring carbon of a phenolic or quinone and other compounds. Covalent bonds involving phenolics form by electrophilic substitution at the ortho and para positions. Covalent bonds involving quinones form by conjugate additions to the unsaturated carbonyl moiety. The differences in the type and position of substitutions are a result of differences in electron densities and distributions in phenolics and quinones. Phenolics may also polymerize by covalent bonds by oxidative processes that generate phenoxyl radicals, or by condensation reactions between polyfunctional nucleophiles (including the anions derived from polyphenols) and quinones. Covalent bonds are irreversible, and may form enzymatically and nonenzymatically.

The formation of oxygen radicals is another important mode of phenolic action. Almost any oxidation of phenolics can generate superoxide anion radicals because the reactive semiquinone can donate an electron to molecular oxygen (Figure 3a). Superoxide anion radicals can generate additional radical species, including hydroxyl radicals, by forming and reacting with hydrogen peroxide

(a)



(superoxide anion radical)

(b)

$$2\,O_2^{\cdot-} + 2\,H^+ \longrightarrow O_2 + H_2O_2 \quad \text{(hydrogen peroxide)}$$

$$O_2^{\cdot-} + H_2O_2 + H^+ \longrightarrow O_2 + H_2O + HO\cdot \quad \text{(hydroxyl radical)}$$

FIG. 3. Oxygen radical formation by phenolic oxidation: (a) formation of superoxide anion radicals by phenolic oxidation; (b) formation of hydrogen peroxide and hydroxyl radicals by reactions of superoxide anion radicals.

(Figure 3b). Radical oxygen species are extremely dangerous to cells because they can cause enzyme inactivation, membrane lipid peroxidation, and strand breaks in DNA (Bendich, 1989; Smith, 1985). Although all cells possess antioxidant enzyme systems to scavenge oxygen radicals, the capacity of these systems to mop up radicals can be exceeded.

Thus, it is clear that the particular mode of action will depend on whether phenolics are protonated, ionized, or oxidized (Table 1 and Figure 2). While hydrogen and hydrophobic bonds require protonated phenolics, ionic bonds require ionized phenolics and oxygen radicals require oxidized phenolics. Covalent bonds can form with either protonated or oxidized phenolics (quinones). The oxidation and ionization of phenolics depend on phenolic structure, the physicochemical conditions under which reactions take place, including the hydrogen ion availability or pH, electron availability or E_h (redox potential), and the concentration of oxidative enzymes, nonenzymatic oxidants and reductants. Phenolics of different structure differ in redox potential under similar experimental conditions (Evans, 1978; Parker et al., 1979). In addition, the stability of phenolate ions, and hence their reactivity with other compounds, depends on phenolic structure. Due to increased resonance stabilization, stability is improved by electron withdrawing ortho and para substitutions, and by increasing the length of those substituents.

Physicochemical conditions influence both the type of binding and the availability of molecules to which the phenolic will bind. For example, under oxidizing conditions or at alkaline pHs, hydrogen bonds will not form, but ionic and covalent bonds will. Similarly, under reducing or acid–neutral conditions ionic bonds will not form, but hydrogen and hydrophobic bonds, and, to a lesser extent, covalent bonds, will. Oxygen radicals will only be formed when phenolics are oxidized; thus, they can occur at any pH, but are more likely under alkaline conditions.

The type of bond formed may be critical to activity. For example, noncovalent binding of chlorogenic acid to dietary proteins in vitro improves enzymatic digestion but covalent binding inhibits it (Barbeau and Kinsella, 1985; Mole and Waterman, 1985).

The activity of enzymes and oxidants that activate phenolics is also influenced by the pH and redox conditions, as is the availability of substrates to which phenolics bind. For example, proteins are more likely to form hydrogen bonds at pHs near their isoelectric point, because protein–protein repulsion is minimized when their net charge is zero. In contrast, proteins are more likely to form covalent bonds at pHs above the pK_a of the amino (9–10) or sulfhydryl (8) groups that participate in the nucleophilic attack on quinones. Thus, even without details of a particular reaction, one can exclude some modes of phenolic action and predict others to occur simply on the basis of general physicochemical conditions in which reactions occur.

Phenolics are readily oxidized in the environment by enzymes and oxidants in plants, soil, sediments, water, and the digestive tracts of herbivores and detritivores (Friend, 1979, 1981; Harborne, 1991a; Vaughn and Duke, 1984; Duffey and Felton, 1989; Felton and Duffey, 1991a; Larson, 1988; Tate, 1987; Stevenson, 1982). There are numerous enzymes produced by plants and microbes that oxidize phenolics, including monophenol oxidases or tyrosinases (EC 1.14.18.1), diphenol oxidases or catechol oxidases (EC 1.10.3.2), laccases (EC 1.10.3.1), and peroxidases (EC 1.11.1.7) (Mayer, 1987; Robb, 1984). Similarly, many molecules produced by plants and microbes can oxidize phenolics, including tocopherols, flavonoids, chromenes, benzofurans, furanocoumarins, organic peroxides, and metal cations. Oxidized phenolics may oxidize other phenolics of lower redox potentials, initiating chain reactions of oxidation and polymerization, such as those proposed to drive the formation of humus in soils and sediments (Dennisov and Khudyakov, 1987; Tate, 1987). Because there are many conditions and compounds that promote autoxidation (e.g., alkaline pH, metal catalysts), phenolic oxidation may proceed rapidly in the absence of enzyme activity.

REALIZED BIOCHEMICAL MODES OF PHENOLIC ACTION

Because phenolics are ubiquitous in plants and their potential modes of action involve oxidation that occurs readily in the environment, one would expect oxidized phenolics to mediate many ecological interactions. This is, in fact, true; in the literature there are numerous examples from diverse systems in which the ecological activity of phenolics depends on oxidative activation. In the next sections I summarize examples for herbivores, detritivores, plant pathogens, plant mutualists, plant competitors, and ecosystems. Herbivores are emphasized because I am most familiar with them and because this is the area in which the importance of oxidative activation has been least appreciated.

Oxidative Activation in Herbivores

Plant phenolics influence the food preference and performance of many different herbivores, including insects (Schultz, 1989; Bernays et al., 1989), isopods, gastropods, and sea urchins (Hay and Fenical, 1988; Steinberg and Van Altena, 1992), crayfish (Lodge, 1991), amphibians (Freda et al., 1990), fish (Boettcher and Targett, 1992; Van Alstyne and Paul, 1990), birds (Jakubas et al., 1989; Jakubas and Gullion, 1990), and mammals (Bernays et al., 1989; Palo and Robbins, 1991). Phenolics may have negative, neutral, or positive effects on herbivores, depending on the organism and situation. Phenolics may

affect the herbivore directly or indirectly through its natural enemies or symbionts. In cases where the biochemical mode of action has been identified, it has often been shown to require oxidatively activated phenolics.

Negative Direct Effects on Herbivores. Phenolics may have direct negative effects on herbivores as feeding deterrents, binding agents, and generators of oxygen radicals. Phenolic feeding deterrency is assumed to result from the astringent sensation of phenolics when they bind to the mucopolysaccharides of saliva, chemosensory cells, and/or epidermis (Haslam, 1989). Feeding deterrency is positively correlated with chemical astringency, the ability of phenolics to precipitate protein (Bate-Smith, 1977). The acid to neutral pH of the oral cavity of most organisms is conducive to hydrogen bond formation, and the strong ecological correlations with astringency suggest that hydrogen bonding may be the primary mode of action in feeding deterrency. However, there are examples of covalent phenolic binding with epidermal proteins. Oxidized urushiol from poison ivy binds covalently to epidermal proteins to cause contact dermatitis (Benezra, 1988; Kalish and Johnson, 1990), although the impact of this on feeding deterrence has not been examined.

Digestion inhibition is usually assumed to result from the formation of hydrogen bonds with dietary protein or digestive enzymes in herbivore guts. This mode of action was proposed by Feeny (1969, 1970) because tannins can precipitate proteins in vitro in this way. Although there are numerous examples of digestion inhibition on phenolic diets (Schultz, 1989; Bernays et al., 1989; Salunkhe et al., 1990; McArthur et al., 1991), there are few demonstrations of the mode of binding, which requires treatment of the complexes with reagents that disassociate bonds of different types. In mammals, there is indirect evidence that hydrogen bond formation is common. Some mammals protect dietary protein by production of salivary "trap" proteins that bind to phenolics by hydrogen bonds stable throughout the digestive tract (Mehansho et al., 1987; Austin et al., 1989; Mole et al., 1990), although their capacity can be exceeded (Robbins et al., 1991). Hydrogen bond formation may often be inhibited by gut surfactants (Martin and Martin, 1984; Martin et al., 1987; Tugwell and Branch, 1992), although high levels of dietary phenolics may precipitate them (De Veau and Schultz, 1992). Alkaline gut conditions (pH $>$ 9) also inhibit hydrogen bond formation, making it unlikely to occur in the digestive tract of many insect herbivores.

Digestion inhibition can also result from the formation of covalent bonds with dietary proteins or digestive enzymes. In caterpillars, covalent bonds of the monomeric phenolic chlorogenic acid with protein inhibit the digestion of amino acids (Felton et al., 1989; Felton and Duffey, 1991b). Chlorogenic acid requires oxidative activation by foliar phenol oxidases, which retain half their activity in the alkaline (pH 8–9) gut (Felton et al., 1989). The observation that phenolic precipitation of gut fluid surfactants is higher at alkaline pHs suggests

that phenolics may also impair lipid digestion by covalent binding (De Veau and Schultz, 1992). In plant glandular hair secretions, covalent binding polymerizes monomeric phenolics, oxidatively activated by foliar enzymes, into sticky polymers that immobilize small insect herbivores (Ryan et al., 1982).

Phenolic toxicity is frequently invoked to explain reductions in growth on phenolic-containing diets when there is no digestion inhibition (Iason and Palo, 1991; Clausen et al., 1990). Oxygen radicals, generated during phenolic oxidation, are thought to disrupt membrane integrity and metabolism in the gut epithelium and are probably responsible for the gut lesions observed in herbivores exposed to novel tannins (Steinly and Berenbaum, 1985; Bernays, 1978; Raubenheimer, 1992; McArthur et al., 1991). Oxidized phenolics can also inhibit antioxidant enzyme systems of herbivores (Lee, 1991), thereby enhancing the effect of oxygen radicals they generate.

Positive Direct Effects on Herbivores. Phenolics can have positive effects on herbivores when they are detoxified and/or used in defense. Some insect herbivores appear to hydrolyze phenolics for nutrients (Bernays et al., 1983), while others promote phenolic oxidation and polymerization to improve food quality. Some aphids polymerize monophenolics with salivary phenol oxidases to strengthen the stylet sheath and facilitate feeding (Peng and Miles, 1988a,b; Miles and Peng, 1989). Some grasshoppers may polymerize and inactivate phenolic resins using midgut phenol oxidases (Rhoades, 1977). We have recently discovered endogenous PPO activity in lepidopteran gut lumens, which may serve a similar purpose (Appel, unpublished). Oxidized plant phenolics are important components of the defensive secretions and immune responses of some herbivores, including grasshoppers (Jones et al., 1986) and beetles (Blum, 1981).

Effects on Natural Enemies and Symbionts of Herbivores. Plant phenolics influence the interactions of herbivores with their microbial pathogens, mutualists, and predators. As with direct effects, phenolics can have negative, neutral, or positive indirect effects. Where examined, the phenolics often require oxidative activation.

Phenolics influence the microbial pathogens of invertebrate herbivores. Several phenolics inhibit infection of insect herbivores by naturally occurring baculoviruses consumed with foliage. The monophenolic chlorogenic acid inhibits infection of several noctuid caterpillars by a baculovirus consumed on tomato (Felton et al., 1987; Felton and Duffey, 1990). Chlorogenic acid forms covalent bonds with the virus following oxidation by tomato phenol oxidases in the gut lumen. Polymeric oak phenolics (tannins) inhibit viral infection of gypsy moth caterpillars, probably by the same mechanism, as phenol oxidase inhibitors reduce the tannin effect (Schultz and Keating, 1991; Appel, unpublished).

The toxicity of the protein delta endotoxin produced by *Bacillus thuringiensis* (Bt) is modified by plant phenolics. Chlorogenic acid increases the toxicity of Bt following oxidative activation by tomato phenol oxidases (Ludlum

et al., 1991). Polymeric phenolics appear to have an effect opposite that of monophenolics on Bt, as tannins reduce Bt toxicity in gypsy moths feeding on oaks and aspen (Appel, unpublished). Tannins also reduce the susceptibility of gall-forming flies on oak to pathogenic fungi (Taper et al., 1986).

Phenolics also influence the parasitoids of insect herbivores. The growth and survival of ichneumonid and tachinid parasitoids of caterpillars are reduced when phenolics are included in the host diet (Bloem and Duffey, 1990; Bourchier, 1991). In these cases, parasitoid growth is reduced by reductions in host growth and not by toxicity to the parasitoid. As there is evidence from other studies for oxidative activation of phenolics in these insects, the impact on the parasitoid is probably indirectly dependent on oxidation.

Phenolics also influence the pathogens and mutualists of vertebrate herbivores. There is a large medical literature on the antimicrobial properties of phenolics (see review by Schultz et al., 1992), but the ecological significance and importance of oxidative activation to pathogenicity has not been examined in nonhuman systems. In humans, oxygen radicals can be mutagenic, carcinogenic, and anticarcinogenic, and they are used in the immune system to destroy pathogens (Smith, 1985; Bendich, 1989). The digestive symbionts of vertebrates are sensitive to dietary phenolics (Barry and Manley, 1986), but some vertebrates possess microbial symbionts capable of degrading tannin–protein complexes (Osawa, 1992; Osawa and Sly, 1992).

Control of Oxidative Activation in Herbivores. The probability of phenolic oxidation will depend on characteristics of the plant and the herbivore. These characteristics include the amount and activity of plant oxidases and oxidants, and the physicochemical conditions of the herbivore gut. There is substantial interspecific, developmental, seasonal, and morphological variation in plant oxidases and oxidants that can influence phenolic activity independent of phenolic concentration, and these are discussed on p. 1536.

Physicochemical conditions in the digestive tracts of herbivores may be influenced by foliage, but at least some insect herbivores regulate gut conditions within fairly narrow margins (Appel, unpublished). Although all digestive tracts have morphologically and functionally specialized regions that differ in pH and redox potential, there is significant variation among herbivore species in those conditions. For example, vertebrate guts do not usually exceed pH 8 (Hofmann, 1989; Horn, 1989; Cork and Foley, 1991) and thus are not likely to promote phenolic oxidation in the absence of active foliar oxidases. However, some herbivorous fish and many insect herbivores, including caterpillars, sawflies, and some Orthoptera (e.g., walking sticks), maintain midgut pHs ranging from 8 to 12 (see Horn, 1989; Appel and Schultz, 1992; Appel, 1993), which promote autoxidation of ingested phenolics. While it would appear at first maladaptive to maintain an alkaline gut in the presence of phenolics, the advantages gained in extracting protein at these pHs (Felton and Duffey, 1991b) and inactivating

foliar enzymes may outweigh the risks. Furthermore, some organisms appear to inactivate ingested phenolics by oxidative polymerization (Rhoades, 1977; Appel, unpublished).

There is significant variation among herbivores in the redox conditions of the gut, ranging from highly reducing to highly oxidizing (Appel and Martin, 1990; Appel, unpublished). Conditions are reducing in fermentation chambers found in some beetles, ruminants, macropods, marsupial arboreal folivores, and certain primates, but are oxidizing elsewhere in the digestive tract (Crowson, 1981; McArthur et al., 1991). Oxidizing conditions in the gut are likely to promote phenolic autoxidation, and reducing conditions prevent it. These differences suggest that herbivores may have multiple strategies for dealing with foliar phenolics: a "reducing strategy" in which reducing conditions in the gut prevent phenolic oxidation, and an "oxidative polymerization strategy" in which phenolics are oxidized and rapidly polymerized. Evolution of one strategy or another may depend on several dietary traits, including phenolic concentration, structure-based differences in the propensity of phenolics to oxidize and polymerize, and nutrient constraints on the production of sulfur- and nitrogen-rich reducing agents. For example, herbivores feeding on foliage with high concentrations of readily oxidized and polymerized phenolics and low concentrations of nutrients (e.g., many trees) may use the oxidative polymerization strategy. Conversely, herbivores feeding on foliage with low concentrations of phenolics but high concentrations of nutrients for reducing potential (e.g., many herbs) may employ the reducing strategy.

Herbivores can influence the activity of oxidized phenolics by conjugation, oxidation, hydrolysis, or antioxidant action. Conjugation of phenolics with glucuronic or sulfuric acid (vertebrates) and glutathione or glucose (invertebrates) increases their hydrophilicity and facilitates egestion and/or excretion (Salunkhe et al., 1990; McArthur et al., 1991; Lindroth, 1991). Phenolics may be methylated at the hydroxyl group (Booth et al., 1959) to prevent the formation of covalent bonds or oxygen radicals, but this may facilitate absorption by enhancing lipophilicity.

The degradation of phenolics by hydrolysis or oxidation is a potentially important detoxification mechanism in herbivores, although hydrolysis can also activate phenolics when they are conjugated as glycosides (Lindroth, 1991; McArthur et al., 1991). The degree to which phenolics are degraded in the gut depends on gut conditions, phenolic structure, and enzyme activity. In general, monomers are more readily hydrolyzed than polymers, and hydrolyzable tannins more readily than condensed tannins, although there is evidence that degradation of all three occurs in some herbivore species (Clausen et al., 1990; Bernays and Chamberlain, 1980; Bourchier, 1991). Hydrolyzable tannins (gallotannins and ellagitannins) are readily hydrolyzed nonenzymatically under acidic and basic conditions (Hagerman and Butler, 1991), such as those encountered in many

herbivore guts. They may also be hydrolyzed by esterases, but this has not been documented. Condensed tannins are oxidatively degraded under strongly acid conditions in the presence of metal catalysts (Hagerman and Butler, 1991), such as those encountered in the stomachs and fermentation chambers of some mammals (McArthur et al., 1991) and fish (Horn, 1989).

Herbivores have enzyme systems that reduce oxidized phenolics or detoxify the oxygen radicals generated during oxidation. Quinone reductases, also known as NADPH dehydrogenases (EC 1.6.99.2) catalyze the two-electron reduction of quinones to hydroquinones (Yu, 1987). There are several antioxidant enzyme systems that scavenge oxygen radicals generated by phenolic oxidation. The first, found in vertebrate and invertebrate herbivores, consists of superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9), and glutathione reductase (EC 1.6.4.2) (Halliwell and Gutteridge, 1989; Lindroth, 1991). The second, found in invertebrates only, consists of ascorbate-free radical reductase (EC 1.6.5.4), dehydroascorbate reductase (EC 1.8.5.1), and glutathione reductase (Felton and Duffey, 1992). The value of an antioxidant enzyme system depends on its location relative to where the oxygen radicals are generated. If radicals are generated in the gut lumen, then a system in the gut epithelium may be of little benefit in preventing membrane damage or enzyme inactivation. Unfortunately, investigators routinely assay enzyme activity of whole insects or specific tissues, and not the gut lumen. I expect luminal activities to be high in herbivores, except where reducing redox conditions prevent oxygen radical formation.

Oxidative Activation in Detritivores

Phenolics reduce the palatability and colonization of detritus by snails, nematodes, earthworms, mites, isopods, and stream invertebrates (Heal and Dighton, 1986; Taylor and Murant, 1966; Lee, 1985; Cameron and LaPoint, 1978; Rietsma et al., 1988; Stout, 1989). They are thought to do this, in part, by slowing microbial conditioning of detritus; palatability increases with microbial conditioning (Anderson and Cargill, 1986; Werner and Dindal, 1986), and high-phenolic leaves are conditioned more slowly (Stout, 1989). Phenolic inhibition of some microbial enzymes is well documented in vitro and is thought to require oxidatively activated phenolics (Tate, 1987). Phenolics may also influence detritivores directly as feeding deterrents, toxins, or binding agents, but this has not been examined (Newman, 1991).

Control of Oxidative Activation in Detritivores. The importance of oxidation to phenolic activity in detritivores depends on oxidative conditions of the food and the gut. As with herbivores, enzymes and oxidants may play a major role in determining the oxidation state, mode of action, and activity of phenolics. Microbial rather than foliar enzymes are active in detritus, as foliar activity is rapidly lost during leaf senescence.

The oxidation state and persistence of phenolics in soils and sediments will depend on rates of release and metabolism, which are influenced by levels in foliage, secretion rate by roots, litter type, soil type, and microbial metabolism (Muller et al., 1989; Schmidt, 1988; Williamson and Weidenhamer, 1990; Blum et al., 1991; Kuiters and Sarink, 1986; Racon et al., 1988). There are numerous opportunities for phenolic oxidation in soils, and thus for oxidative activation. There is likely to be substantial spatial and temporal variation in soil constituents influencing phenolic oxidation, but this is rarely investigated.

A large number of soil microorganisms produce phenolic-oxidizing enzymes extracellularly to acquire nutrients or inactivate phenolic defenses (Szklarz et al., 1989). Although oxidized phenolics can inhibit microbial enzymes, oxidation may be necessary for substrate digestion or defense inactivation. Microbial oxidation of phenolics is modified by interactions with soil particles (Ruggiero and Radogna, 1988; Claus and Filip, 1990), phenolics (Baldwin et al., 1983; Harrison, 1971), and metals (Illman, 1991). Phenolic activity can be influenced by shifts in the microflora composition from phenolic-sensitive to phenolic-tolerant species (Shafer and Blum, 1991). This may also be true for the microfauna of soils, although it has not been examined.

Microbial decomposers secrete phenol oxidases active towards a variety of phenolic substrates (Tate, 1987; Stevenson, 1982), which are thought to reduce phenolic inhibition of microbial enzyme activity. These enzymes may oxidize phenolics released from detritus by leaching or feeding. Like foliar enzymes, they have a pH optimum of about 7.0 and may be active prior to ingestion or after ingestion in the detritivore gut. Phenol oxidase and peroxidase activity have been obtained from homogenates of detritivores as diverse as earthworms, isopods, diplopods, mollusks, oligochaetes, and insects with activity at neutral to alkaline pHs, although it is not clear whether the enzymes are ingested or endogenous (Hartenstein, 1982). There is an extensive literature on the activity of microbial enzymes in the guts of invertebrate detritivores (Martin, 1987), although the activity of phenol oxidases has not been examined.

The oxidation state of detrital phenolics may also be influenced by nonenzymatic oxidants present in detritus and associated soil particles. Metal cations are catalysts of nonenzymatic oxidations of soil phenolics (Tate, 1987) and are used by brown-rot fungi to generate oxygen radicals for cellulose depolymerization (Illman, 1991). Although there are no quantitative estimates of their significance, they are likely to be important.

The gut conditions of detritivores are not well characterized, but gut pH appears to vary significantly among groups. Isopod, stone fly (Plecoptera), and caddisfly (Trichoptera) larvae have neutral to slightly alkaline midguts (Martin et al., 1980, 1981a,b; Kukor and Martin, 1986; Barlocher, 1983), which are not likely to promote autoxidation of phenolics. However, cranefly (Tipulidae), mosquito, and blackfly (Simuliidae) larvae have highly alkaline guts, ranging

from pH 8–11 (Martin et al., 1980; Dadd, 1975; Lacey and Federici, 1979; Undeen, 1979) in which autoxidation of phenolics is likely. Gut redox conditions may also be important to phenolic activity but have not been determined in any detritivores.

Oxidative Activation in Plant Pathogens, Parasites, Mutualists, and Competitors

Phenolics have diverse effects within plants and on the interactions of plants with other organisms. Real and proposed functions within plants include regulating photosynthesis and gene expression (Peters and Verma, 1990) and ethylene production (Elstner et al., 1976), screening ultraviolet radiation, preventing water loss, and metabolic storage (Seigler and Price, 1976). In addition to the interactions of plants with herbivores and detritivores covered in the previous sections, phenolics influence interactions between plants and pathogens, mutualists, and competitors (allelopathy). In many cases in which the biochemical mode of phenolic action has been identified, it has been shown to require oxidative activation.

Effects on Plant Pathogens. Phenolics are important to the defense strategy of plants at several stages, including host cell death and necrosis, accumulation of toxins, modification of cell walls, and synthesis of specific antibiotics (Matern and Kneusel, 1988). Their participation in each stage varies with plant and pathogen (Nicholson and Hammerschmidt, 1992). Host cell death and necrosis can be caused by phenolic-generated oxygen radicals (Adam et al., 1990; Doke et al., 1991). Phenolics can act directly as toxins at the site of infection, probably by enzymatic oxidation and covalent binding of foliar quinones to microbial proteins (Mink and Saksena, 1971; Byrd et al., 1960; Friend, 1979; Dix, 1979; Blakeman and Atkinson, 1981). Modification of cell walls requires enzymatically oxidized phenolics as substrates for lignin biosynthesis, resulting in numerous positive correlations between pathogen resistance and activity of phenol oxidases and peroxidases (e.g., Graham and Graham, 1991; Southerton and Deverall, 1990; Bashan et al., 1987; Ye et al., 1990; Smith et al., 1991). Many antibiotics synthesized in response to pathogens are phenolic, including some phytoalexins (Kumar et al., 1991; Phan, 1991; Friend, 1981). Although the biochemical mode of action of phenolic phytoalexins has not received much attention (Anderson, 1991), recent evidence suggests that cytotoxicity depends on autoxidation by transition metals (Stipanovic et al., 1991). Phenolics produced in response to wounding may also serve as within-plant signals of infection (Doke et al., 1991; Daub and Hangarter, 1983) and stimulate the expression of virulence genes in pathogens (Peters and Verma, 1990).

Effects on Plant Parasites, Mutualists, and Competitors. Oxidized phenolics are required for host recognition by some parasitic plants. Seed germi-

nation and haustoria formation of several parasitic angiosperms are regulated by oxidatively activated phenolics (Fate et al., 1990; Smith et al., 1990; Lynn and Chang, 1990). Phenolics are also important in host recognition by microbial symbionts, although the importance of phenolic oxidation state has not been investigated (Peters and Verma, 1990).

A large number of plant phenolics has been proposed to be allelopathic by inhibiting the germination and/or growth of other plants (Rice, 1984; Einhellig, 1986; Putnam and Tang, 1986). Unfortunately, most studies are not designed to evaluate allelopathic effects in the field and are thus difficult to interpret in an ecological context (Dao, 1987). The importance of oxidation state has been investigated for two allelopathic phenolics, juglone (Rietveld, 1983) and sorgoleone (Einhellig and Souza, 1992). Produced in a reduced form in the plant, they are rapidly oxidized to the more stable and active quinone form when released as tissue leachates or root exudates.

Control of Oxidative Activation in Plants. There are substantial differences among genotypes, developmental stages, and tissues in plant constituents influencing phenolic oxidation. Phenol oxidase and peroxidase enzymes exist in many different isozymic forms, with different substrate specificities, pH optima, localization, and response to stress. Activities may vary as much as sevenfold among genotypes, as much as eightfold between young and mature leaves and fruits, and up to eightfold between epidermal and mesophyll tissues and between intracellular and extracellular compartments (Lanker et al., 1987; del Grosso et al., 1987; Lee et al., 1990; Felton et al., 1989; Janovitz-Klapp et al., 1989; Bassuk et al., 1981; Gentile et al., 1988; Kojima and Conn, 1982; van den Berg and Huystee, 1984; van Huystee and Cairns, 1980). Furthermore, these enzymes are inducible by stresses, including pathogens (Patra and Mishra, 1979; Ye et al., 1990; Bashan et al., 1987; Southerton and Deverall, 1990). There is also variation in the pH optimum and pH dependence of phenoloxidases that influences their activity under different physicochemical environments. Although pH optima can vary genotypically and seasonally (Sanchez-Ferrer et al., 1989), most are near neutrality but retain significant activity up to pH 9 (Felton et al., 1989; Owusu-Ansah, 1989; Fujita and Tono, 1988; Oda et al., 1989). In addition, conformational changes from inactive to active forms can be pH dependent (Valero and Garcia-Carmona, 1992).

There is also substantial variation in the levels of oxidants and reductants (antioxidants). Species differ up to fourfold in ascorbate content, and up to 70-fold in β-carotene (Duke and Atchley, 1986). In addition, levels of oxidants and reductants vary with stress and time of day. Glutathione, a major reductant involved in cellular redox cycling (Meister, 1981), increases in concentration in response to extremes of temperature, drought, herbicides, and air pollutants (Alscher, 1989) and can increase twofold on a diurnal basis (Koike and Patterson, 1988). Chlorogenic acid, a ubiquitous and readily oxidized monophenolic

in plants, can vary up to 15-fold with genotype, development, and stress (drought, nitrogen deficiency, UV exposure) (del Moral, 1972; Cole et al., 1987; Parfit et al., 1986; Cilliers et al., 1990; Cherney et al., 1990; Griffiths et al., 1992).

The activity of antioxidant enzyme systems also varies substantially with genotype, development, and stress. In general, levels are lower in older tissue and higher in plants under pathogen and oxidative stress (extreme temperatures, anaerobiosis, UV radiation, high light intensity, elevated SO_2 and O_3) (Scandalios, 1990; Anderson et al., 1992).

Ecosystems and Nutrient Cycling

Phenolics have an important influence on the rates of nutrient cycling in terrestrial and aquatic environments (Schlesinger, 1991). Because they can complex toxins as well as nutrients, their effects may be positive or negative, depending on the substance bound and the consequences of that binding for the organisms involved. Their modes of action are comparatively well documented, and most require oxidation.

Phenolics are a major component of humus and are responsible for most of its physicochemical and biological properties. By complexing nutrients, phenolic (and phenolic acid) monomers and humic polymers influence rates of productivity and nutrient turnover in soils, sediments, lakes, streams, estuaries, and oceans (Munster and Chrost, 1990; Aiken et al., 1985; Wetzel, 1991). Humus formation requires oxidatively activated phenolics. Quinones, oxidized by microbial enzymes or mineral catalysts, covalently bind with amino acids, sugars, and minerals to form a matrix recalcitrant to microbial digestion (Stevenson, 1982; Tate, 1987; Flaig, 1988; Hayes, 1989).

Phenolic acids not incorporated in humus regulate the cation exchange capacity of soil and water by weathering and complexing minerals (Kalisz and Stone, 1980; Gillman, 1985; McColl et al., 1990). Phenolics increase the availability of phosphorus to terrestrial plants and phytoplankton by competing for anion adsorption sites on humus and clay and by binding with soluble aluminum, iron, and manganese that would otherwise bind phosphate (Kafkafi et al., 1988; Tan and Binger, 1986; Lopez-Hernandez et al., 1979; Aiken et al., 1985). By binding to aluminum, phenolics reduce the aluminum toxicity of soils and water (McColl and Pohlman, 1986; Driscoll et al., 1985; Hue et al., 1986; Freda et al., 1990). Phenolic binding to minerals is largely responsible for the high productivity of estuaries; the flocculation, deposition, and retention of minerals in estuaries is caused by the replacement of hydrogen ions on the exchange sites of phenolic acids with cations of seawater (Schlesinger, 1991). Phenolic binding to minerals sometimes requires oxidative activation; mineral complexation occurs either by adsorption, a process involving hydrophobic/electrostatic interactions between minerals and nonoxidized phenolics, or by organometallic complex formation, a process involving ionic binding (Tate, 1987).

The ability of oxidized phenolics to complex nutrients is beginning to be used to human advantage in the management and recovery of ecosystems. Nutrient retention of soils in agroecosystems is being improved by use of phenolic-containing mulch crops (Fox et al., 1990; Palm and Sanchez, 1991). Predictions of the metabolism and movement of toxins of human origin through soil and water can be improved by accounting for phenolic complexation (Bollag, 1991; Chiou, 1990). Similarly, the ability of phenol oxidases to polymerize and precipitate phenolics is being tested in the removal of phenolic-based toxins from aquatic systems (Shannon and Bartha, 1988; Davis and Burns, 1990).

DISCUSSION

Oxidative Activation of Phenolics is Common

With the exception of studies of phenolics in nutrient cycling, the ecological consequences of phenolic oxidation have been largely ignored and remain peripheral to current theory. In this paper, however, I provide numerous examples from a diversity of systems that indicate that biological activity resulting from phenolic oxidation is widespread. Oxidatively activated phenolics influence critical aspects of the survival, growth, and reproduction of organisms, including dietary preferences, host recognition, nutrient acquisition, disease resistance, predation and parasitism, and competitive interactions.

There are situations in which oxidative activation is not important to phenolic activity, and some of those situations are described in individual sections above. To understand variable phenolic activity in these, it will be necessary to identify other characteristics promoting an alternative mode of action; this may be the absence of conditions promoting oxidation or something else altogether. There may be situations in which activity results from both reduced and oxidatively activated phenolics in a single organism. There may also be situations in which factors other than phenolics are overriding in importance.

Oxidative activation of phenolics in ecological interactions has several important consequences. First, it means that variation in oxidative conditions can regulate phenolic activity and can be used to explain some of the variation we observe. Second, it means that the design of laboratory and field experiments must include components that normally accomplish this oxidation.

Oxidative Conditions May Regulate Phenolic Activity

Basic chemistry dictates that the physicochemical conditions of the immediate environment will determine the oxidation state of phenolics, and hence their activity when oxidative activation is required. This means that the oxidative conditions of the immediate environment are as important to phenolic activity

as are the levels of phenolics themselves. Thus, in herbivores, phenolic activity can vary with the levels of oxygen and of foliar and herbivore enzymes and oxidants. In detritivores, activity can vary with the levels of oxygen and microbial/soil and detritivore enzymes and oxidants. Similarly, in plants, activity can vary with the level of oxygen and foliar and microbial/soil enzymes and oxidants, and in ecosystems, with the level of oxygen and microbial/soil enzymes and oxidants. While these represent a large number of individual factors and possible interactions, they are likely to be major ones that drive phenolic oxidation and thus predict their activity.

Using Oxidative Conditions to Predict Phenolic Activity

Oxidative conditions may be described by either summary characteristics of oxidative strength or the levels of specific enzymes and oxidants. The availability of electrons exchanged in redox reactions can be measured with pH and redox probes, respectively. Although these values represent the sum of all redox reactions in the system, they provide an indication of the likelihood of particular ones, including phenolic oxidation. Electrodes for pH and redox measurements are widely available in macro to ultramicro sizes.

This approach has been used by soil scientists to predict the turnover of inorganic metals in soils, as metal ions differ in mobility in soils of different pH and redox potentials (Bartlett, 1986). I am using this approach in my own work on plant phenolics and insect herbivores. By characterizing the oxidative conditions in the guts of herbivores, I can predict the mode of action of ingested phenolics, and likely physiological adaptations to them. Once demonstrated, the mode of action and inactivation become the basis for testable predictions about the evolution of adaptations to dietary phenolics.

Specific enzymes and oxidants must also be measured once their importance to phenolic activity has been identified. There are spectrophotometric, gas chromatographic, and liquid chromatographic techniques to quantify enzyme activity and oxidant levels. We can expect oxidation to be more likely in the presence of enzymes like phenol oxidases, tyrosinases, laccases, and peroxidases. Similarly, we can expect oxidation to be more likely at alkaline pHs, and in the presence of oxidants, like tocopherols, chromenes, benzofurans, furanocoumarins, organic peroxides, metal cations, and phenolics with lower redox potentials. Felton et al. (1989) have used this approach to predict the activity of monomeric phenolics in insect herbivores. Having shown that the phenolics require oxidative activation by foliar phenol oxidases, they can predict phenolic activity on the basis of phenolic and enzyme levels. We are also using this approach to characterize the impact of oak phenolics on the gypsy moth, a system in which both plant and insect enzymes appear to be important (Appel and Schultz, 1992).

Oxidative Activation Influences Experimental Design

A failure to appreciate the importance of oxidation to phenolic activity can lead to significant flaws in experimental design. If phenolic activity requires oxidation, then our measures of phenolics must include measures of compounds that oxidize them. In other words, levels of oxidative enzymes, oxidants, and reductants of foliage, litter, or soil may need to be measured in addition to phenolics to accurately assess phenolic activity in correlative studies. The discovery that plant enzymes retain activity in the digestive tracts of herbivores (Felton et al., 1989) makes their inclusion in phenolic studies imperative.

The importance of enzymes and oxidants to phenolic activity also means that experimental studies in which phenolic levels are manipulated must include the components that normally oxidize them in the field or in vivo. Foliar or microbial enzymes and oxidants and the pH and redox conditions of digestive tracts must be incorporated into the experimental design of laboratory and in vitro studies. For example, experiments with tissue culture or sterile soil lacking the constituents responsible for oxidation in situ may provide no information on phenolic activity in ecological contexts. Similarly, the widespread use of artificial diets may provide little information on the actual activity of phenolics consumed in leaves unless the diets contain foliar enzymes and oxidants.

Given the potential importance of enzymes and oxidants to phenolic activity and the rarity of their inclusion in experimental design, it is not surprising that correlative, or even manipulative, studies have failed to provide a clear picture of phenolic action. Indeed, the best predictive understanding of phenolics is in nutrient cycling, in which the importance of oxidative activation in humus formation is recognized.

A New Understanding of Phenolics

Phenolics clearly have more diverse and variable effects on ecological interactions than originally conceived. Their treatment as generalized defenses of woody plants with a uniform mode of action is no longer appropriate. Not only are their effects and modes of action diverse, but they include small phenolic molecules produced by plants of all growth forms.

Phenolics also clearly require oxidative activation for much of their activity. Their treatment as allelochemicals operating independently of other foliage or soil constituents is not tenable in most situations. Their activity must be examined in the context of the components that activate them.

The potential biochemical interactions of phenolics with other compounds are diverse and complex. As the biochemistry of most organisms in an ecological context is poorly known, characterization of the metabolism of phenolics in any detail is often impossible, except in rare collaborations of ecologists with biochemists or toxicologists. However, in the absence of detailed biochemical knowl-

edge, an appreciation for the mode of phenolic action may provide the only means to understand and predict the activity of these ubiquitous, diverse chemicals in ecological interactions.

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POTENTIAL ROLE OF ASCORBATE OXIDASE AS A PLANT DEFENSE PROTEIN AGAINST INSECT HERBIVORY

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Abstract—Ascorbic acid is essential for both nutritive and antioxidant functions in phytophagous insects; however, maintaining sufficient quantities of reduced ascorbate may be problematical for them. In this investigation, we show that the plant enzyme ascorbate oxidase retains activity in the digestive system of the herbivore *Helicoverpa zea*. High levels of the enzyme are present in several host plants of *H. zea*, including cotton, tomato, soybean, crimson clover, and vetch. The enzyme oxidizes L-ascorbic acid to dehydro-L-ascorbic acid, a potentially toxic product. The oxidation of ascorbic acid also produces active oxygen species such as the highly reactive hydroxyl radical. The nutritional quality of protein for larval *H. zea* was significantly reduced by treatment with ascorbate and ascorbate oxidase. Oxidative damage to the protein was indicated by decreased lysine content, increased carbonyl formation, and the occurrence of protein fragmentation and polymerization. Furthermore, the oxidative loss of ascorbate in the herbivore's digestive system prevents ascorbate from functioning as an important antioxidant against a plethora of dietary prooxidants.

Key Words—Ascorbic acid, ascorbate oxidase, plant resistance, plant defense, oxidative stress, *Helicoverpa zea*, Lepidoptera, Noctuidae, cotton, tomato, soybean.

INTRODUCTION

Ascorbic acid (i.e., vitamin C) is an essential nutrient for many insects, including the highly polyphagous noctuid, *Helicoverpa zea* (Vanderzant et al., 1962;

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Dadd, 1985). Ascorbate is required for normal growth, molting, and reproduction (Kramer and Seib, 1982; Dadd, 1985; Bruins et al., 1991). The specific functions of ascorbate in insects are not established; it may be involved in tyrosine metabolism, collagen synthesis, steroid synthesis, carnitine synthesis, phagostimulation, neuromodulation, and/or detoxification reactions (Kramer and Seib, 1982; Dadd, 1985; Englard and Seifter, 1986).

The factors that determine the relative bioavailability of ascorbate in insects are essentially unknown. A comparative wealth of information from mammalian and plant systems indicates that its bioavailability may be affected by the ease with which ascorbate can switch from antioxidant to prooxidant activity (e.g., Englard and Seifter, 1986). Ascorbate may be rapidly oxidized in biological systems in the presence of catalysts such as transition metals and alkaline pH or during a variety of redox reactions. In plant tissues, ascorbate may be directly oxidized by ascorbate oxidase (EC 1.10.3.3), a widely occurring enzyme whose physiological function is poorly understood (Wayner et al., 1986; Baysal et al., 1989; Lin and Varner, 1991; Dean and Eriksson, 1992). The enzyme oxidizes L-ascorbic acid in a two-step reaction to form dehydro-L-ascorbic acid:

L-ascorbic acid → ascorbate free radical → dehydro-L-ascorbic acid

The oxidation product, dehydro-L-ascorbic acid, may partially or fully substitute for dietary ascorbate in insects (Kramer and Seib, 1982).

Consequently, ascorbate bioavailability in herbivorous insects may be hindered via several dietary and physiological factors. Green plants are normally considered adequate sources of ascorbate for herbivores, but obtaining and/or maintaining sufficient quantities of reduced ascorbate could be especially problematical for lepidopterans. First, the alkaline conditions of the midgut of lepidoptera greatly accelerate the rate of ascorbate autoxidation (Slight et al., 1990). Second, significant conversion of ascorbate to dehydro-L-ascorbic acid may occur if ascorbate oxidase retains activity in the digestive system. Third, oxidative stress from dietary plant prooxidants may deplete ascorbate. The enzymatic oxidation of phenolics and the resulting formation of quinones in the midgut of *H. zea* (Felton et al., 1989) would require that significant amounts of ascorbate be oxidized to dehydro-L-ascorbic acid (Janovitz-Klapp et al., 1990).

Ascorbate oxidation may be detrimental to herbivore growth and survival. First, ascorbate is an essential nutrient for most insects. The oxidation product, dehydro-L-ascorbic acid, must be chemically or enzymatically reduced to ascorbate in order for it to function as a nutrient. However, dehydro-L-ascorbic acid is particularly unstable and irreversibly degrades to nutritionally inert products such as 2,3-diketogulonic acid (Clemetson, 1989). Second, dehydro-L-ascorbic acid and diketogulonic acid are cellular toxins (Bianchi and Rose, 1986; Rose et al., 1992), capable of forming covalent adducts with proteins and amino acids (Slight et al., 1990). Two molecules of dehydro-L-ascorbic acid react with one

molecule of amino acid to form CO_2, an aldehyde, and a chromophore (Tolbert and Ward, 1982). Third, the oxidation of ascorbic acid produces reactive oxygen species (e.g., hydroxyl radicals) capable of causing further chemical damage to proteins, lipids, and DNA (Chiou, 1984; Halliwell et al., 1987a; Uchida et al., 1989; Baysal et al., 1989). In fact, hydroxyl radical is the most reactive substance known and can damage virtually any biomolecule (Halliwell et al., 1987a). Finally, the oxidation of ascorbate prevents its functioning as an antioxidant against aqueous peroxyl and alkoxyl radicals, hydrogen peroxide, singlet oxygen, quinones, vitamin E oxidation, etc. (Frei et al., 1989). In light of these adverse effects of ascorbate oxidation, we initiated this investigation to examine the potential role of ascorbate oxidase in plant defense against *Helicoverpa zea*.

METHODS AND MATERIALS

Insects and Plants. Eggs of *H. zea* were obtained from insect-rearing facilities at the University of Arkansas. Larvae were maintained on artificial diet described by Chippendale (1970) unless otherwise noted.

Tomato (var. Roma VF), cotton (var. DPL 50), and soybean (var. Forrest) plants were grown in the greenhouse in 1-gal containers. Soybean plants were also grown in the field at 5 plants/row ft in Fayetteville, Arkansas. When plants reached the five- to six-node stage (all preflowering), a 1.0-g fresh weight (fwt) sample of leaf tissue was removed from leaves on the two uppermost nodes for ascorbate oxidase determination. Leaf tissue was also collected from preflowering crimson clover (*Trifolium incarnatum*) and vetch (*Vicia americana*) in May 1991 from feral populations in Fayetteville, Arkansas. Eight replicates were tested per species.

Assay of Ascorbate Oxidase. Leaf tissue (1.0 g fwt) was homogenized in 10 ml ice-cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 1.0% insoluble polyvinylpyrrolidone. Homogenates were filtered through cheesecloth and centrifuged at 10,000*g* for 20 min. Supernatants were used immediately as the enzyme source.

Ascorbate oxidase activity was measured by following the decrease in ascorbate absorbance at 265 nm using a SLM-AMINCO 3000 Array UV-Vis spectrophotometer (Urbana, Illinois). The reaction mixture contained 100 ml enzyme homogenate, 1.9 ml 0.1 M potassium phosphate buffer (pH 7.0) with 0.15 mM ascorbate, 0.5 mM EDTA, and 0.002% metaphosphoric acid (Esaka et al., 1989). An extinction coefficient of 14.7 mM was used. One unit of activity is defined as 1.0 mmol/min. Enzyme rates were determined using SLM-AMINCO rate analysis and enzyme kinetics software. The oxidation of ascorbate may also be due to the hydrogen peroxide-dependent enzyme, ascorbate peroxidase. The addition of 0.5 mM EDTA in the reaction buffer inhibits ascor-

bate oxidation by ascorbate peroxidase (Dalton et al., 1986). Preliminary assays were conducted with the addition of 100 units of purified catalase (Sigma Chemical Co.) to remove endogenous hydrogen peroxide in the leaf homogenates, but ascorbate oxidase activity was reduced by less than 10%. These data indicate that the majority of ascorbate oxidation occurring in the leaf extracts was due to ascorbate oxidase activity and not ascorbate peroxidase.

Determination of Ascorbate Oxidase Activity in Larval Midgut. To determine if ascorbate oxidase from ingested foliage is active in the larval digestive system, newly molted fifth instar *H. zea* larvae were placed individually on either cotton plants or tomato plants in the greenhouse. Plants were grown as described above and used at the five- to six-node stage. After 48 hr feeding, larvae were removed from the respective plants, half the larvae from each plant species were used for regurgitate collection and the other half for collection of midgut lumen contents. For each plant species, a total of six larvae were used for regurgitate collection and six larvae for lumen contents. Regurgitate was collected on parafilm, weighed, and added immediately to ice-cold 0.1 M potassium phosphate buffer, pH 8.0, at the equivalent of 100 mg/500 ml buffer. Midguts were removed from larvae and gut wall, and peritrophic membranes were removed from the lumen contents. Lumen contents were added to buffer as described above. The regurgitate and lumen samples were homogenized and centrifuged at 10,000*g* for 20 min. The supernatant was used immediately for measurement of ascorbate oxidase activity. The ascorbate oxidase activity of regurgitate and lumen contents from six larvae maintained on artificial diet was also determined as a control.

To measure the ascorbate oxidase activity of tomato or cotton foliage used in the feeding assays, 0.5-g samples of foliage were homogenized in buffer and centrifuged as described above. Six replicates from each plant species were analyzed.

Determination of Ascorbate and Dehydroascorbic Acid in Midgut Lumen and Plant Tissue. To determine if ascorbate and dehydroascorbic acid are present in the midgut lumen of larvae feeding on excised soy foliage, a spectrophotometric assay following the modifications of Law et al. (1983) by Cakmak and Marschner (1992) was used. Total ascorbate (ascorbic acid + dehydroascorbic acid) was measured after reduction of dehydroascorbic acid by 10 mM dithiothreitol. Ascorbate was estimated in the absence of dithiothreitol. Dehydroascorbic acid concentrations were estimated from the difference of total ascorbate and ascorbate. Briefly, leaf tissue or midgut lumen contents were extracted (1 : 10 w/v) with 5% metaphosphoric acid and centrifuged at 20,000*g* for 15 min. Reaction mixture for total ascorbate contained 0.2 ml tissue aliquot, 0.5 ml 150 mM sodium phosphate (pH 7.4) containing 5 mM EDTA, and 0.1 ml 10 mM dithiothreitol. After 10 min at room temperature, 0.1 ml 5 mM *N*-ethylmaleimide was added to remove dithiothreitol. Color was developed with 0.4 ml 10%

TCA, 0.4 ml 44% orthophosphoric acid, 0.4 ml 4% α'-dipyridyl in 70% ethanol, and 0.2 ml 3% $FeCl_3$. The reaction mixtures were incubated at 40°C for 40 min, and the absorbance was read at 525 nm. The assay for ascorbate omitted the dithiothreitol and *N*-ethylmaleimide using 0.2 ml water as a replacement.

Larvae and foliage were treated as follows: Twenty newly molted fifth instar larvae were transferred from artificial diet to soybean foliage for 48 hr. Fresh foliage was provided after the initial 24 hr feeding. After a total of 48 hr feeding on foliage, midguts were removed and lumen contents collected. Lumen contents were weighed to the nearest 0.1 mg and transferred to centrifuge tubes containing ice-cold 5% metaphosphoric acid. Groups of five midgut contents were pooled for a total of four replicates. Six replicate 1-g samples of excised foliar tissue from plants used for feeding were placed individually in tubes containing ice-cold 5% metaphosphoric acid. Lumen contents or soy foliar tissue were homogenized and then centrifuged as described above. Larvae feeding on artificial diet were also tested with the same number of replicates as used in the foliage assays. Six 1-g samples of artificial diet were also assayed for ascorbate concentrations. To estimate the ascorbate oxidase activity of the leaves and insect midgut lumen contents, a total of seven larvae were placed on excised foliage, and the ascorbate oxidase activity in the gut and leaf was determined as described above.

Preparation of Soy Foliar Protein and Bioassays. Six hundred grams of fresh weight leaf tissue was removed with a razor blade from V4–V6 stage greenhouse-grown soybean plants (var. Forrest). Tissue was homogenized in 2 liters of ice-cold 0.1 M potassium phosphate, pH 7.0, containing 0.5 mM EDTA, and 2.0% polyvinylpyrrolidone. The homogenate was filtered through cheesecloth and centrifuged for 30 min at 10,000*g*. The supernatant was removed and kept on ice while ammonium sulfate was slowly added to 80% saturation. Protein preparations were held on ice for 60 min following ammonium sulfate application and then centrifuged for 30 min at 10,000*g*. The pellets were resuspended in 0.1 M potassium phosphate, pH 8.0, at 1 g protein/100 ml buffer and divided into six equal suspensions. Ascorbate oxidase was quantified from the suspensions as described above. The six suspensions were gently stirred at room temperature for 2 hr. To simulate the possible effect of ascorbate oxidase on dietary protein in the larval digestive system, three of the suspensions received 1 mg/ml of ascorbate at time zero, while the other three were not treated with ascorbate. Following the 2-hr incubation, the suspensions were dialyzed at 10,000 mol wt cutoff for 48 hr against repeated exchanges of ddH_2O.

The samples were then frozen, freeze-dried, and incorporated into artificial diet. A 100-g preparation of artificial diet contained the following ingredients: 1.000 g soy foliar protein, 5.215 g cellulose, 0.685 g Vanderzant vitamins, 2.400 g agar, 0.200 g wheat germ oil, 3.370 g dextrose, 2.750 g wheat germ, 0.900 g Wesson salts, 0.425 g alginic acid, 0.365 g ascorbate, 0.180 g choles-

terol, 0.090 g choline chloride, 0.010 g streptomycin, 0.120 g aureomycin, 0.200 g methyl paraben, 0.090 g sorbic acid, and 82 ml distilled water.

Twenty neonate *H. zea* larvae were individually placed in 18.5-ml clear plastic cups containing diet with the appropriately treated protein. The experiment was replicated three times with a total of 60 larvae per treatment. Larvae were weighed to the nearest 0.1 mg after 13 days.

Chemical Characterization of Soy Foliar Protein. The protein content of the foliar isolates obtained above was determined using Coomassie brilliant blue G-250 reagent following Stoschek (1990) except that 1 mg/ml of soluble polyvinylpyrrolidone was added to the reagent to minimize phenolic–protein interferences.

Carbonyl formation was used as an indicator of oxidative damage in the protein isolates. Protein carbonyls are formed when active oxygen species are generated that oxidize amino acid residues at or near the cation binding site of proteins (Levine et al., 1990). Soy foliar proteins from treatments described above were suspended in 2 N HCl with and without 0.1% 2,4-dinitrophenylhydrazine (DNP) at 1 mg/ml. After 1 hr incubation at 25°C, the proteins were precipitated with 500 ml 20% trichloroacetic acid/ml. The pellet was washed and centrifuged at least four times with 1:1 ethanol–ethyl acetate to remove lipids and unreacted reagent. The final precipitate was dissolved in 6 M guanidine HCl. Carbonyl groups were estimated by measuring 370-nm absorbance of the DNP-treated proteins and subtracting the absorbance of the untreated sample (Levine et al., 1990). Data were presented as nanomoles of carbonyl groups per milligram protein using a millimolar extinction coefficient of 21.

To determine if ascorbate oxidation decreased the free amine content (primarily e-NH_2 of lysine) in the treated protein, the free amine content of the protein was determined using 2,4,6-trinitrobenzene sulfonic acid (TNBS) following Fields (1972).

Electrophoretic Analysis of Soy Foliar Protein. To determine if ascorbate oxidation resulted in protein fragmentation and/or polymerization, automated SDS polyacrylamide gel electrophoresis was performed on the protein isolates using the LKB PhastSystem and PhastImage Analyzer and Software (Pharmacia LKB Biotechnology, Piscataway, New Jersey). Samples were heated at 100°C in 2.5% SDS and 5.0% mercaptoethanol for 5 min and then ca. 25 mg protein aliquots were separated on 8–25% gradient PhastGel media (PhastSystem Separation Technique File No. 110). Protein bands were visualized with Coomassie blue and analyzed with PhastImage Analyzer and software (PhastSystem Development Technique File No. 200). The molecular weight and percent of total protein of each visible band were determined. Molecular weight markers were obtained from Sigma Chemical Co., St. Louis, Missouri. Three replicates for each treatment were performed.

Effect of Ascorbate Oxidation on Protein Digestibility. To determine if

ascorbate oxidation can impair the digestibility of protein, ascorbate and ascorbate oxidase or the oxidation product dehydro-L-ascorbic acid were incubated with azocasein. Two milliliters of a 1.0% (w/v) solution of azocasein in 0.2 M potassium phosphate, pH 8.0, were incubated with 0, 0.25, 0.5, or 1.0 mg/ml ascorbate with 3.0 units ascorbate oxidase/ml for 30 min. Azocasein was also treated with 0, 0.25, 0.50, or 1.0 mg/ml dehydroascorbic acid for 30 min.

The treated proteins were then incubated with 25 mg bovine trypsin/ml for 30 min. Protein digestion was terminated with 2 ml 10% trichloroacetic acid. Samples were centrifuged for 20 min at 10,000*g*, the supernatants were removed, and an equal volume of 0.1 N NaOH was added and mixed. The amount of digested protein was determined by measuring the absorbance at 440 nm due to the release of the chromophore *p*-aminobenzenesulphonamide (Broadway and Duffey, 1986). Five replicates were tested for each protein treatment.

Effect of Ascorbate Oxidase on Hydroxyl Radical Formation. To determine if the enzymatic oxidation of ascorbate produces hydroxyl radicals, the deoxyribose method for detecting hydroxyl radicals was used (Halliwell et al., 1987b). The method is sensitive and specific for the hydroxyl radical (Halliwell et al., 1987b). Hydrogen peroxide was omitted, and the solutions were incubated at 25°C for 60 min. All treatments were tested with 0.125 mg ascorbate/ml. Ascorbate oxidase treatments contained 2 units/ml of ascorbate oxidase activity. Mannitol was used as a hydroxyl radical scavenger in selected treatments at 10 mM. The treatments included ascorbate alone; ascorbate and mannitol; ascorbate, 50 mm $FeSO_4$, and 100 mm EDTA; ascorbate, 50 mm $FeSO_4$, 100 mm EDTA, and mannitol; ascorbate and ascorbate oxidase; and ascorbate, ascorbate oxidase, and mannitol.

RESULTS

Survey of Ascorbate Oxidase in Host Plants. Ascorbate oxidase was present in leaf tissue from all of the host plants of *H. zea* examined (Figure 1). The highest activity was noted with vetch, while the lowest occurred with cotton. However, the differences in activities may be partly attributable to the manner in which the plants were grown. Ascorbate oxidase activity was higher in the field-grown plants (soy, vetch, and clover) than in the greenhouse-grown plants (tomato, soy, and cotton).

Determination of Ascorbate Oxidase Activity in Larval Midgut. Ascorbate oxidase activity was not detectable in midgut lumen contents of larvae feeding on artificial diet. The regurgitate of larvae feeding on tomato or cotton possessed ca. 17% and 9%, respectively, of the activity of ingested leaf tissue (Figure 2). Larvae ingesting tomato foliage retained 3.9% of the ascorbate oxidase activity in the midgut lumen while larvae ingesting cotton foliage retained 2.8%. The

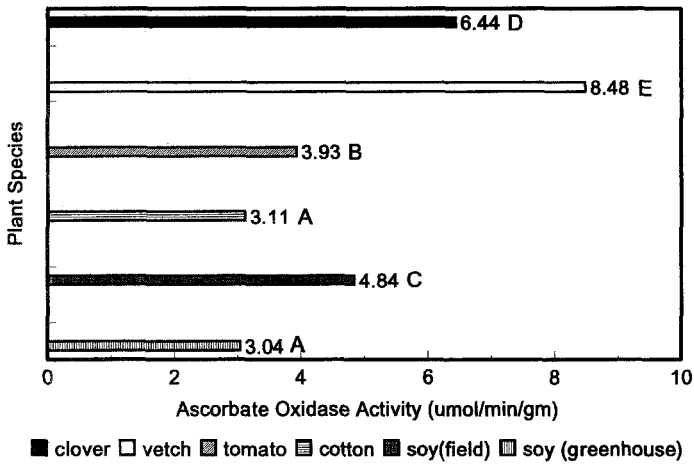

FIG. 1. Survey of ascorbate oxidase among host plants of *Helicoverpa zea*. Means not followed by the same letter are significantly different at $P < 0.05$ by comparison of 95% confidence limits.

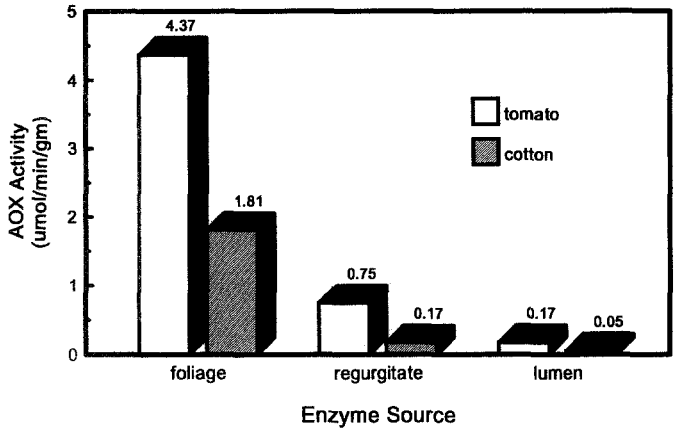

FIG. 2. Ascorbate oxidase activity in foliage, larval regurgitate, and midgut lumen.

specific activity of ascorbate oxidase from midgut lumen contents of larvae ingesting tomato foliage was 170 nmol/min/g compared to 50 nmol/min/g for cotton-fed larvae.

Determination of Ascorbate in Midgut Lumen and Plant Tissue. Ascorbate content in leaf tissue averaged 0.458 μmol/g leaf wet weight (wwt) (Table 1). This value is lower than previously published values for soybean foliage (e.g., Lee, 1991) but may reflect loss of ascorbate occurring during the 24-hr period

following excision of leaf tissue. The dehydroascorbic acid level in foliage averaged 0.285 μmol/g leaf. Ascorbate in the midgut lumen from foliage-fed larvae was not detectable, indicating substantial oxidation of ascorbic acid in the midgut. The activity of ascorbate oxidase in the midgut was 0.4 units/g lumen content and likely contributed to the observed loss in ascorbate. Other oxidative processes in the midgut lumen may also contribute to ascorbate oxidation (e.g., phenolic oxidation by polyphenol oxidases). Dehydroascorbic acid level in midgut lumen was 0.056 μmol/g content. In experiments with artificial diet, ascorbate levels in diet averaged 1.961 μmol/g, and dehydroascorbic acid was 0.070 μmol/g. Lumen contents from larvae feeding on artificial diet averaged 0.702 μmol/g ascorbate and 0.030 μmol/g of dehydroascorbic acid.

Effect of Ascorbate Oxidation on Protein Quality. Larval growth on diet containing foliar protein treated with ascorbate and active ascorbate oxidase activity was significantly ($P < 0.01$) reduced by more than 30% compared to larvae ingesting diet containing control untreated protein (Table 2). Treatment

TABLE 1. ASCORBATE OXIDASE AND ASCORBATE CONTENTS IN FOLIAGE AND LARVAL MIDGUT LUMEN OF LARVAE FED SOYBEAN FOLIAGE[a]

Source	ASC (μmol/g)	DHA (μmol/g)	AOX (units/g)
Artificial diet			
Diet	1.961a	0.070a	-0-
Midgut lumen	0.702b	0.030b	-0-
Soybean foliage			
Foliage (excised)	0.458	0.285a	3.07a
Midgut lumen	not detectable	0.056b	0.40b

[a] ASC, ascorbic acid; DHA, dehydroascorbic acid; AOX, ascorbate oxidase. Numbers in columns for each diet treatment not followed by the same letter are significantly different at $P < 0.05$ by Student's t test.

TABLE 2. EFFECT OF ASCORBATE OXIDATION ON NUTRITIONAL QUALITY OF SOY FOLIAR PROTEIN[a]

Treatment	Larval weight (mg)	Protein carbonyls[b]	Lysine[c]
Control	127.7a	94.0a	100.0a
With ascorbate	87.6b	107.5b	96.4b

[a] Numbers in columns not followed by the same letter are significantly different at $P < 0.05$ by Student's t test.
[b] Protein carbonyls expressed as nmol/mg protein following Levine et al. (1990).
[c] Percent relative lysine content determined following fields (1972).

of protein with ascorbate and ascorbate oxidase resulted in oxidative damage as indicated by an increase in protein carbonyl groups of 13.5 nmol/mg protein (Table 2). Also, the lysine content of the treated protein was significantly ($P < 0.05$) reduced by 3.6%. The ascorbate oxidase activity averaged 256 nmol/min/mg protein (95% confidence limit = ±32 nmol) in the six protein suspensions prepared above.

Electrophoretic analysis indicated that substantial modification of the treated protein occurred as shown by changes in protein molecular weights (Figure 3). Three proteins at 31, 135, and 151 kDa in the untreated sample were not detectable in the ascorbate-treated protein homogenate. Several novel protein bands occurred in the ascorbate treatment and included proteins of 56, 65, 169, and 217 kDa. The unique bands in the ascorbate and ascorbate oxidase treatment indicated that protein polymerization and/or fragmentation occurred. It is difficult to determine the relative importance of fragmentation vs. polymerization because of the multiple number of leaf proteins present in each treatment. The bands at 234 and 231 kDa in the control and ascorbate treatments, respectively, were not considered significantly different because the error rate for molecular weight determination with the protein standards averaged ca. 3–5% depending upon electrophoretic run.

Effect of Ascorbate Oxidation on Protein Digestibility. The treatment of azocasein with ascorbate and ascorbic acid oxidase significantly ($P < 0.05$) reduced the in vitro digestibility of azocasein by as much as 15% (Figure 4). At 0.5 and 1.0 mg/ml of ascorbate with ascorbate oxidase, a significant reduction in digestibility was observed. When dehydro-L-ascorbic acid was tested, a significant reduction was observed with the 1.0 mg/ml concentration of dehydro-L-ascorbic acid.

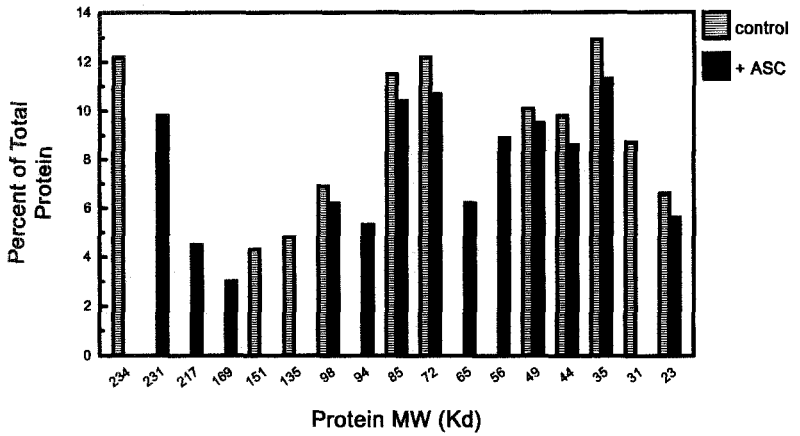

FIG. 3. Effect of ascorbate oxidation on molecular weights of soybean foliar protein.

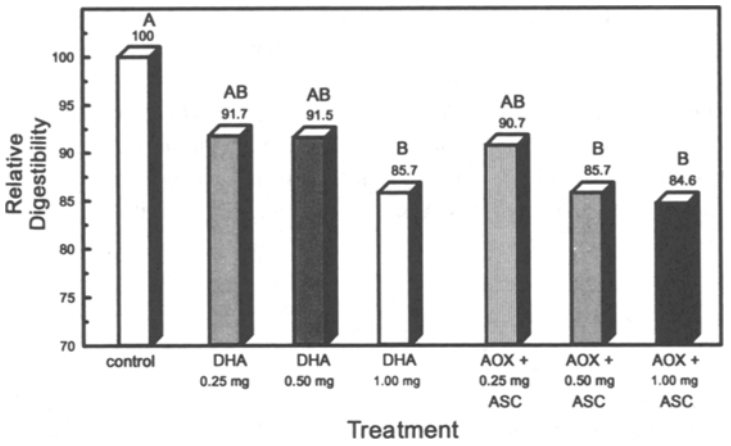

FIG. 4. Effect of ascorbate oxidase and dehydroascorbic acid on the in vitro digestibility of azocasein by bovine trypsin. AOX, ascorbate oxidase; DHA, dehydroascorbic acid; ASC, ascorbate. Means not followed by the same letter are significantly different at $P < 0.05$ by comparison of 95% confidence limits.

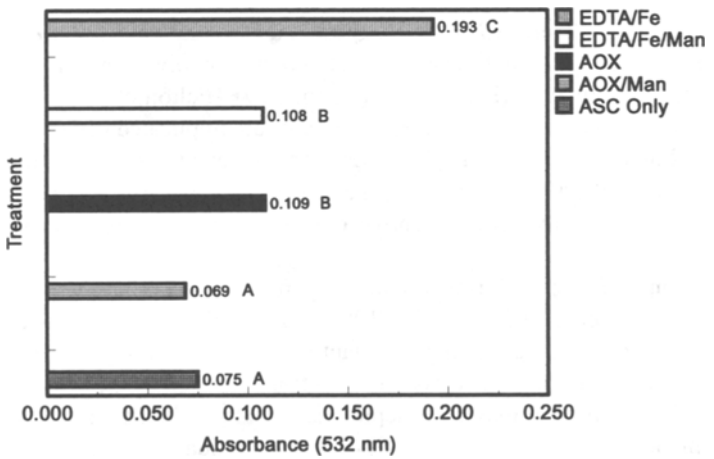

FIG. 5. Effect of ascorbate oxidation on hydroxyl radical formation. MAN, 10 mM mannitol; FE, 50 mm $FeSO_4$; EDTA, 100 mm EDTA; ASC, 0.125 mg/ml ascorbate; AOX, 2 units/ml ascorbate oxidase. Means not followed by the same letter are significantly different at $P < 0.05$ by comparison of 95% confidence limits.

Effect of Ascorbate Oxidase on Hydroxyl Radical Formation. Maximal hydroxyl radical formation occurred with ascorbate, ferrous sulfate, and EDTA (Figure 5). The addition of 10 mM mannitol, a hydroxyl radical scavenger, decreased hydroxyl radical formation in all treatments ($P < 0.05$). In the treatment with EDTA, ascorbate, and ferrous sulfate, mannitol decreased hydroxyl radical formation by 44%. Hydroxyl radical formation by ascorbate and ascorbate oxidase was indicated because the addition of mannitol reduced radical formation by nearly 37%.

DISCUSSION

Defense-related proteins in higher plants have received considerable attention as important mediators of microbe–plant interactions (e.g., Bowles, 1990; Ryan, 1990; Bol et al., 1990). Defense proteins are divided into several classes, including proteins that directly alter the properties of the plant extracellular matrix by strengthening the wall environment (e.g., hydroxyproline-rich glycoproteins), proteins with direct antimicrobial activity (e.g., proteinase inhibitors, chitinases), and finally, proteins whose appearances are correlated with a defense response but are of unknown function (i.e., pathogenesis-related proteins) (Bowles, 1990; Bol et al., 1990). Substantially less attention has been given to plant proteins as mediators of insect–plant associations.

Protease inhibitors and lectins have received the most attention as sources of resistance against insect pests (e.g., Shukle and Murdock, 1983; Ryan, 1990; Chrispeels and Raikhel, 1991). We suggested that oxidative plant enzymes may also represent an important class of plant proteins involved in defense against certain insect herbivores (Duffey and Felton, 1991; Felton et al., 1992). Polyphenol oxidases, peroxidases, and lipoxygenases are implicated in defense against insects (Shukle and Murdock, 1983; Hildebrand et al., 1988; Felton et al., 1989; Mohri et al., 1990; Duffey and Felton, 1991). We propose that ascorbate oxidase may be another component of oxidative defense against insect herbivores.

The antioxidant and prooxidant properties of ascorbate are becoming increasingly appreciated (Frei et al., 1989; Baysal et al., 1989). Our data indicate that ascorbate may act as a prooxidant in the alkaline midgut of *H. zea* due to the presence of ascorbate oxidase activity from ingested foliage. Furthermore, other oxidative stresses may also deplete ascorbate. When herbivores feed on certain plant species (e.g., tomato), polyphenol oxidase is very active in the digestive system and would cause further loss of ascorbate. The ability of the herbivore to maintain the redox properties of ascorbate will ultimately determine if ascorbate oxidation can reduce herbivore growth. We have previously identified ascorbate-free radical reductase and dehydro-L-ascorbic acid reductase in

the midgut of *H. zea* as components of an enzymatic system for maintaining ascorbate in a reduced state (Felton and Duffey, 1992; Summers and Felton, 1993). The peptide glutathione may also prevent autoxidation of ascorbate, but the efficiency of the redox couple between glutathione and ascorbate may be insufficient to maintain ascorbate levels in tissues (pH 7.4) exposed to oxidative stress (Winkler, 1987). The greater alkalinity of the herbivore midgut (pH 8.0–9.8) would further diminish the ability of glutathione to protect ascorbate. Protection from ascorbate oxidation may also be provided by metal-binding proteins, ascorbate-binding peptides/proteins, and high ionic strength (Fleming and Bensch, 1983a,b). We suggest that when herbivores feed on plant tissues with high ascorbate oxidase levels and/or polyphenol oxidase, they will be unable to maintain adequate amounts of reduced ascorbate. In these instances, the rate of ascorbate oxidation will greatly exceed the rate at which the insect can reduce the ascorbate free radical or dehydroascorbic acid. Consequently, the products of ascorbate oxidation would accumulate and be free to react with both dietary and endogenous proteins.

We have shown that ascorbate oxidase and ascorbate can reduce the nutritional quality of dietary protein. Protein quality may be reduced by one or more of the following mechanisms: (1) modification of lysine side chains, (2) modification of arginine side chains, and (3) hydroxyl radical damage to protein resulting in carbonyl formation and/or protein fragmentation and polymerization (Lin and Varner, 1991). Any of these processes can interfere with the utilization of dietary protein. The in vitro digestibility of protein treated with dehydro-L-ascorbic acid was significantly reduced (Figure 3), which may be explained by a partial blockage of the arginine/lysine side chains. These side chains are the cleavage sites for trypsin (Dunn, 1989), the principal digestive protease of *H. zea* (Lenz et al., 1991). Our data indicated that ascorbate oxidase activity resulted in hydroxyl radical formation in vitro, protein carbonyl formation, protein polymerization and fragmentation, and decreased protein lysine content.

In this investigation, we did not determine if enzymatic oxidation of ascorbate could contribute to dietary deficiency of ascorbate. The insect possesses dehydro-L-ascorbic acid reductase and ascorbate free radical reductase activities in the midgut wall (Felton and Duffey, 1992), but the enzymes are not sufficiently active in the midgut luminal fluid to maintain ascorbate in its reduced state when larvae feed on foliage containing appreciable ascorbate oxidase (unpublished data). The ascorbate free radical and dehydro-L-ascorbic acid are most likely to be reduced following uptake across the gut wall. Because of the very short half-life of dehydro-L-ascorbic acid in physiological conditions (Clemetson, 1989), it is possible that significant amounts of ascorbate in the digestive system are irreversibly lost to breakdown products such as diketogulonic acid. The pharmacokinetics and pharmacodynamics of ascorbate in these systems are in need of investigation.

In summary, we propose that ascorbate oxidase has the ability to reduce the bioavailability of ascorbate for nutritional and antioxidant functions. Ascorbate oxidase may cause indirect harm to the insect through deprivation of ascorbate and/or damage to dietary proteins. Direct damage to the insect may also result via formation of oxidative products (i.e., dehydro-L-ascorbic acid) and active oxygen species. Furthermore, ascorbate oxidase may complement the action of other oxidative plant defenses. Ascorbate can inhibit the action of lignin-forming enzymes (e.g., lignin-specific peroxidases) by eliminating hydrogen peroxide or reducing the enzymatic products of peroxidase (Dean and Eriksson, 1992; Takahama and Oniki, 1992). Thus, the rapid oxidation of ascorbic acid by ascorbate oxidase may facilitate lignin biosynthesis and phenolic oxidation implicated in defense against pest invasion (Dean and Eriksson, 1992; Felton et al., 1989). The enzymatic removal of ascorbate may also accelerate the hypersensitive reaction to pest attack in plants (Adam et al., 1989). The depletion of ascorbate may enhance the toxic action of polyphenol oxidases, peroxidases, and lipoxygenases because ascorbate will be unavailable to chemically reduce the enzymatic products of these reactions or act as a chain-breaking antioxidant (Felton and Duffey, 1992). Our investigations on ascorbate oxidation are in the early stages, but they suggest that ascorbate oxidation may be an important component of oxidative plant defense.

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SPRING MIGRATION OF DAMSON-HOP APHID, *Phorodon humuli* (Homoptera, Aphididae), AND SUMMER HOST PLANT-DERIVED SEMIOCHEMICALS RELEASED ON FEEDING

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Abstract—Behavioral studies using an olfactometer demonstrated that spring migrants of the damson-hop aphid, *Phorodon humuli*, respond to semiochemicals released by spring migrants feeding on hop leaves. Samples of the total volatiles released on feeding were analyzed by coupled gas chromatography–single cell recording techniques and showed the presence of several active components. Three compounds were identified, using coupled gas chromatography–mass spectrometry, as methyl salicylate, (*E*)-2-hexenal and β-caryophyllene. These three compounds elicited responses from separate olfactory receptors on the antenna. In the olfactometer, both (*E*)-2-hexenal and β-caryophyllene gave positive responses from spring migrants, and a mixture of the two compounds in the natural ratio was more attractive than (*E*)-2-hexenal alone. Addition of methyl salicylate eliminated the response to the active binary mixture.

Key Words—Damson-hop aphid, *Phorodon humuli*, Homoptera, Aphididae, methyl salicylate, (*E*)-2-hexenal, β-caryophyllene, semiochemicals, aggregation, attractant.

INTRODUCTION

Most aphid species aggregate in colonies of varying densities, although the mechanisms regulating colony cohesion are unknown. A general description of

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the phenomenon has been given after studies on *Aphis varians* (Turchin and Kareiva, 1989) and is expressed as "lower mobility in the vicinity of an aphid aggregate." Negative effects of heterospecific feeding were observed in experiments with cereal aphids (Chongrattanameetekul et al., 1991a,b). The advantage of aggregated feeding has been demonstrated for *Brevicoryne brassicae* (Way, 1973; Way and Cammel, 1970), but the active stimuli involved in keeping the colony together were not identified. Tactile stimuli have been implicated in the aggregation of the sycamore aphid, *Drepanosiphum platanoides* (Dixon and Logan, 1972; Kennedy and Crawley, 1967), and an aggregation pheromone has been demonstrated for *Aphis fabae* in studies using a catwalk olfactometer (Kay, 1976).

The current study was made on the damson-hop aphid, *Phorodon humuli* (Schrank), for which the sex pheromone has been identified and its ecological role investigated (Campbell et al., 1990). Evidence is presented for semiochemical regulation of spring migrant colonization on the secondary host, the perennial hop *Humulus lupulus* L. (Cannabaceae).

METHODS AND MATERIALS

Insects. Spring morphs of *P. humuli* were produced from eggs laid on damson, *Prunus insititia* (Rosaceae), and the nymphs were raised on damson leaves at 20°C under a 16:8 hr day–night regime. Adult spring migrants were maintained on hops, *H. lupulus* (hop progeny 64/84/15).

Bioassay. Behavioral assays were done in a Perspex olfactometer, as described by Pettersson (1970), having a weak airstream directed towards the center from each of four side arms. The odor stimulus was placed at the end of one of the arms. *P. humuli* spring migrants (10) were introduced into the center of the chamber, and their positions were noted at 3-min intervals for 30 min. Differences in humidity between airstreams were avoided by applying damp filter paper to all four arms of the olfactometer. Each experiment was replicated four to eight times, and results were analyzed by paired t tests; the numbers observed in the treatment arm were compared with the mean of the numbers in the three control arms. If aphids in the arena showed little activity, the experiment was terminated and the aphids changed. However, such problems were substantially reduced by following the diurnal rhythm and carrying out the experiments between 10:00 AM and 3:00 PM.

Isolation of Volatiles. Volatiles produced by hop leaves or by *P. humuli* spring migrants feeding on hops were collected by air entrainment, using Porapak Q as the adsorbent (Blight, 1990). The compounds were eluted from the Porapak with freshly distilled diethyl ether, and the sample was concentrated under a stream of nitrogen at room temperature and stored sealed in glass under

nitrogen at −20°C. (*E*)-2-Hexenal, β-caryophyllene, and methyl salicylate were obtained commercially (Aldrich) and diluted in hexane.

Gas Chromatography (GC). Air entrainment volatiles of *P. humuli* spring migrants on hops were separated on an AI 93 gas chromatograph equipped with a cold on-column injector, a flame ionization detector (FID), and a 50 m × 0.3 mm ID HP-1 bonded phase fused silica capillary column. The oven temperature was maintained at 40°C for 1 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. The carrier gas was hydrogen.

Gas Chromatography–Mass Spectrometry (GC-MS). A capillary GC column (50 m × 0.32 mm ID HP-1) was directly coupled to the MS and integrated data system (70-250 VG Analytical). Ionization was by electron impact at 70 eV, 230°C. The GC was maintained at 30°C for 5 min and then programmed at 5°/min to 180°C. Tentative identifications were confirmed by comparison with authentic samples and then by peak enhancement on GC.

Electrophysiology. Recordings from the olfactory cells associated with the primary rhinaria of *P. humuli* spring migrants were obtained using tungsten microelectrodes. The indifferent electrode was positioned in the first antennal segment, and the recording electrode was then brought into contact with the multiporous plate of the rhinarium until impulses were recorded. Permanent copies of the action potentials generated by the olfactory cells were obtained by standard methods (Wadhams et al., 1982).

Gas Chromatography–Single Cell Recording (GC-SCR). The GC-SCR system, in which the antennal preparation is directly coupled to the capillary column gas chromatograph, has been described previously (Wadhams, 1990). During each experiment, simultaneous records of the FID response and of the action potential frequencies were obtained by detecting with a level discriminator and plotting by means of a voltage/frequency convertor.

RESULTS AND DISCUSSION

Host plant location and selection by aphids is mediated by a complex of olfactory, visual, and gustatory stimuli (Pickett et al., 1992). Evidence is now accumulating that aggregation of migratory morphs on host plants is mediated by "pioneer" colonizers (Pettersson, 1993; Campbell et al., 1993). This paper presents the first identification of volatile semiochemicals implicated in this important aspect of aphid behavior.

Spring migrants of *P. humuli* responded positively in the olfactometer to volatiles from hop leaves and, in a comparative study, showed a preference for leaves bearing settled spring migrants (Table 1). An air entrainment sample of volatiles produced by spring migrants feeding on hop leaves was also attractive.

Biologically active components in the air entrainment sample of *P. humuli*

spring migrants were investigated using the coupled GC-SCR technique with recordings from the primary rhinaria, and a number of peaks were associated with activity (Figures 1 and 2). Three predominantly active compounds were subsequently identified by coupled GC-MS and GC as methyl salicylate, (*E*)-2-hexenal, and β-caryophyllene, which were present in the ratio 20:78:2 as determined by GC-FID from integrated peak areas. No correction was made

TABLE 1. RESPONSE OF *Phorodon humuli* SPRING MIGRANTS IN OLFACTOMETER TO HOP LEAVES AND FEEDING APHIDS: CUMULATIVE COUNTS OVER 30 MIN

Treatment	Control	Mean no. aphids		Significance[a]
		Treated arm	Each control arm	
Hop leaf	solvent blank	29.0	21.7	<0.02
Hop leaf + 5 spring migrants	hop leaf (no aphids)	33.5	17.6	<0.01
Entrainment sample of spring migrants on hop leaf (10 μl)	solvent blank	32.2	18.3	<0.01

[a] Student's *t* test.

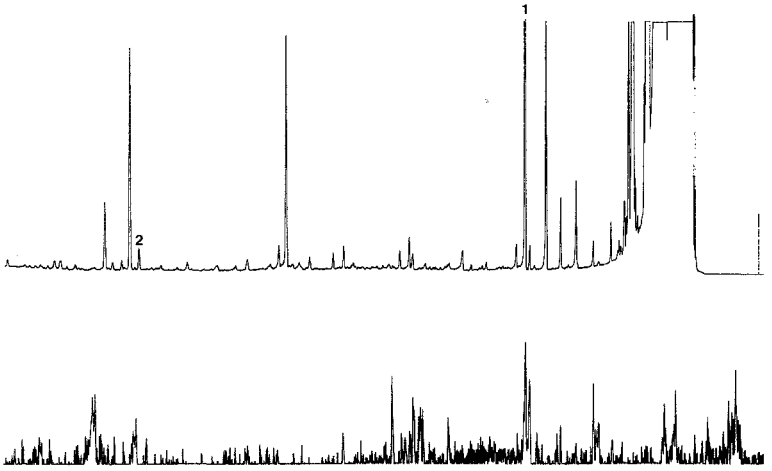

FIG. 1. GC-SCR of volatiles entrained from *Phorodon humuli* spring migrants feeding on hop leaves. Upper trace: FID response; lower trace: action potential summation from cells on the proximal primary rhinarium. (1) (*E*)-2-Hexenal, (2) β-caryophyllene.

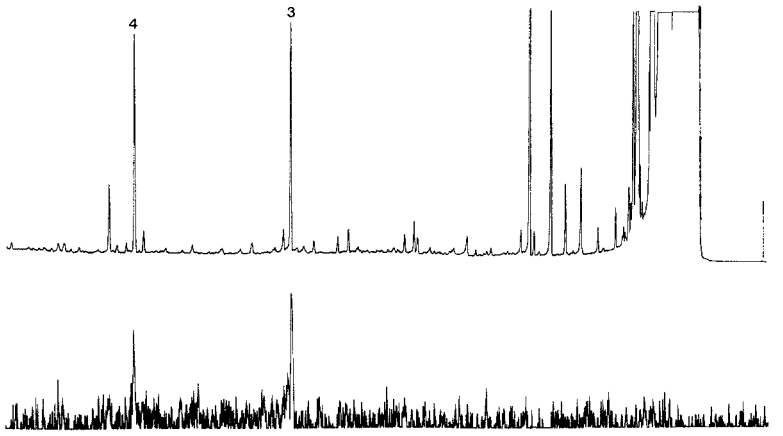

FIG. 2. GC-SCR of volatiles entrained from *Phorodon humuli* spring migrants feeding on hop leaves. Upper trace: FID response; lower trace: action potential summation from cells on the distal primary rhinarium. (3) Methyl salicylate, (4) (*E*)-β-farnesene.

for the differing FID response for each compound. However, the mixture, when made up in the ratio 20:78:2 by weight and checked by GC-FID, gave 17.5:79:3.5, which is well within experimental error for this study. These three compounds elicited responses from different olfactory cells on the antenna: the receptors for methyl salicylate are located on the distal primary rhinarium, while (*E*)-2-hexenal and β-caryophyllene are perceived by separate olfactory cells on the proximal primary rhinarium. (*E*)-β-Farnesene, the alarm pheromone for this aphid (Pickett and Griffiths, 1980), was also present in the sample and was active by GC-SCR (Figure 2).

In the olfactometer, both (*E*)-2-hexenal and β-caryophyllene elicited positive responses from spring migrants (Table 2) and, in a comparative bioassay, a mixture of hexenal and β-caryophyllene in the natural ratio was more attractive than hexenal alone (hexenal + β-caryophyllene = 25.5, hexenal alone = 13.5; $P < 0.04$). These compounds also showed activity in preliminary field trials and, again, a mixture of the two was more attractive than the individual components (C.A.M. Campbell, unpublished data). However, although the binary mixture of (*E*)-2-hexenal and β-caryophyllene in the natural proportions was behaviorally active, a 1:1 mixture was not active in olfactometer tests (Table 2). As the aphid has separate receptors for these compounds, it would be capable of discriminating between different ratios, and it appears that the correct ratio is essential for full behavioral activity.

In both olfactometer assays (Table 3) and field trials (C.A.M. Campbell, unpublished data), addition of methyl salicylate to the active binary mixture,

TABLE 2. RESPONSE OF *Phorodon humuli* SPRING MIGRANTS IN OLFACTOMETER TO (*E*)-2-HEXENAL AND B-CARYOPHYLLENE: CUMULATIVE COUNTS OVER 30 MIN (CONTROL = SOLVENT ALONE)

Treatment	Mean no. aphids		Significance[a]
	Treated arm	Each control arm	
Hexenal (1 μg)	32.2	15.9	<0.01
Caryophyllene (1 μg)	31.8	17.7	<0.007
Hexenal (0.97 μg) + caryophyllene (0.03 μg)[b]	42.8	14.4	<0.0005
Hexenal (0.5 μg) + caryophyllene (0.5 μg)	27.0	18.8	NS

[a] Student's *t* test. NS = not significantly different at $P = 0.05$.
[b] At the approximate natural ratio determined by GC-FID.

TABLE 3. RESPONSE OF *Phorodon humuli* SPRING MIGRANTS IN OLFACTOMETER TO (*E*)-2-HEXENAL, β-CAROPHYLLENE, AND METHYL SALICYLATE IN APPROXIMATE NATURAL RATIO (78:2:20) FROM GC-FID: CUMULATIVE COUNTS OVER 30 MIN (CONTROL = SOLVENT ALONE)

Treatment (total applied 1 μg)	Mean no. aphids		Significance[a]
	Treated arm	Each control arm	
Hexenal + caryophyllene	28.8	15.1	<0.04
Hexenal + caryophyllene + methyl salicylate	15.8	15.1	NS

[a] Student's *t* test. NS = not significantly different at $P = 0.05$.

although in the proportions present in the entrainment sample, eliminated the positive response. The role of this compound in host plant selection by *P. humuli* spring migrants is not yet understood. Air entrainment studies with spring migrants feeding on hop leaves showed an increase in the proportion of methyl salicylate produced as aphid numbers increased (Wadhams, unpublished data). While aggregated feeding can be advantageous (Way and Cammel, 1970), over-exploitation may have deleterious effects and different strategies have evolved to meet this problem (see Begon et al., 1986). In bark beetles, regulation of population density is mediated by semiochemicals (Borden, 1989). Many species utilize an aggregation pheromone, emitted by the pioneers, to draw other beetles of both sexes to the host material, causing a rapid build-up of population and a

consequent release of more pheromone. However, as colonization proceeds, an antiaggregation pheromone is frequently produced that terminates attraction, allowing new sites to be exploited. For *P. humuli*, methyl salicylate may function in a similar manner to space the aphid population or to shift the focus of colonization. Indeed, different behavioral roles for the individual active compounds could be rationalized by their being representative of three biosynthetically distinct phytochemical groups [(*E*)-2-hexenal, a fatty acid oxidation product; β-caryophyllene, an isoprenoid; methyl salicylate, a phenolic], which may convey information on different aspects of host plant physiology.

Since the air entrainment sample was active in the bioassay and yet the mixture of (*E*)-2-hexenal and β-carophyllene with the methyl salicylate was not attractive, it appears that there is a contribution from other active compounds. Additional electrophysiologically active peaks were detected but at levels that have so far precluded their identification. However, work is in progress to isolate and identify these compounds and to determine their role in the chemical ecology of the hop aphid.

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THE EFFECTS OF CHRONIC TANNIC ACID INTAKE ON PRAIRIE VOLE (*Microtus ochrogaster*) REPRODUCTION

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Abstract—The hypothesis was tested that the reproductive performance of voles would be reduced when fed diets containing tannins either because of increased metabolic rates, decreased intake, or decreased digestive efficiency. We fed a ration containing 4% tannic acid (TA) (dry mass basis) to 24 pair of prairie voles (*Microtus ochrogaster*) and compared reproductive performance (litter size, birth weights, body mass of the young until weaning, and mass changes in the dams) to that of 24 pair of prairie vole fed a control ration. We also compared the intake rates, digestive efficiency, and metabolic rates [as measured by Vo_2 consumption (cm^3/hr)] of dams and young fed both rations. Voles consuming 4% TA diets produced litters of similar size and mass as did voles fed the control ration. Furthermore, the mass of the young of dams fed the tannic acid ration were similar to the young of dams consuming the control ration through day 19 postpartum. However, dams consuming the tannic acid ration lost mass throughout lactation while the control dams maintained mass. Because the Vo_2 rates of both treated and control dams and litters were similar, we conclude increased metabolic costs were not the reason for the observed mass loss but, rather, reduced digestive efficiency, reduced intake of digestible dry mass, and apparent digestible nitrogen.

Key Words—Tannins, tannic acid, polyphenolics, plant defense, microtines, *Microtus ochrogaster*, reproduction, resting metabolic rate, digestibility reduction, energetics, growth rates.

INTRODUCTION

Previous studies have shown that ingestion of dietary tannins, a class of polyphenolic polymers, reduces microtine growth rates and digestive efficiency.

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Ingestion of diets containing 3–6% tannins reduced growth, intake, and digestive efficiency in subadult prairie voles (*Microtus ochrogaster*) (Lindroth and Batzli, 1984). Ingestion of digallic acid, a metabolite of tannic acid (a hydrolyzable tannin), increased resting metabolic rates (as measured by VO_2) in meadow voles (*Microtus pennsylvanicus*) (Thomas et al., 1988).

Both effects could reduce microtine reproductive performance. Production energy (that available for growth or reproduction) is the difference between energy assimilated (intake rate × digestive efficiency) and that used for maintenance (resting metabolic rate) (Maynard et al., 1979; Robbins, 1983). Tannins could reduce reproductive performance in voles by reducing intake and/or digestion of nutrients and energy and/or increasing resting metabolic rates due to detoxification expenditures.

To test that hypothesis, we fed one group of paired adult prairie voles a synthetic diet containing tannic acid and another group of paired adult voles a control diet. We predicted that ingestion of tannic acid by dams would reduce intake rates, reduce digestive efficiency, and/or increase metabolic maintenance costs resulting in reduced litter size, mass of young at birth, reduced growth rates of young until weaning, and reduced mass of dams during lactation.

METHODS AND MATERIALS

We established our breeding colony by obtaining 50 prairie voles from an outbred laboratory colony maintained at the University of Illinois. Founders of the colony were live-trapped in old field habitats in central Illinois, and wild voles were periodically introduced to maintain genetic variability (G. Batzli, personal communication). Vole pairs used in our experiments were selected to ensure similar age (16–24 weeks) and lack of relatedness (breeding records showed neither member of a pair shared a parent or grandparent).

Experimental Diets and Environment. Twenty-four pair of male and female prairie voles were randomly assigned to an experimental group fed the control diet while another 24 pair received the 4% tannic acid (Sigma Chemical, St. Louis, Missouri) diet (40 g tannic acid replaced 40 g corn starch/kg diet; Table 1). The diets were a modification of a nutritionally complete synthetic ration formulated in powdered form for prairie voles by Lindroth et al. (1984). While synthetic diets do not have the physical characteristics of natural forage, we chose protein concentrations that best matched those of prairie vole diets in old field habitats during the breeding season (Cole and Batzli, 1979).

Voles were housed in plastic shoebox cages containing wood shavings for bedding and cotton for nesting material. The experimental rations and water were provided ad libitum. Temperature was 23 ± 1°C and the lighting schedule was 15:9 hr light–dark. Males from each mated pair were removed within a

TABLE 1. COMPOSITION OF SYNTHETIC DIETS FED PRAIRIE VOLES THROUGHOUT REPRODUCTION EXPERIMENT

Component (g/kg diet)	Control diet	Tannic acid diet
Casein[a]	148.0	148.0
DL-Methionine[a]	2.0	2.0
Corn starch[a]	236.2	196.2
Sucrose[a]	200.0	200.0
Fiber[a]	300.0	300.0
Corn oil[a]	65.0	65.0
Vitamin mix[a,b]	15.0	15.0
Mineral mix[a,c]	15.0	15.0
$CaCO_3$[d]	8.4	8.4
$Ca(H_2PO_4)_2H_2O$[d]	10.4	10.4
Tannic acid[d]	0.0	40.0

[a]Ingredients from Teklad, Inc., Madison, Wisconsin.
[b]Vitamin mix catalog, No. 40060.
[c]Mineral mix catalog, No. 79055.
[d]Ingredients from Sigma Chemical, St. Louis, Missouri.

day of the birth of the litter. At birth, litters and dams were weighed, and reweighed every three to four days until the young were weaned on day 19.

Measurement of Resting Metabolism ($\dot{V}O_2$). We measured the $\dot{V}O_2$ of lactating dams and their litters for 48 hr one to five days postpartum using an open flow system respirometer. Each dam and litter was housed individually in airtight stainless-steel chambers (23 × 12 × 13.5 cm). A nest box (8 × 8 × 8 cm), containing cotton for bedding, was included in each chamber. Light was admitted to the cage via its glass lid. Urine and feces fell through a 6.5-mm screen floor. Food and water were provided ad libitum. Air, preconditioned to a dew point of 0°C, entered each cage through three holes in the glass lid and was drawn out through three holes in the sides. A switching manifold caused the air from each chamber to be exhausted through a ventilation pump for 24 of every 30 min. For the remaining 6 min, air from a chamber was drawn through a series of sensors. First, dewpoint was measured with a model 911 Digital Humidity Analyzer (EG&G; Waltham, Massachusetts), then mass flow with a Linde mode FM-4550 mass flowmeter (Union Carbide Corp., Somerset, New Jersey). Following desiccation by Drierite, the air was analyzed for O_2 content using a two-channel model S-3A oxygen analyzer (Applied Electrochemistry Inc., Sunnyvale, California) and for CO_2 content using a model 864 infrared analyzer (Beckman Instruments Inc., Fullerton, California). Analog output from all sensing devices was converted, using previously derived calibration relationships, to true values of dewpoint, mass flow, O_2 content, and CO_2 content,

which were then stored by an Apple IIe computer. Washout time from the switching manifold through the sensors was approximately 45 sec. Values from the first 2 min of each 6-min sampling period were discarded to prevent carryover from one chamber to the next. Using these values, V_{O_2} was calculated every hour. The three lowest V_{O_2} measurements for each dam and litter each day were used as a measure of resting metabolic rate (RMR). Daily metabolic rates (DMR) were estimated by summing hourly V_{O_2} measurements during the first 24 hr of the trials.

Measurement of Intake and Digestive Efficiency. Feeding trials were conducted for 72 hr 4–10 days postpartum (one day following the termination of the respirometry trials). We inserted 6.5-mm wire mesh into the plastic shoe box cages during fecal collections and intake measurements to facilitate collection of feces and uneaten food (orts). Food and water were provided ad libitum. Nest boxes were provided in all cages. Twice during the feeding trials, dams and litters were placed in metabolic cages (Maryland Plastics, Beltsville, Maryland) for 12 hr (collections encompassed a complete 24-hr period) to collect feces uncontaminated by urine for nitrogen analysis.

Following the feeding trials, feces and orts were dried at 50°C and sorted. The dry matter (DM) intake, dry matter digestibility [DMD; 100 × (g DM consumed − g fecal DM)/g DM consumed], and apparent nitrogen (N) digestibility [100 × (g N consumed − g fecal N)/g N consumed] of the two ration groups were compared. Nitrogen content of the samples was measured using the micro-Kjeldahl technique.

Statistics. We used a two-way ANOVA analysis to test whether litter size (3 or 4 young/litter) or diet (control or tannic acid) caused a difference in either litter mass or dam mass after parturition. We tested only the effects of diet on metabolic rate (V_{O_2}), intake rates, and digestive efficiency (Student's t test) (Ostle and Mensing, 1975) as equal numbers of litter sizes of three and four were used in each experimental group.

RESULTS

Seventeen of the 24 pair of voles fed the control ration produced litters (71%) with a mean litter size of 3.7 ± 0.3 (±1 SE). Similarly, 16 of the 24 pair (67%) fed the tannic acid ration produced litters with a mean litter size of 3.4 ± 0.2. We only included dams that produced litters with three or four young in the experiment as similar numbers of this litter size occurred in each experimental group (one tannic acid dam produced one young, one control dam produced two young, eight tannic acid dams and seven control dams produced three young, six tannic acid dams and five control dams produced four young, and three control dams and one tannic acid dam produced five young). While

total birth masses of litters containing three young were significantly less than litters containing four young (Figure 1), diet had no effect on the birth masses of litter sizes of three or litter sizes of four. In addition, there was no significant difference in total body mass of litter sizes of three or four on days 4, 7, 14, or 19 postpartum, nor was there a difference in litter mass due to the diet fed on those days.

The tannic acid diet did have a significant effect on the mass balance of dams during lactation (Figure 2). At parturition the mean body mass of control dams was similar to dams fed the tannic acid ration. Dams consuming the tannic acid ration lost mass throughout lactation (day 4, $F = 4.21$; $P = 0.06$; day 7, $F = 6.05$, $P = 0.028$; day 14, $F = 13.29$, $P = 0.003$; day 19, $F = 14.86$; $P = 0.002$). By the end of lactation, the dams fed tannic acid ration had lost $>10\%$ of their original mass, while the control dams lost $<3\%$. Litter size had no effect on dam mass balance when fed either diet.

The resting Vo_2 rates of the dams and litters were similar on day 1 and day 2 of the respirometry trials and were only slightly (although not significantly) elevated by the tannic acid diet (Table 2). Furthermore, the summed 24-hr Vo_2 rates of litters and dams were also similar (Table 2).

There was also no significant effect of diet on dry matter intake (DMI, $t = 0.13$, $P = 0.93$) (Table 3). However, dams consuming the tannic acid ration had reduced efficiency in digesting dry matter ($t = 5.01$, $P = 0.001$) and nitrogen ($t = 3.07$, $P = 0.013$). Multiplying DMI by the digestion coefficients for dry mass and nitrogen for each dam, significantly lower quantities of digestible dry matter [control ($N = 5$) 172 $\pm$ 7 mg/g body wt/day vs. tannic acid

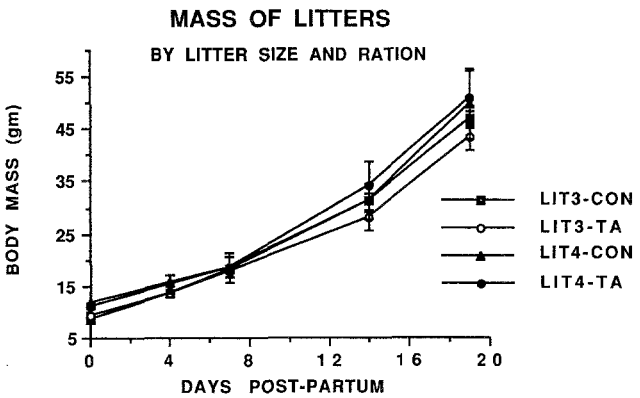

FIG. 1. Body mass of litters (three and four young per dam) from birth through weaning. The birth mass of litter size 3 was significantly less than litter size 4 ($P < 0.05$); however, mass was similar on days 4–19. The ration fed (4% tannic acid vs. control) had no effect ($P < 0.05$) on litter mass throughout lactation (days 1–19 postpartum).

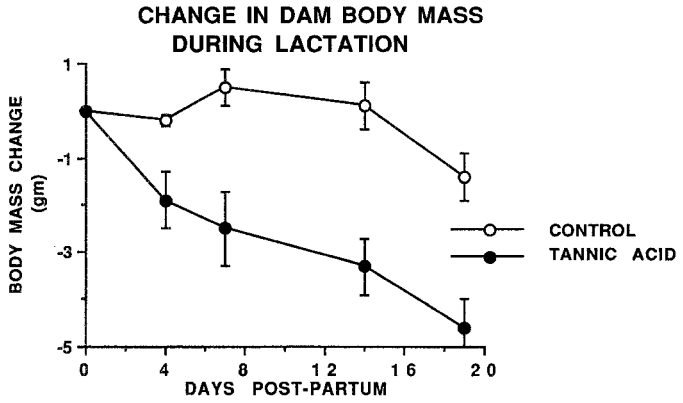

FIG. 2. Body mass changes in lactating dams fed tannic acid or control rations. While mass of dams was similar on the day of birth (day 0), dams consuming the tannic acid diet lost mass thereafter ($P = 0.06$ day 4; $P < 0.05$ day 7; $P < 0.01$ day 14–19).

TABLE 2. BODY MASS AND RESTING, AND DAILY OXYGEN CONSUMPTION RATES ($\bar{X} \pm$ SE) PRAIRIE VOLE DAMS AND LITTERS FED CONTROL AND TANNIC ACID RATIONS[a]

	Resting O_2 consumption rates				Average daily metabolic rate
	Day 1		Day 2		
Diet	VO_2 (cm^3/hr)	Litter and dam body mass (g)	VO_2 (cm^3/hr)	Litter and dam body mass (g)	Day 1 (cm^3/day)
Control ($N = 6$)[b]	82.6 ± 2.1	50.8 ± 1.4	82.6 ± 3.0	54.8 ± 1.9	2200 ± 30
Tannic acid ($n = 6$)[b]	85.0 ± 2.4	51.7 ± 1.3	85.0 ± 1.9	53.4 ± 1.5	2250 ± 40

[a] Measurements made one to five days postpartum.
[b] N = Number of dams and litters.

($N = 6$) 144 ± 9 mg/g body wt/day; $t = 2.40$, $P < 0.05$] and slightly, although not significantly lower quantities of digestible nitrogen [control ($N = 5$) 22 ± 2 mg/g body wt/day vs. tannic acid ($N = 6$) 20 ± 1 mg/g body wt/day; $t = 1.88$, $P < 0.10$) were consumed by the tannic acid dams.

TABLE 3. INTAKE RATES AND DIGESTIVE EFFICIENCIES ($\bar{X} \pm 1$ SE) of PRAIRIE VOLE DAMS FED CONTROL AND TANNIC ACID RATIONS 4–8 DAYS POSTPARTUM

Diet	Body mass dam (g)	Dry matter intake (mg/g body wt/day)	Dry matter digestion (%)	Apparent protein digestion (%)
Control ($N = 5$)	37.6 ± 0.6	239 ± 14	70.8 ± 0.8	90.1 ± 1.4
Tannic acid ($N = 6$)	36.9 ± 1.5	238 ± 19	62.4 ± 1.0[a]	85.6 ± 0.7[b]

[a] $P < 0.01$ Student's one-tailed t test.
[b] $P < 0.05$ Student's one-tailed t test.

DISCUSSION

We found that preweanling prairie vole growth rates were unaffected by 4% tannic acid when diets contained 15% protein. Lindroth and Batzli (1984) found weaned prairie vole young (20–42 days old) fed 3% and 6% tannic acid diets containing ≥ 12% protein grew at the same rate as young fed control diets; however, those consuming tannic acid diets containing 8% protein and 3% and 6% tannic acid grew significantly more slowly than those fed an 8% protein control diet. Toxicity due to gallic acid absorption was suspected. The concentration of protein in this study diet (15% CP) apparently protected preweanling prairie voles from any negative effects despite the fact that the tannic acid dose the lactating dams received was likely twice as great as nonlactating voles (lactating voles increase intake rates approximately two- to threefold to compensate for increased energy requirements) (Migula, 1969; Innes and Millar, 1981).

Dams consuming tannic acid diets did lose mass, while those consuming control rations did not. The resting metabolic rates (RMR, measured by Vo_2) of dams and litters fed both diets were similar, indicating increased metabolic energy demands due to detoxification of absorbed metabolites of dietary tannic acid did not occur during the measurements. However, RMR may not have been measured during the most critical period. The trials were run one to five days postpartum to maximize the respiratory contribution of the lactating dams (as the nursing pups grow they contribute an increasing amount of respiratory gases to the chamber and meet their nutritional needs primarily from milk). Intake rates of three other species of lactating voles (*Microtus arvalis*, *M. pennsylvanicus*, and *Clethrionomys gapperi*) (Migula, 1969; Innes and Millar, 1981) approximately doubled between day 5 and day 15 postpartum. Thus, the tannic acid dose received by lactating dams one to five days postpartum may have been half of that received during late lactation. This lower dose may have been insufficient to increase RMR one to five days postpartum; however, RMR may

have increased, along with the tannic acid dose, during late lactation. Nonreproductive adult meadow voles (*Microtus pennsylvanicus*) increased RMR ~20% when fed rations containing 5% digallic acid, a breakdown metabolite of tannic acid (Thomas et al., 1988). Since tannic acid is comprised of seven to nine digallic acid monomers (mol wt 332.2) esterified to a glucose molecule (mol wt 180.2), our diets contained ~3.7% digallic acid. It is also possible that the prairie vole absorbs less digallic acid than does the meadow vole, and/or less digallic acid is available for intestinal absorption if tannic acid is refractory to hydrolysis (i.e., bound to dietary proteins).

Reduced dry matter and nitrogen digestive efficiency and dry matter intake likely contributed to the mass loss observed in dams fed tannic acid diets. As digestible dry matter is essentially equal to digestible energy in rodents fed concentrate diets (Robbins, 1983), dams fed tannic acid diets consumed significantly less energy than control dams even though their requirements (as measured by V_{O_2}) were similar. Lindroth and Batzli (1984) found subadult prairie voles fed dietary tannic acid increased intake rates to compensate for reduced digestive efficiency. A compensatory increase did not occur in this experiment, indicating that the energy demands of lactation, given the type of diet fed, had already increased intake to the maximum rate of which the dams were physiologically capable.

Few studies have documented the effects of plant secondary metabolites on mammalian reproduction. We propose that the experimental design used in this study could be used to screen the effects of many secondary metabolites on mammalian reproduction.

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ARISTOLOCHIC ACIDS FROM *Thottea* SPP. (ARISTOLOCHIACEAE) AND THE OSMETERIAL SECRETIONS OF *Thottea*-FEEDING TROIDINE SWALLOWTAIL LARVAE (PAPILIONIDAE)

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Abstract—Two aristolochiaceous plants in the genus *Thottea* were shown to contain aristolochic acids. Larvae of two Malaysian troidine swallowtail butterflies, *Troides (Troides) amphrysus* and *Pachliopta (Losaria) neptunus*, that fed on *Thottea* leaves were found to sequester corresponding aristolochic acid analogs in the osmeterial glands.

Key Words—Aristolochic acid, Aristolochiaceae, *Thottea*, osmeteria, swallowtail butterfly, Papilionidae, *Troides*, *Pachliopta*, Lepidoptera, defense.

INTRODUCTION

A large number of swallowtail butterflies (Papilionidae) feed on the plant family Aristolochiaceae. Most of these species exhibit aposematic coloration and are presumed to serve as unpalatable models for a variety of insect mimics (Rothschild, 1973). Several troidine species have been shown to sequester toxic aristolochic acids (AAs) from their host plants, thus acquiring protection from predators (Euw et al., 1968; Urzúa et al., 1983; Urzúa and Priestap, 1985; Nishida and Fukami, 1989a). These species feed exclusively on the pipevine

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genus *Aristolochia*, which has been recognized as the only source of aristolochic acids in plants (Mix et al., 1983). Nishida and Fukami (1989a,b) have demonstrated that AAs act as specific host-finding cues for a Japanese pipevine swallowtail, *Parides (Atrophaneura) alcinous* Klug, both for oviposition and larval feeding.

Some of the troidine species in Southeast Asia, however, utilize a group of shrubby aristolochiaceous plants in the genus *Thottea* instead of or in addition to plants of the genus *Aristolochia*. It was therefore of interest to examine whether or not *Thottea* leaves contain AAs and, if so, whether the *Thottea*-feeding troidines utilize these compounds as allomones. We report here the occurrence of AAs in the leaves of two *Thottea* species in Malaysia and also demonstrate sequestration of the corresponding AA analogs in the defensive secretions from larval osmeterial glands of two *Thottea*-feeding troidine species, *Troides (Troides) amphrysus* (Cramer) (Figure 1) and *Pachliopta (Losaria) neptunus* (Guérin-Méneville) (in this paper, our use of subgeneric names follows the classification of Miller, 1987).

METHODS AND MATERIALS

Instruments. Mass spectra (MS) were measured with a Hitachi M-80 mass spectrometer at 70 eV. UV spectra were obtained with a Beckman DU-64 spectrophotometer in ethanol. The ^{1}H NMR spectrum was recorded with a JEOL

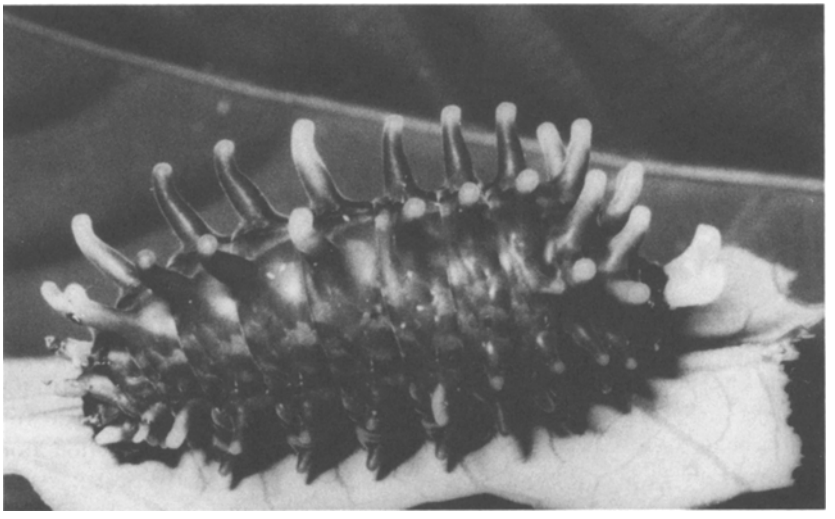

FIG. 1. A *Troides amphrysus* larva everting its osmeterium (right side) in response to mechanical disturbance.

JNM FX-90Q spectrometer (90 MHz) in d_6-dimethylsulfoxide using TMS as an internal standard.

Extraction and Isolation of AAs from Thottea *Leaves.* Fresh leaves of *Thottea* sp.-1 (see *Notes* section below) (approximately 10 g) were collected in the Sungai Menyala Forest Reserve near Port Dickson, Negri Sembilan, Malaysia (September 1987), and extracted successively with ethanol (15 ml) and acetone (30 ml × 3). The combined ethanol–acetone solution was evaporated in vacuo to yield an oily material (~0.5 g), which was dissolved in water (5 ml) and washed with 30% methyl ethyl ketone in ether (10 ml × 3). The upper layer was extracted with saturated sodium bicarbonate (10 ml × 2). The aqueous layer thus obtained was washed with ether (10 ml), acidified to pH 2 with hydrochloric acid, and extracted with 30% methyl ethyl ketone in ether (10 ml × 3). The acidic upper phase was washed with saturated sodium chloride and dried over anhydrous sodium sulfate. Removal of the solvent gave a brown solid mass of acidic components (2 mg). The yellow powder of an AA analog (approximately 0.3 mg) precipitated when the solid was dissolved in a small quantity of a mixture of benzene and ether and held at 5°C. From dried leaves of *Thottea* sp.-2 (see *Notes*) (2.15 g of dry weight, collected from Air Hitam Forest Reserve, Selangor, Malaysia, in July 1987), 9.3 mg of the acidic fraction was obtained in a similar manner to that described above. A yellow crystalline mass of an AA analog (approximately 0.5 mg) was obtained from the acidic fraction.

Voucher specimens of the two *Thottea* species (see *Notes*) have been deposited in the herbarium of the L.H. Bailey Hortorium, Cornell University (sterile specimens with photographs of flowers: Weintraub 1987-NGS-1 and Weintraub 1987-SEL-1) and the herbarium of University Pertanian Malaysia, Serdang, Selangor, Malaysia (flowers in FAA, specimen numbers as above).

Notes on the Identity of the two Malaysian Thottea *spp. Studied.* There appears to be some confusion regarding the identity of the *Thottea* spp. utilized as larval hosts by *Troides amphrysus* and *Pachliopta (Losaria) neptunus*. The two species involved are *T. tricornis* Maingay *ex* Hooker*f.* and *T. dependens* (Planch.) Klotzsch (see Hou, 1984). These can only be distinguished with certainty on the basis of floral morphology, although Hou (1981) has described leaf surface ultrastructure characters that may facilitate identification of sterile specimens. This usually makes field identification difficult, as *Thottea* species typically flower for a short period of time, and the majority of plants encountered are in sterile condition. In the absence of data on floral morphology and/or leaf surface ultrastructure, AA profiles seem to be useful for distinguishing these two closely related species. Previous collections of the *Thottea* (sp.-1) utilized by *Troides amphrysus* at the Sungai Menyala Forest Reserve have been identified as *Thottea tricornis* [e.g., KEP/FRI series #76188 as determined by KMK (= K.M. Kochummen)]. This determination is probably incorrect, as photographs of flowering *T.* sp.-1 taken at Sungai Menyala F.R. agree with the illustration

accompanying the original description of *T. dependens* (Planchon, 1847). *Thottea* sp.-2 from Air Hitam Forest Reserve has been previously identified as *T. dependens* by R. Kiew (Kiew, 1984) and voucher specimens from the Air Hitam population have been deposited in the herbarium of University Pertanian Malaysia [e.g., Kiew 1028 (UPM)]. This determination is also questionable, as photographs of *T.* sp.-2 taken at Air Hitam F.R. show that the flower shape of this taxon differs significantly from the illustration accompanying the original description of *T. dependens*. It is possible that *T. tricornis* Maingay *ex* Hooker*f.* will prove to be a junior taxonomic synonym of *T. dependens* (Planch.) Klotzsch, and *T.* sp.-2 an as yet undescribed species. Accurate identification of these larval hosts must await comparison of flowering material from both populations with type specimens and/or chemical analysis of type specimens preserved in the herbaria of the Royal Botanical Gardens, Kew, and The Natural History Museum [formerly British Museum (Natural History)].

Isolation of AAs from Larval Osmeterial Secretions. Using a glass capillary tube, fluid (approximately 0.6 μl) was collected from the extruded osmeterium of a fifth-instar larva of *Troides amphrysus* (Figure 1) that was found feeding on *T.* sp.-1 at the Sungai Menyala Forest Reserve on September 2, 1987. In the same manner, osmeterial fluid (approximately 0.3 μl) was collected from a fourth-instar larva of *Pachliopta neptunus* feeding on *T.* sp.-2 in the Air Hitam Forest Reserve in August/September 1987. The osmeterial secretions were dissolved separately in ethanol. Portions of the ethanolic solutions, after elution from a short reverse-phase column (ODS-W, microbead silica gel 5D, 100–200 mesh, Fuji-Davison Chemical Ltd.) in 70% ethanol, were chromatographed on a high-performance liquid chromatograph (HPLC) using Shiseido Capcell pak (S-5 mm, 250 mm × 4.6 mm ID), eluted with a mixture of methanol, water, and acetic acid (66:33:1) at 1 ml/min (detector: JASCO UVIDEC-100-II, at 254 nm).

RESULTS

The major crystalline acidic components contained in the leaves of *Thottea* sp.-1 and sp.-2 were identified as AA-I and AA-II, respectively, from their spectrometric data as shown below: AA-I (from *T.* sp.-1). MS: *m/z*(%) 341(15), 280(23), 267(14), 241(100), 237(15), 196(23), 193(16), 165(16), 150(39), 138(39), 87(20). UV(nm): λ_{max} 251, 316, 386. AA-II (from *T.* sp.-2). MS: *m/z*(%) 311(20), 265(100), 237(18), 209(20), 207(20), 163(44), 151(73), 75(21). UV(nm): λ_{max} 251, 299. ^{1}H NMR: δ 6.51(2H, singlet), 7.81(1H, singlet), 7.8–8.0(2H, multiplet), 8.28(1H, double doublet, J = 9 and 2 Hz), 8.59(1H, singlet), 9.09(1H, broad doublet, J = 9 Hz).

The osmeterial secretions of the two troidine species were analyzed by

means of HPLC monitored by UV detection (254 nm) (Figure 2). A substantial amount of AA-I (R_t = 11.5 min) was detected in the osmeterial secretion of the *Troides amphrysus* larva feeding on *T.* sp.-1 (Figure 2A). The chemical identity of AA-I was confirmed by its diagnostic UV absorption bands and mass spectrum, in which the molecular ion peak was found at *m/z* 341 (19%). The concentration of AA-I was estimated to be as high as 2%. The other UV-positive components revealed by HPLC (Figure 2A), however, remain to be identified. AA-II was isolated from the secretion of the *Pachliopta neptunus* larva feeding on *T.* sp.-2 (R_t = 8.6 min, Figure 2B). The UV and mass spectra (M^+, *m/z* 311, 16%) were identical to those of an authentic sample. The concentration of AA-II in the osmeterial fluid of *P. neptunus* was approximately 1.5%. Since *T.* sp.-1 and *T.* sp.-2 contain AA-I and AA-II, respectively, as the major acidic constituents, the chemical profiles of AAs in the osmeteria of *T. amphrysus* and *P. neptunus* appeared to reflect those found in their host-plant leaves.

DISCUSSION

The family Aristolochiaceae comprises seven genera, namely *Aristolochia (s.l.)*, *Paraistolochia*, *Holostylis*, *Euglypha*, *Thottea* (= *Apama*), *Asarum (s.l.)*, and *Saruma* (Hou, 1984). *Thottea* is known only from tropical Asia (Hou, 1981,

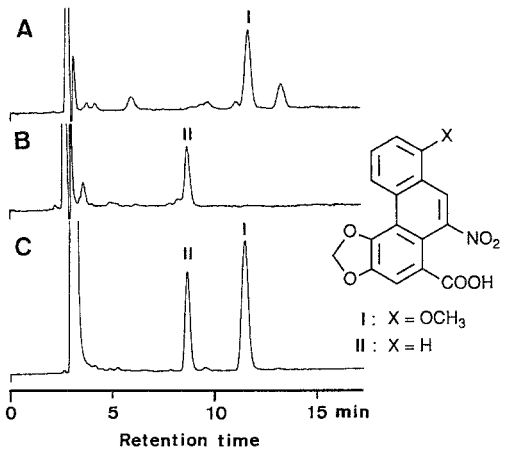

FIG. 2. Liquid chromatograms of aristolochic acids in the osmeterial secretions of *Troides amphrysus* (A) and *Pachliopta neptunus* (B), and an authentic mixture of aristolochic acid I and II (C), monitored by UV absorption at 254 nm.

1984). Several troidine species in the genera *Pachliopta*, *Troides*, and *Parides* utilize *Thottea* as their major larval hosts (Leefmans, 1934; Bachelor, 1959; Straatman and Nieuwenhuis, 1961; Straatman, 1968; Corbet and Pendlebury, 1978).

We have demonstrated here the presence of aristolochic acids in the leaves of *Thottea* species for the first time. A previous chemical analysis of *Thottea siliquosa* (Lamk) Ding Hou, a species used medicinally in peninsular India and Sri Lanka (Chopra et al., 1956; Gowda Balakrishna et al., 1988), demonstrated the presence of isoaristolochic acid in the roots [Manjunath and Shankara Rao, 1938 (as *Bragantia wallachii*, a synonym of *T. siliquosa*)]. In the present study, however, we were unable to detect AAs in a sample of *Thottea corymbosa* (Griff.) Ding Hou that was collected from Templer Park, Selangor, Malaysia (September 1987), even though this species has been documented as a host plant for some aposematic troidine species (Corbet and Pendlebury, 1978; J.D. Weintraub, unpublished data). The two *Thottea* species analyzed in the present study have previously been identified as *T. tricornis* (*T.* sp.-1) and *T. dependens* (*T.* sp.-2), but there appears to be some confusion regarding the identity and correct nomenclature of these taxa (see *Notes* above for additional comments). Both species are fairly widespread in lowland forests of the Malay Peninsula, and at least one of the two species ranges northward to the Thai peninsula (Hou, 1984).

Osmeterial secretions of both *Troides amphrysus* and *Pachliopta neptunus* larvae exhibit characteristic faint odors, similar to those of other troidine larvae but unlike the strong fatty acid odors of final-instar *Papilio* larvae (Eisner and Meinwald, 1965; Honda, 1980a, 1981). Larvae of an *Aristolochia*-feeding swallowtail butterfly, *Parides (Atrophaneura) alcinous*, produce volatile sesquiterpenes (Honda, 1980b). They also accumulate a series of AA analogs (aristolochic acids I, II, III, B, C, D, and E) in the osmeterial secretion, the composition of which is similar to that of the acidic fraction of its host plant, *Aristolochia debilis* Sieb. et Zucc. (Nishida and Fukami, 1989a). The compositions of AA analogs in the osmeterial secretions of the two *Thottea*-feeding troidines appear to be simple compared to that of *P. alcinous*, probably reflecting directly the AA compositions of the host-plant leaves. The total AA contents in all these three species were found in a range of 1.5–2.5% of the secretion.

Adults of several *Aristolochia*-feeding papilionid butterflies have been known to sequester AAs in the body tissues (Euw et al., 1968; Rothschild et al., 1972; Urzúa et al., 1983; Urzúa and Priestap, 1985; Nishida and Fukami, 1989a). *P. alcinous* also has been shown to sequester AAs in the eggs as well as in the tissues of all other stages (Nishida and Fukami, 1989a). Although the presence of AAs in the adult and egg stages of *T. amphrysus* and *P. neptunus* has not yet been examined, our results suggest that troidine swallowtails are able to feed on *Thottea* species without losing the ecological advantages of

aristolochic acid sequestration that were previously thought to be associated only with *Aristolochia*-feeding.

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ERRATUM

There was a typographical error in Table 4 of Ando, T. and Ohsawa, H. 1993. Sex pheromone candidates with a conjugated triene system: Synthesis and chemical characterization. *J. Chem. Ecol.* **19:** 119–132. Under B. Conjugated trienes (ppm), change:

Configuration factor[b]			to	Configuration factor[d]		
ZE	EZ	ZZ		ZEE	EZE	EEZ

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STRUCTURE–ACTIVITY RELATIONSHIP OF SYNTHETIC PHEROMONE COMPONENTS IN SEX COMMUNICATION OF CLICK BEETLES (Coleoptera, Elateridae)

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Abstract—It has been shown earlier that various geranyl and (*E*,*E*)-farnesyl esters are major components of natural sex pheromones of click beetles, *Agriotes*. In addition, some isomeric terpene esters have an inhibiting or synergistic influence upon the sex communication of some species of *Agriotes*. In this paper the influence of synthetic terpene esters in pheromone compositions on the sex communication of different *Agriotes* species occurring in different climatic zones has been studied. The relationship between the biological activity of sex attractants and their chemical structure has been established.

Key Words—Synthetic sex attractant, geranyl and farnesyl esters, *Agriotes*, Coleoptera, Elateridae, click beetles, structure–activity relationship.

INTRODUCTION

The synthetic compounds that are very similar in structure to sex pheromones often have a noticeable effect on the sex communication of insects. According to their biological activity, such compounds may be divided into sex attractants, synergists, and inhibitors. The sex pheromones of various species of *Agriotes*

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contain aliphatic terpene alcohol esters, mainly those of geraniol and farnesol (Borg-Karlson et al., 1988; Oleshchenko et al., 1976; Yatsy'nin et al., 1980).

The method of synthesizing terpene esters used by us (Lääts, 1957), as well as other known methods, affords isomeric by-products that are absent in natural pheromones. A study of their biological effect on the sex communication of the above pests is of great practical importance.

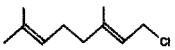
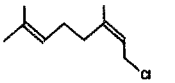
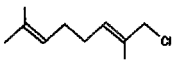
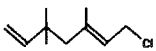
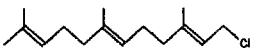
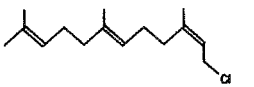
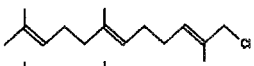
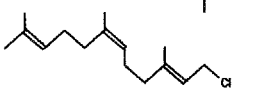
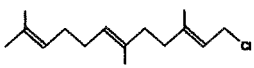
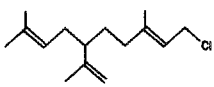
METHODS AND MATERIALS

The synthesis of pheromone components was performed via the terpenic and sesquiterpenic chlorides obtained by $SnCl_4$-catalyzed haloalkylation of 3-methyl-1,3-butadiene (isoprene) with its allylic monohydrochlorides (1-chloro-3-methyl-2-butene–3-chloro-3-methyl-1-butene, 9:1) (Lääts, 1957). The target compounds of the above reaction are primary allylic chlorides whose chemical structures and contents in the reaction product are presented in Table 1. For their separation from the mixtures with other isomers, a regiospecific reaction with dimethylphenylamine in the presence of methanol was used (Lääts, 1960a; Lääts et al., 1979). The water-soluble quaternary ammonium salts of primary allylic chlorides obtained after their separation from unreacted chlorides were thermally decomposed to the starting chlorides and dimethylphenylamine according to Scheme 1.

3-Methyl isomers (**1**, **2**, **4**, **5**, **6**, **8**, **9**, and **10**) were separated regioselectively from 2-methyl isomers (**3** and **7**) by their partial reaction with dimethylphenylamine without the presence of methanol. The content of quaternary ammonium salts of 2-methyl isomers (**3** and **7**) did not exceed 1% of the content of quaternary ammonium salts of 3-methyl isomers (Siirde et al., 1991). The quaternary ammonium salts obtained were subjected to subsequent partial thermal decomposition, allowing the separation of 3-methyl-2 (*Z*) isomers (**2** and **6**) and traces of 2-methyl isomers (**3** and **7**) due to the lower thermal stability of their quaternary ammonium salts. In the case of terpenic chlorides, the above decomposition was carried out at 60°C and with separation of the decomposition products by distillation at 3 mm Hg (Lääts et al., 1990a) and in case of sesquiterpenic chlorides at 55°C and with separation of the decomposition products by extraction with heptane (Lääts et al., 1990b). The primary allylic chlorides separated were transformed to esters via a stereospecific alkylation of Na salts of carboxylic acids by the corresponding quaternary ammonium salts with dimethylphenylamine according to Scheme 2 (Lääts, 1960b). The final purification of esters was performed by rectification on an efficient bristle-rotor distillation column (Mihkelson et al., 1980) at 1 mm Hg.

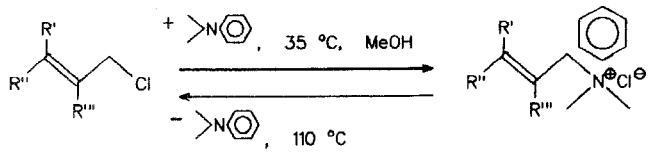
The terpenic and sesquiterpenic esters synthesized from the haloalkylation product of isoprene are presented in Table 2. The purity of some of the com-

TABLE 1. PRIMARY ALLYLIC CHLORIDES OF ISOPRENE MONOHYDROCHLORIDE ADDUCTS WITH ISOPRENE

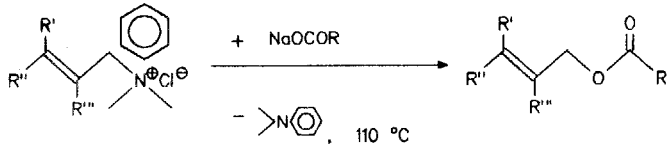
Compound No	Adduct	Content
	1 : 1 adducts[a]	60% of total product % of 1 : 1 adducts
1		54.1
2		3.5
3		5.2
4		1.2
	2 : 1 adducts[b]	18% of total product % of 2 : 1 adducts
5		35.0
6		3.1
7		1.9
8		3.1
9		3.7
10		8.8
	higher adducts	22% of total product

[a] A. Erm et al., 1981
[b] K. Siirde et al., 1987

pounds synthesized by the above scheme was not sufficient to establish their biological activity, and therefore known methods for the synthesis of esters from alcohols and corresponding acid chlorides were used. For example, neryl butanoate **(12)** was also synthesized through nerol obtained by reduction of synthetic



SCHEME 1.



SCHEME 2.

citral (Lääts and Teng, 1967). 2,6-Dimethyl-2(*E*),6(*E*)-octadienyl 1,8-di-(3-methylbutanoate) **(16)** was synthesized via (*E*)-8-hydroxygeraniol obtained from pure geranyl ethanoate by regioselective SeO_2 oxidation with subsequent reduction of the mixture of aldehyde and alcohol with $NaBH_4$ and saponification of hydroxygeranyl ethanoate into diol (Teng et al., 1984a). (*E*,*E*)-Farnesyl ethanoate **(20)** with 95% purity was synthesized from the isoprene haloalkylation product with geranyl chloride (1) (Siirde et al., 1986).

The synthons and final products were analyzed using GC and ^1H and ^{13}C NMR spectroscopy (Erm et al., 1981; Teng et al., 1984b).

Gas chromatographic conditions for monoterpenic chlorides analysis were: Chrom 5 gas chromatograph (Laboratorne Pristroje Chechoslovakia) with FID, 38-m glass capillary column (ID 0.2 mm) modified with $BaCO_3$ surface and coated with TCEP (1,2,3-tris-(2-cyano-ethoxy)-propane, column temperature 50°C, injection port temperature 150°C, carrier gas (Ar) flow rate 0.4 cm^3/min.

Conditions for ester analyses were: Chrom 5 gas chomatograph with FID, 23-m fused silica capillary column (ID 0.22 mm) coated with methylsilicone OV-101 fixed phase, column oven temperature 120–170°C, injection port temperature 220–270°C, carrier gas (He) flow rate 1.5 cm^3/min. The concentration of isomeric trace compounds was determined with an accuracy of $\pm 0.1\%$ using internal standards.

Field tests were used to establish the biological activity of the compounds separated, attracting the male insects and counting those caught in the traps. The compounds under study were carried to the rubber or porous carriers, which were placed in the traps specially designed for click beetles (Oleshchenko et al., 1987). The traps were placed directly on the ground, the distance between them being 20 m. In Table 3 results of establishing the influence of isomeric butanoates **(11, 12, 13)** on the attraction male *A. gurgistanus* are presented. The

TABLE 2. BIOLOGICAL ACTIVITY OF SYNTHETIC ATTRACTANTS COMPONENTS FOR MALE *Agriotes*

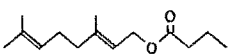
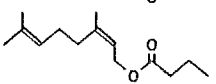
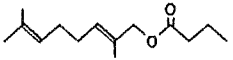
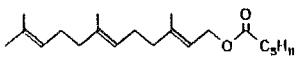
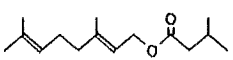
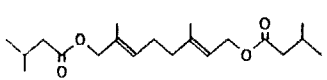
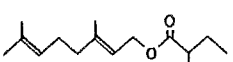
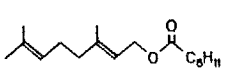
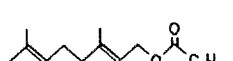
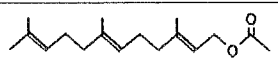
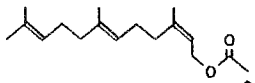
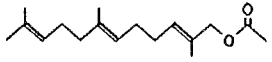
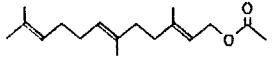
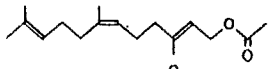
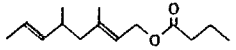
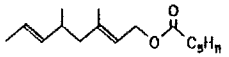
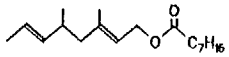
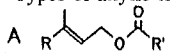
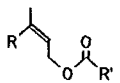
No.	Compound	Amount of allylic isomers[a]	Biological activity			
			Attractant	Inhibitor[b]	Synergist[b]	No Response
11		A–99.3 B–0.5 C–0.2	*A. gurgistanus* *A. sputator*			
12		B–97.0 A–2.6		*A. gurgistanus* >0.3	*A. sputator* ~5	
13		C–85.6 A–8.8 B–4.3		*A. gurgistanus* >1.0	*A. sputator* ~10	
14		A–84.3 B–4.6 C–3.9			*A. sputator* ~15	
15		A–98.5 B–0.8 (17)–0.2	*A. tauricus*	*A. gurgistanus*		*A. sputator*
16		A–88.8 B–3.4				
17		A–93.8 (15)–2.2		*A. gurgistanus* *A. tauricus* >1.0		*A. sputator*
18		A–95.8 B–0.8 C–2.3	*A. obscurus* *A. lineatus*[c]			*A. lineatus*[d]
19		A–94.5 B–0.8 C–2.0				

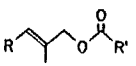
TABLE 2. CONTINUED

No.	Compound	Amount of allylic isomers[a]	Biological activity: Attractant	Inhibitor[b]	Synergist[b]	No Response
20		A–95.0 B–1.2 C–0.8	*A. ustulatus* *A. lineatus*[d]			
21		B–50.0 A–38.0 C–10.5		*A. ustulatus* >20	*A. ustulatus* <10	*A. lineatus*[d]
22		C–24.0 A–61.7 B–11.1		*A. ustulatus* >1.0		*A. lineatus*[d]
23		A–30.0 (20)–68.5		*A. lineatus*[d]		*A. ustulatus* <10
24		A–51.0 (20)–39.3 (21)–2.8 (22)–1.9				*A. ustulatus*
25		A–94.5	*A. gurgistanus* *A. sputator*			
26		A–93.2	*A. obscurus* *A. lineatus*			
27		A–93.0				

[a] Types of allylic isomers

A  (3-methyl-2(*E*)-)

B  (3-methyl-2(*Z*)-)

C  (2-methyl-2(*E*)-)

[b] Amount of compound, relative to attractant (%)

[c] Occurring in the northern region

[d] Occurring in the Western Ukraine

TABLE 3. ATTRACTION OF MALE *A. gurgistanus* TO DIFFERENT MIXTURES OF PHEROMONAL COMPONENTS AND RELATED COMPOUNDS

	Ratio (%) of compounds 11:13:12	Males caught[a] by one trap	Standard error $S_{\bar{x}1}$	Interval $t_{0.95}S_{\bar{x}1}$
1.1	99.3:0.5:0.2	47.8	4.3	10.5
1.2	2.3:0.0:97.0	5.1	3.3	8.1
1.3	8.8:85.6:4.3	3.3	1.5	3.7
2.1	99.3:0.5:0.2 + 2.3:0.0:97.0	6.0	3.9	9.5
2.2	99.3:0.5:0.2 + 8.8:85.6:4.3	7.0	4.7	11.5
3.1	99.0:0.7:0.3	44.1	5.5	13.5
3.2	98.4:0.8:0.8	40.4	5.2	12.7
3.3	98.5:1.1:0.4	49.5	4.1	10.0
3.4	97.1:1.1:1.0	21.8	4.4	10.8
3.5	98.0:1.3:0.7	15.4	3.9	9.5
3.6	97.2:1.2:1.6	7.1	4.2	10.2
3.7	97.1:1.6:1.3	6.9	4.7	11.5
3.8	96.5:1.8:1.7	8.1	3.6	8.8

[a] Mean value of six experiments.

amount of active compounds used in tests was 5 mg. Entries 1.1–1.3 (Table 3) describe the attractivity of pure compounds **(11, 12, 13),** entries 2.1–2.2 the simultaneous influence of two pure compounds in two carriers in the same trap. In entries 3.1–3.8, the attractivity of mixtures with different ratios of compounds **(11, 12, 13)** is given. In Table 4 the results of attraction of *A. sputator* with the same compounds **(11, 12, 13)** and their mixtures are presented. The results of experiments are summarized in Table 2.

RESULTS AND DISCUSSION

The inhibiting and synergistic effects can be observed in the sex communication of *A. sputator* and *A. gurgistanus* using synthetic isomeric by-products in the pheromone composition.

The main natural pheromone component of both the neighboring click beetle species is geranyl butanoate 3,7-dimethyl-2(*E*),6-octadienyl butanoate **(11)** (Table 2) (Oleshchenko et al., 1976). If the content of 3,7-dimethyl-2(*Z*),6-

TABLE 4. ATTRACTION OF MALE *A. sputator* TO DIFFERENT MIXTURES OF PHEROMONAL COMPONENTS AND RELATED COMPOUNDS

	Ratio of compounds 11:13:12	Males caught[a] by one trap	Standard error $S_{\bar{x}1}$	Interval $t_{0.95}S_{\bar{x}1}$
1	99.3:0.5:0.2	93.4	16.5	40.4
2	90.3:4.4:5.2	127.1	23.9	58.5
3	85.8:8.6:5.6	230.0	35.5	86.9
4	83.1:10.2:6.6	364.9	61.4	150.2
5	74.0:14.5:11.5	260.7	39.2	95.9
6	65.3:19.2:15.4	238.3	39.9	97.6
7	50.9:28.2:20.8	138.6	12.9	31.6
8	10.7:57.4:31.8	69.4	11.1	27.2
9	8.8:85.6:4.3	31.8	4.8	11.7
10	2.3:0.0:97.0	24.4	5.6	13.7

[a]Mean value of six experiments.

octadienyl butanoate **(12)** and/or 2,7-dimethyl-2(*E*),6-octadienyl butanoate **(13)** in geranyl butanoate **(11)** is higher than 1.3%, the sex communication of *A. gurgistanus* is suppressed. In the case of *A. sputator*, these synthetic by-products act as a synergist. If their content in geranyl butanoate **(11)** is about 5%, the trapping of male click beetles is four times as effective as that carried out with pure geranyl butanoate **(11)**. Geranyl 3-methylbutanoate **(15)**, the main sex pheromone component of *A. tauricus*, serves as a sex inhibitor for *A. gurgistanus*, but has no effect on *A. sputator*.

Geranyl hexanoate **(18)**, the main sex pheromone component of *A. obscurus* (Borg-Karlson et al., 1988), does not affect the sex communication of the *A. lineatus* occurring in the same area. The main sex pheromone component of this species is geranyl octanoate **(19)**, and in this case it was possible to elaborate a composition for trapping pests by the same trap.

For *A. ustulatus* and the *A. lineatus* occurring in the West Ukraine, the main sex pheromone component is (*E*,*E*)-farnesyl ethanoate **(20)** (Yatsy'nin et al., 1984). At a concentration lower than 10% in the pheromone composition, 2(*Z*),6(*E*)-farnesyl ethanoate **(21)**, a by-product of synthetic (*E*,*E*)-farnesyl ethanoate **(20)**, acts as a synergist, and if its content is higher than 20%, it acts as a sex inhibitor with respect to *A. ustulatus* but has no influence on *A. lineatus*. The sex communication of *A. lineatus*, on the contrary, is suppressed by the 6-methyl isomer, 3,6,11-trimethyl-2(*E*),6(*E*),10-dodecatrienyl ethanoate **(23)**, while that of *A. ustulatus* is not affected.

It must be pointed out that synthetic 3,5-dimethyl-2(*E*),6(*E*)-octadienyl

butanoate **(25)** and natural pheromone, geranyl butanoate **(11),** are similar in sex activity in the case of *A. gurgistanus*.

In conclusion, it can be said that various species of *Agriotes* react differently to the changes taking place in the structure of the pheromone component, especially in its acyl group or allylic fragment. The isomeric fragments located far from the ester group do not practically affect the sex attractivity in case of the pests under study. In the same area, the sex attractant of one species may act as a sex inhibitor with respect to the other. The area of *Agriotes gurgistanus* seems to be attacked by *A. sputator* from the north and by *A. tauricus* from the south, whose sex pheromones are more complicated than that *A. gurgistanus* (geranyl butanoate). We suppose that the latter species developed and inhabited the area earlier than the others.

As a result, synthetic sex attractant compositions have been elaborated for monitoring *Agriotes sputator*, *Agriotes gurgistanus*, *Agriotes tauricus*, *Agriotes obscurus*, *Agriotes lineatus*, and *Agriotes ustulatus* (Kudryavtsev et al., 1993). Methods of synthesizing and analyzing pheromone compounds also have been developed.

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DETERMINATION OF DISTRIBUTION OF HARMFUL CLICK BEETLE SPECIES (COLEOPTERA, ELATERIDAE) BY SYNTHETIC SEX PHEROMONES

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Abstract—By means of pheromone traps containing synthetic sex pheromones, areas of the most harmful click beetle species, *Agriotes obscurus*, *A. lineatus*, *A. sputator*, *A. gurgistanus*, *A. ustulatus*, *A. tauricus* Heyd, and *A. lineatus*, occurring in southern regions and differing biologically from the so-called northern species, have been specified and charted in the European and central Siberian areas of the former USSR.

Key Words—Click beetle, *Agriotes gurgistanus*, *A. sputator*, *A. obscurus*, *A. lineatus*, *A. tauricus* Heyd, *A. ustulatus*, Coleoptera, Elateridae, monitoring, sex pheromones, trap.

INTRODUCTION

It is known that harmful click beetle species are distributed according to climatic zones. In the former USSR, *Agriotes gurgistanus* and *A. tauricus* Heyd occur widely in the country's southern regions; *A. sputator*, inhabiting the central and northwestern climatic zones (Dolin, 1964), is also widespread.

The first report of geranyl butanoate as a sex attractant of *A. gurgistanus*

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(Oleshchenko et al., 1980) stimulated us to develop synthetic sex attractant compounds for the widespread click beetle species. On the basis of structure-activity relationships of attractant components established (Siirde et al., 1993) we suggested attractant mixtures for six *Agriotes* species. We also designed a baited pheromone trap, "Estron," for monitoring and trapping them (Oleshchenko et al., 1987). Since click beetle species and their distribution had been earlier determined only by low-efficiency soil sampling, monitoring by means of pheromone traps was of great interest.

METHODS AND MATERIALS

The following synthetic sex attractant compositions and their carriers were used as attractant baits: for *A. obscurus* and *A. lineatus*—a mixture of geranyl hexanoate (4 mg) and geranyl octanoate (1 mg) on a rubber disk (0.5 g) (Oleshchenko, et al., 1986a); for *A. sputator*—a mixture of geranyl (8.5 mg), neryl (0.4 mg), and (*E*)-2,7-dimethyl-2,6-octadienyl (0.8 mg) butanoates on a rubber disk (0.5 g) (Lääts et al., 1990a); for *A. gurgistanus*—geranyl butanoate (5 mg), purity 99%, on a rubber disk (0.5 g) (Oleshchenko et al., 1979a); for *A. tauricus* Heyd—a mixture of geranyl 3-methylbytanoate and (*E*,*E*)-dimethyl-2,6-octadien-1,8-di-(3-methylbutanoate) (4 and 1 mg, respectively) on expanded polyurethane particles (5 × 5 × 10 mm) (Oleshchenko et al., 1979b); for *A. ustulatus*—a mixture of (*E*,*E*)-farnesyl ethanoate with its (*E*,*E*)-6-methyl and (*Z*,*E*)-isomers (85, 11, and 2%, respectively; 10 mg in all) on expanded polyurethane particles (5 × 5 × 10 mm) (Lääts et al., 1990b); and for *A. lineatus* occurring in the western Ukraine—a mixture of (*E*,*E*)-farnesyl ethanoate and neryl 3-methylbutanoate (9.9 and 0.1 mg, respectively) on expanded polyurethane particles (5 × 5 × 10 mm) (Oleshchenko et al., 1979c).

The synthesis of above attractant components is discussed in our preceding papers (Siirde et al., 1991, 1993). The larval density in the soil was assessed by soil samples, consisting of digging control holes 25 × 25 × 30 cm (eight holes per 100 ha), screening the soil, and counting the larvae.

Depending on the click beetle species, the mating flight usually begins in the end of April or early May for *A. sputator* and in early to middle May in the case of *A. obscurus* and *A. lineatus*. For the southern beetle species, *A. gurgistanus*, *A. tauricus* Heyd, and *A. ustulatus*, the mating flight begins in the end of June and early July. The beginning of the mating flight was determined by means of control traps placed in the fields 10–15 days before this period. The Estron pheromone traps (Figure 1) were placed directly on the earth. After the first males had appeared in control traps, a calculated number of traps (one per 10 ha) was distributed uniformly in the fields to be monitored. Trapping was usually carried out for about 35–40 days.

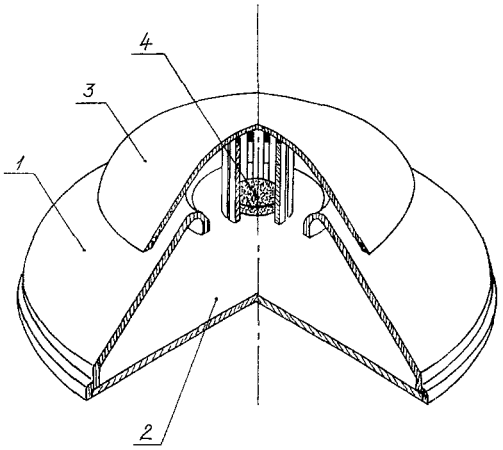

FIG. 1. Estron pheromone trap. 1, body; 2, bottom (160 mm diam.); 3, removable container for attractant bait; 4, attractant bait.

During 1983–1989, pheromone monitoring was carried out in 420 control stations, using 300,000 traps placed in corn fields over an area of 3 million ha. The results of monitoring are illustrated in Figure 2.

RESULTS AND DISCUSSION

The analysis of results of trapping and soil sampling obtained in different regions and for the different click beetle species with their different population densities showed straight statistical correlations between population density and the number of males trapped. These correlations are presented in the form of nomograms and equations and are recommended for the estimation of pest population densities by number of males caught by Estron traps baited with synthetic attractants (Oleshchenko et al., 1986b).

It should be pointed out that the data published on the distribution of *A. sputator* in northwestern regions of the former USSR (Oleshchenko et al., 1983) are incorrect. The northern border of distribution of this species runs along the parallel Brest–Moscow–Tyumen–Barnaul (Siberia). *A. obscurus* and *A. lineatus* prevail north of this line (see Figure 2).

Based on pheromone monitoring results, it has been established that two different species of *A. lineatus* inhabit the European part of the former USSR: one secreting the sex attractant geranyl octanoate and occurring in the northern region (Borg-Karlson et al., 1988), and the other, whose sex attractant is (*E*,*E*)-

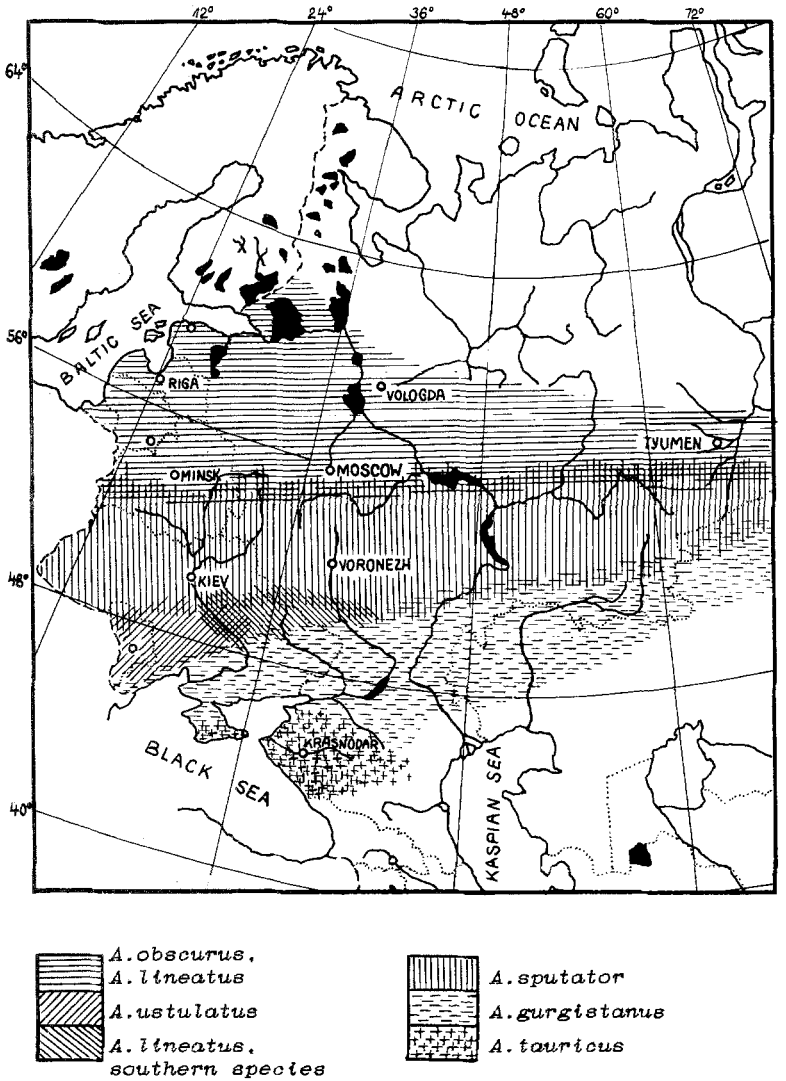

FIG. 2. Occurrence of click beetles *Agriotes* in the European part of the former USSR.

farnesyl ethanoate, occurring in the western Ukraine (Yatsy'nin and Lebedeva, 1984; Lääts, 1982). An extrapolation of the data enables us to predict the distribution of *A. obscurus* and *A. lineatus* in northern Europe, northern Poland, Germany, and France, *A. sputator* in Central Europe; and *A. ustulatus* may prevail in Romania and Bulgaria.

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CYCLIC HYDROXAMIC ACID ACCUMULATION IN CORN SEEDLINGS EXPOSED TO REDUCED WATER POTENTIALS BEFORE, DURING, AND AFTER GERMINATION

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Abstract—Cyclic hydroxamic acids are innate compounds associated with pest resistance in several grass species. The major cyclic hydroxamic acids of corn, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-2H-1,4-benzoxazin-3-one (DIBOA), were measured in seedlings after exposure to various water stress treatments. Both DIMBOA and DIBOA were found in greater quantities in plants experiencing a water deficit stress than in nonstressed plants. The increased cyclic hydroxamic acid coincided with a reduction in seedling growth, suggesting that cyclic hydroxamic acids are stress metabolites. Plants grown under conditions that restrict growth, such as water deficit stress, contain higher cyclic hydroxamic acids, which should make them more resistant to herbivorous pests and pathogenic microorganisms.

Key Words—*Zea mays*, corn, cyclic hydroxamic acids, DIMBOA, DIBOA, water stress, drought, osmotic priming, insect resistance, disease resistance.

INTRODUCTION

Cyclic hydroxamic (Hx) acids are secondary plant metabolites found in several species of the Gramineae, including such major agricultural crops as wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.). These compounds have natural

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biological activity against many insects, fungi, and bacteria (Niemeyer, 1988). DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one) is the major cyclic Hx acid of corn and wheat, while DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3-one), the demethoxy analog of DIMBOA, is the major cyclic Hx acid of rye, but a minor compound in corn and wheat. In addition to their toxic properties, the cyclic Hx acids have also been associated with detoxification of triazine herbicides (Hamilton, 1964), with iron acquisition by plant roots (Tipton and Buell, 1970), inhibition of plant growth regulators (Hasegawa et al., 1992), and may even have allelopathic properties (Pèrez and Ormeño-Nuñez, 1991).

Corn cultivars resistant to the first brood European corn borer (*Ostrinia nubilalis* Hübner) reflect high DIMBOA levels during the seedling stage when this insect is most damaging (Klun and Robinson, 1969). Cyclic Hx acids in corn tassels, although much reduced compared to seedling levels, were related to resistance to the corn leaf aphid (*Rhopalosiphum maidis* Fitch) (Beck et al., 1983). Correlations have also been reported between the level of DIMBOA and resistance of corn to numerous microorganisms, including *Erwinia chrysanthemi* (Corcuera et al., 1978), *Helminthosporium turcicum* (Couture et al., 1971), and *Fusarium moniliforme* (BeMiller and Pappelis, 1965).

The accumulation of cyclic Hx acids in a corn seedling is a dynamic process, with peak levels occurring a few days after germination and declining rapidly as the seedling matures (Klun and Robinson, 1969). The rapid accumulation and equally rapid disappearance of these compounds might limit their function as a biochemical control agent, especially against organisms that invade corn at later stages of development (Niemeyer, 1988). Equally important, the growing environment of the plant may influence the accumulation of these resistance compounds.

Information pertaining to environmental effects on cyclic Hx acid levels is limited to only a few studies. It has been demonstrated that high light intensity (Manuwoto and Scriber, 1985; Thompson et al., 1970), long photoperiod (Epstein et al., 1986), and elevated growth temperatures (Epstein et al., 1986; Thompson et al., 1970) can reduce the cyclic Hx acid content of seedlings. However, another major limiting factor of corn at the germination and seedling stage is adequate soil moisture. Soil moisture may also influence the synthesis or accumulation of cyclic Hx acids; yet, there have been no attempts to determine the affects of water stress on these compounds. The following experiments examine the influence of water stress at different times during germination and seedling development on the subsequent accumulation of cyclic Hx acids in the corn seedling.

METHODS AND MATERIALS

Plant Material. The cultivar Yellow Trucker's Favorite was utilized in all studies. Preliminary screening experiments indicated that this line contained moderately high levels of the two major cyclic Hx acids of corn (DIMBOA and DIBOA).

Standards of Cyclic Hx Acids. Synthetic DIMBOA [UV absorption, λ_{max} (MeOH) 263 with a shoulder at 282; 70 eV mass spectral analysis, *m/z* 211 (M+), 166 (28), 165 (100), 150 (65), 106 (36)] was obtained from Dr. J. Atkinson (Ottawa-Carleton Institute of Chemistry, University of Ottawa, Ottawa, Ontario Canada KIN 6N5). DIBOA [UV absorption, λ_{max} 254 with a shoulder at 282; 70 eV mass spectral analysis, *m/z* 181 (M+, 63), 152 (32) 136 (29), 135 (41), 108 (53), 79 (100)] was extracted from rye seedlings according to previously described procedures (Bailey and Larson, 1989). The aqueous DIBOA extract was further purified on a Ferric-SP-Sephadex column (Corbett and Chipko, 1978). The aqueous eluant was extracted with ethyl acetate, dried and concentrated, and the DIBOA crystallized from acetone with the addition of hexane (mp 155–157°C).

Cyclic Hx Acid Extraction and HPLC Analysis. Freshly harvested plant tissues were extracted following the procedures of Lyons et al. (1988). Dried extracts were dissolved in HPLC grade methanol (ca. 1 mg/ml) and analyzed on a Hewlett Packard model 1050 liquid chromatograph system using an ODS-Hypersil (C_{18}) reverse-phase column (Hewlett-Packard, 5μm, 200 × 4.6 mm) protected by a C_{18} guard column (Hewlett-Packard, 5 μm, 20 × 4.0 mm). A binary mobile phase consisting of 1% glacial acetic acid in H_2O (solvent A) and absolute methanol (solvent B) was passed through the column in a stepwise gradient at a flow rate of 1 ml/min (Lyons et al., 1988). The gradient system consisted of: step 1, 90% A to 50% A, 8-min linear gradient; step 2, 50% A for 5 min; step 3, 50% B to 100% B, linear gradient for 3 min. Solvents were then returned to the initial conditions and equilibrated for 2 min between injections. The column eluant was monitored at 280 nm with a variable wavelength detector. A peak area–response curve and retention time was established for each cyclic Hx acid standard. The Hx acids of the corn extracts were quantified using the external standard method.

Water Stress during Germination. Corn kernels were surface sterilized in full-strength Clorox (5.25% sodium hypochlorite) for 15 min, rinsed three times with sterile distilled water, and dried (room temperature for 1 hr). The kernels were germinated on solutions of polyethylene glycol (PEG, mol wt = 20,000; J.T. Baker, Inc., Phillipsburg, New Jersey) corresponding to water potentials ranging from −0.01 MPa to −1.07 MPa, as determined by thermocouple psychrometry (Brown and Oosterhuis, 1992). Petri dishes (9.0 cm) were lined with a single Whatman No. 4 filter paper and 20 ml of an appropriate PEG solution was added to each dish. Ten corn kernels were randomly spaced in each dish and treatments were replicated six times. Dishes were maintained in complete darkness at 25°C.

Germinated seedlings of three replicates were harvested after five days and separated into shoot, radicle, and seminal roots. Similar tissues within each Petri dish were combined and rinsed with distilled water, fresh weighed, and immediately extracted for cyclic Hx acids. Seedlings from three replicates of each

treatment also were harvested, rinsed with distilled water, separated into various organs, and fresh weights recorded. Those samples were then dried in a forced-air oven at 80°C for 48 hr and the dry weight was determined. The average percent moisture in the dried samples was used to estimate the dry weight yields of seedlings harvested for chemical analysis.

Water Stress after Germination. Ten seed each were germinated in 15-cm-diam. pots containing a well watered potting soil (Fafard Mix 3-B, Fafard, Inc., Springfield, Massachusetts). Pots were placed in a growth chamber under an 18-hr light (250 $\mu E/m^2$), 6-hr dark photoperiod and an average day/night temperature of 32 $\pm$ 1°C and 21 $\pm$ 2°C, respectively. Upon emergence of the coleoptile (successful germination), water was withheld from half of the pots, while the other half continued to receive water daily. Three replicate pots were harvested daily for five days from both the well-watered and dry-down treatments. The harvested seedlings were fresh weighed and extracted for cyclic Hx acids as described. Two seedlings were subsampled from each pot for dry weight determination and fresh weights obtained from harvested seedlings were adjusted to dry weight based on the subsample. Plant water potential was also determined from the subsample using thermocouple psychrometry techniques (Brown and Oosterhuis, 1992). A 1.0-cm section was excised from the base of the coleoptile and immediately sealed in a thermocouple psychrometer chamber (Merrill 84-2VC, J.D. Merrill, Logan, Utah). The sample was equilibrated in a water bath for 4 hr at 25°C, and the total water potential determined using the dew point method (Brown and Oosterhuis, 1992). Each psychrometer was calibrated against KCl solutions of defined osmolality.

After five days of water stress, soil water potential (determined by thermocouple psychrometry) of the stressed pots averaged -1.92 ± 0.13 MPa. The remaining stress pots were then rewatered to near field capacity, allowing the seedlings to rehydrate for three days. Previously stressed plants and controls were then harvested and analyzed for water potential and cyclic Hx acids.

Osmotic Priming. A Whatman No. 4 filter paper was placed in a 9.0-cm Petri dish and saturated with 10 ml of a 325 g/liter or 400 g/liter polyethylene glycol solution (PEG 8000, average mol wt 7000–9000, J.T. Baker Chem. Co., Phillipsburg, New Jersey). The water potentials of the solutions were -1.09 MPa (325 g) and -1.57 MPa (400 g), as determined by thermocouple psychrometry. Fifty corn seed were randomly spaced in each Petri dish and placed in darkness at 20°C for six days.

After six days, the kernels were removed from the solutions, washed with water, and air dried on the lab bench for 12 hr. Ten kernels were planted in potting mix in each of four replicate 20-cm-diam. pots per treatment. Also included were pots containing control seed that had been maintained in normal storage. Seedlings were harvested at six and 14 days after emergence and analyzed for cyclic Hx acids.

Statistical Treatment. Treatment replications are described in the above sections and all experiments were conducted at least two times. Data were analyzed by analysis of variance techniques of a completely randomized design and a least significant difference was calculated to compare means within each experiment.

RESULTS

Water Stress during Germination. The highest concentrations of cyclic Hx acids in 5-day-old germinating seedlings occurred in the shoot, followed by the radicle, and lowest concentrations in the seminal roots (Figure 1). The concentration of DIBOA in seminal roots was below the detectable limits during most sampling days and is therefore not reported. The total cyclic Hx acid content

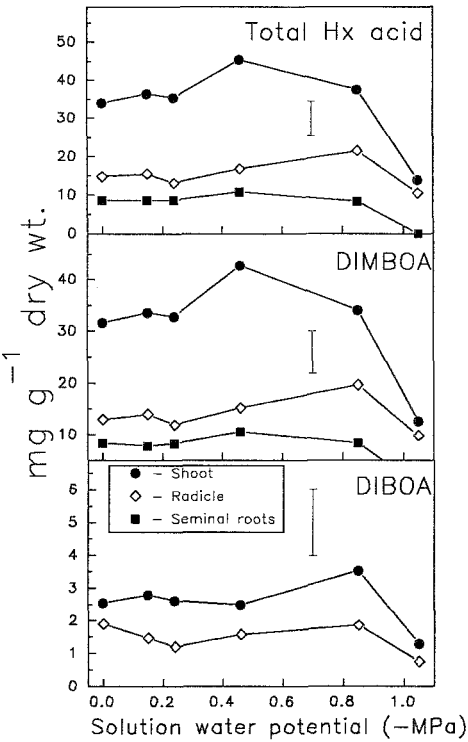

FIG. 1. DIMBOA, DIBOA, and total cyclic hydroxamic acid (Hx) content of corn seedlings germinated for five days on solutions of PEG 20,000 corresponding to water potentials ranging from −0.01 MPa to −1.10 MPa. Vertical bar represents the LSD ($P = 0.05$) for comparing means.

(DIMBOA plus DIBOA) in the germinating seedling was influenced by water stress, with the greatest cyclic Hx acid content observed in the shoot and radicle of seedlings germinated at a water potential of −0.47 MPa (Figure 1). DIMBOA accumulation in shoots was also greatest when seed were germinated at −0.47 MPa. Cyclic Hx acid accumulation in both the shoot and radicle was significantly lower than controls when seed were germinated on a water potential of −1.07 MPa, as the concentration of total Hx dropped to about 40% of the controls. The cyclic Hx acid content in the seminal roots was low, and there were no significant differences over the range of water potential tested. However, there was a general trend for DIMBOA to increase at intermediate levels of water stress and decrease at severe levels of stress (Figure 1).

Seedling biomass was significantly reduced by increased water stress, especially shoot dry weight (Figure 2). Dry weight of the shoot was reduced approximately 25% at the highest concentration of PEG compared to the controls. Radicle dry weight production was less sensitive to water stress at intermediate water potentials, but it was reduced as the water potential fell below −0.85 MPa. There was a slight, but insignificant, increase in seminal root production at intermediate levels of water stress, but seminal roots were severely inhibited at −0.85 MPa and were completely absent at −1.07 MPa.

Water Stress after Germination. The effects of water stress were apparent

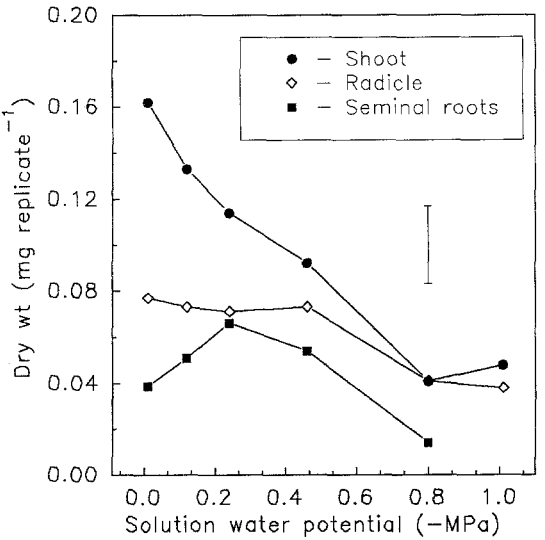

FIG. 2. Dry weight of corn seedlings germinated for 5 days on solutions of PEG 20,000 corresponding to water potentials ranging from −0.01 MPa to −1.10 MPa. Vertical bar represents the LSD (P = 0.05) for comparing means.

within one day after water was withheld. Significant differences in total plant water potential were observed between nonstressed and stressed seedlings at day 1 of water stress, while differences in seedling dry weight were not statistically significant until day 3 (Figure 3). After four days of water stress, plant water potential of stressed seedlings had reached −2.80 MPa and plants were severely wilted. However, rehydration of stressed plants for three days led to similar values of water potential between nonstressed and stressed seedlings. Although dry weight production of the stressed seedlings lagged behind those of the controls, growth recovered quickly after water stress was relieved and previously stressed seedlings appeared healthy.

The DIMBOA and total cyclic Hx acid content of stressed seedlings were significantly higher than nonstressed seedlings after one day of water stress (Figure 4). Differences in cyclic Hx acids between stressed and nonstressed plants became more distinct as the stress continued, primarily reflecting a sharp decrease in DIMBOA in the nonstressed seedlings. Although DIBOA levels also decreased rapidly in the nonstressed seedlings, their disappearance from stressed seedlings occurred at a comparable rate to nonstressed seedlings and there was no statistical difference between water treatments.

The total cyclic Hx acid content of stressed seedlings was between two and three times greater than the nonstressed seedlings after four days of stress and

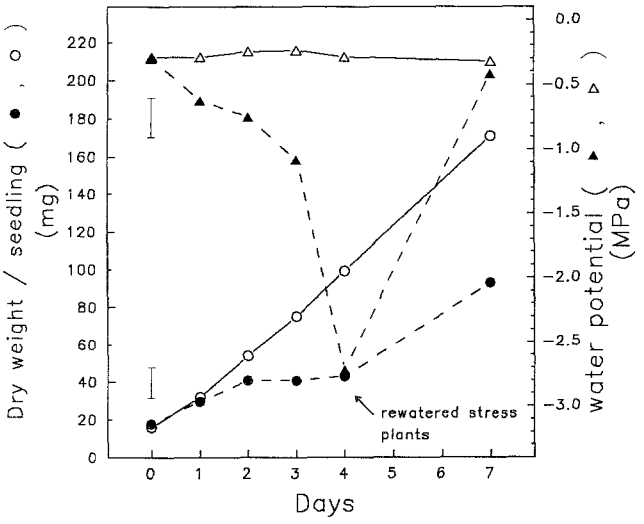

FIG. 3. Dry weight and total water potential of corn seedlings over a period of seven days. Closed symbols represent plants from which water was withheld to induce a water deficit stress. Previously stressed seedlings were watered to near field capacity on day 4 and allowed to rehydrate for three days. Vertical bar represents the LSD ($P = 0.05$) for comparing means.

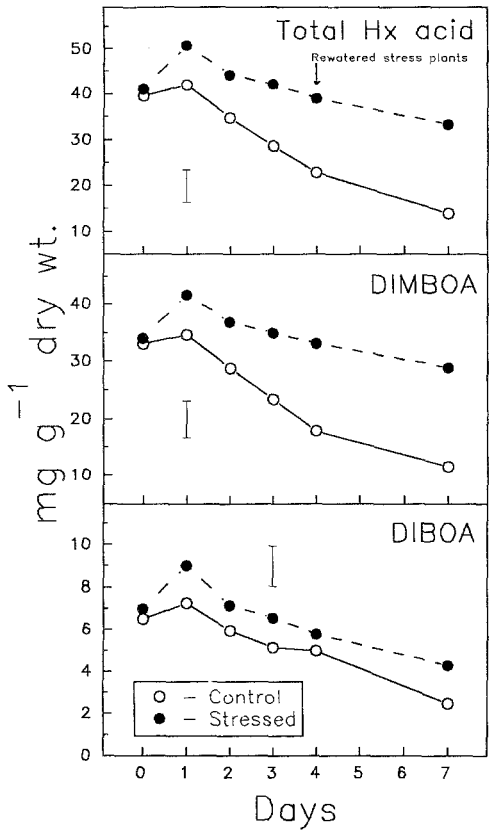

FIG. 4. DIMBOA, DIBOA, and total cyclic hydroxamic acid (Hx) content of corn seedlings under nonstressed conditions (○) or stressed conditions (●) beginning on day 0 and continuing for four days. Previously stressed seedlings were watered to near field capacity on day 4 and allowed to rehydrate for three days. Vertical bar represents the LSD (P = 0.05) for comparing means.

upon rewatering (Figure 4). At day 4, DIMBOA concentration of stressed seedlings remained at approximately 80% of the maximum level observed in those plants. Nonstressed plants experienced an approximately 70% decrease in Hx acid concentration on day 7 compared to the maximum levels attained during the experiment. Rehydrating stress plants for three days did not cause a significant decrease in DIMBOA concentrations of the stressed seedlings, as concentrations were still maintained at about 70% of the maximum (Figure 4).

Osmotic Priming. Osmotic priming delayed germination of this corn cultivar, as emergence of primed seed occurred approximately one day after control

seed (data not shown). To prevent confounding the data based upon plant age, seedlings within each treatment were harvested at six and 14 days after emergence. Osmotic priming significantly decreased the accumulation of cyclic Hx acids on the first sampling period, day 6 (Figure 5), although the differences between nonprimed and osmotically primed kernels were small. The different osmotic water potentials did not influence total cyclic Hx acid levels, but seedlings from both treatments had significantly lower total cyclic Hx acids than seedlings from nonprimed seed. However, by day 14, total cyclic Hx acid content of nonprimed and osmotically primed treatments were not significantly different (Figure 5).

DISCUSSION

Cyclic Hx acid accumulation in corn seedlings was significantly influenced by water availability, regardless of when water levels were altered. Interestingly, a moderate water deficit experienced during or after germination increased the cyclic Hx acid content of this corn cultivar, suggesting that plants growing under a reduced water potential should have increased protection against predators. Although previous research has shown that water-stressed plants [e.g., *Sorghum halepense* (L.) Pers. and *Trifolium repens* L.] are often more resistant to pests (Rhoades, 1979), the specific role of cyclic hydroxamic acids or other secondary

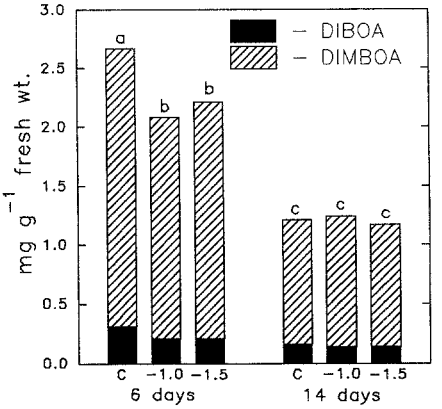

FIG. 5. Cyclic hydroxamic acid content of 6-day-old and 14-day-old corn seedlings whose kernels had been osmoprimed at either −1.0 MPa or −1.5 MPa for six days prior to germination. Control (C) seedlings were germinated from seed that had not been osmoprimed prior to planting. Data are means of six replicates and different letters within a group indicate significant difference in total cyclic hydroxamic acid according to LSD ($P = 0.05$).

compounds was not addressed by those investigators. From the present data, it appears that an increase in secondary metabolites may contribute to that resistance.

The increase in cyclic Hx acids in stressed plants coincided with a reduction in seedling growth, suggesting that Hx acids accumulate under growth-limiting conditions. The cyclic Hx acids should, therefore, be considered stress metabolites. These results are the first to report on moisture as an abiotic factor or inducer of this specific stress metabolite. Previous investigations regarding abiotic effects on cyclic Hx acids include that of Epstein et al. (1986), who observed a decrease in cyclic Hx acids and an increase in growth of wheat seedlings grown under long photoperiod and increased temperatures. In another study (Thompson et al., 1970), atrazine tolerance and DIMBOA content in corn were reduced when plants were cultured under high light intensity and elevated temperature, factors which should enhance growth rate in corn. The accumulation of the phenolic compound, chlorogenic acid, in *Helianthus annuus* L. under drought stress has also been reported (del Moral, 1972). In all of these studies, the secondary compound of interest was found in greatest concentration when conditions were less favorable for growth, suggesting that these compounds are stress metabolites.

It is interesting to note that when water was withheld from corn seedlings after successful germination, significant changes in the Hx acid content occurred before a significant reduction in seedling biomass was observed (see Figures 3 and 4). Although secondary metabolites are generally synthesized from intermediates of primary metabolism, they are produced more readily when primary metabolism and growth are limited (Lindsey and Yeoman, 1983). However, when significant differences in Hx acid concentration were first observed in this experiment (day 1), it was plant water potential, rather than seedling growth, that exhibited symptoms of water stress (Figure 3). This suggests that other mechanisms that are sensitive to subtle changes in plant water status, such as protein synthesis or hormone production, may also play a role in regulating the metabolism of these secondary metabolites. Nevertheless, the observed inverse relationship between seedling growth and cyclic Hx acid accumulation warrants further investigation.

Although we successfully increased cyclic Hx acids in corn seedlings by reducing seedling growth, water-limiting conditions are stressful and will likely diminish the overall health of the plant. However, "nonstress" methods that inhibit growth might be a viable alternative to increase hydroxamic acid production in young corn seedlings without harmful side effects. The osmotic treatment of a dormant seed is an example of a nonstress, growth-inhibiting technique, since metabolism initiates in the dormant seed and germination is repressed (Bradford, 1986). Unfortunately, in this study, osmotic priming did not have a positive effect on germination and the subsequent accumulation of cyclic Hx

acid (Figure 5). The literature regarding osmotic priming of corn seed is conflicting, as both enhanced and delayed germination have been observed following PEG treatments (Bradford, 1986). Excess PEG may have some unknown deleterious effects on corn seed metabolism, since cyclic Hx acids were also severely reduced in another experiment (Figure 1) when PEG concentrations exceeded 300 g/liter. Therefore, other pre-sow hydration treatments may be more useful in corn.

Environmental stress often plays an important role in host susceptibility to pathogens, and the growing environment needs to be more carefully considered when developing alternative pest management systems. The present experiments demonstrate that factors such as water stress can significantly alter the accumulation of natural defense chemicals of corn seedlings. Other biotic or abiotic factors that affect the synthesis of these compounds during the seedling stage or that alter the peak production interval should also influence their effectiveness against seedling pathogens and herbivores and will be the subject for future research.

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METABOLIC FATE OF DIETARY TERPENES FROM *Eucalyptus radiata* IN COMMON RINGTAIL POSSUM (*Pseudocheirus peregrinus*)

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Abstract—Arboreal marsupials consume terpenes in quantities that are toxic to other mammals, indicating that they possess special detoxification mechanisms. The metabolic fate of dietary terpenes was studied in the common ringtail possum (*Pseudocheirus peregrinus*). Three animals were fed *Eucalyptus radiata* leaf for 10 days. Leaf consumption increased over three days to an average steady state of about 10–15 mmol total terpenes per day. GC-MS analysis identified six urinary terpene metabolites, which were dicarboxylic acids, hydroxyacids, or lactones. Another nine metabolites could only be shown to be terpene-derived but of unknown structure. The amounts excreted were estimated by GC-FID, using response factors based on carbon content. Total 24-hr excretion of terpene-derived metabolites increased to 6.2–7.6 mmol on days 5–10, while glucuronic acid excretion remained constant at about 1.5 mmol. No other conjugates of terpene metabolites were found. The strategy used by the possum to detoxify dietary terpenes seems to be to polyoxygenate the molecules forming highly polar, acidic metabolites that can be readily excreted. Conjugation is minimal, perhaps to conserve carbohydrate and amino acids.

Key Words—Terpene, possum, *Pseudocheirus peregrinus*, *Eucalyptus radiata*, metabolism, foliage, diet, urinary acids, glucuronic acid.

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INTRODUCTION

Although *Eucalyptus* is the dominant genus of trees in 90% of Australian forests, few vertebrate herbivores eat the foliage. This is largely due to a combination of a low nutritional quality and the presence of significant quantities of allelochemicals, in particular, polyphenols and volatile oils (Foley and Hume, 1987; Foley, 1987; Cork, 1986; Cork and Foley, 1991). *Eucalyptus* leaf contains up to 22% dry weight of volatile oil (Morrow and Fox, 1980), a complex mixture of mainly C_{10} terpenoids whose composition varies with species of *Eucalyptus*, leaf age, locality, and season (Eberhard et al., 1975). Nonetheless, some species of arboreal marsupials, including koalas (*Phascolarctos cinereus*), greater gliders (*Petauroides volans*), common brushtail possums (*Trichosurus vulpecula*), and common ringtail possums (*Pseudocheirus peregrinus*) do eat *Eucalyptus* foliage in varying amounts, and the high terpene content of *Eucalyptus* leaf presents them with a considerable toxic challenge. The koala, for example, can ingest several milliliters of *Eucalyptus* oil daily (Eberhard et al., 1975), a dose that has been fatal in humans despite their 10-fold larger body weight (Martindale, 1989). The different extent to which arboreal marsupials feed on *Eucalyptus* leaf may be due to their varying abilities to handle this potentially toxic load.

There is evidence that the terpenes of *Eucalyptus* oil are well absorbed from the gut and require metabolism before excretion in the urine, as would be expected for lipophilic substances. Eberhard et al. (1975) found that only 15% of the ingested oil from *E. punctata* appeared in the feces of koalas, and 1% was excreted unchanged in the urine. Administration of pure *Eucalyptus* terpenes to the brushtail possum (*Trichosurus vulpecula*) resulted in only trace amounts being excreted unchanged in urine and feces (Southwell et al., 1980). In the greater glider (*Petauroides volans*) and brushtail possum, *Eucalyptus* oils are almost completely absorbed before reaching the hindgut, which is the principal site of microbial activity in these species (Foley et al., 1987). Thus the gut flora are unlikely to be important in terpene metabolism in these animals.

Early reports of glycosuria in koalas and possums suggested that *Eucalyptus* terpenes are ultimately excreted as conjugates with glucuronic acid; sulfate conjugation was found to be very low (Hinks and Bollinger, 1957a,b; Roy, 1963). Southwell (1975) reported some novel monoterpenoid lactones in the urine of the koala fed *E. punctata*. The author considered that these metabolites were probably formed during isolation by cyclization of hydroxycarboxylic acids released as hydrolysis products of labile urinary glucuronides. The hydroxyacids were thought to be derived from α- and β-pinene, two of the major leaf terpenes of *E. punctata*. When the brushtail possum was dosed with pure α- or β-pinene, different metabolites were found: a carboxylic acid (myrtenic acid) and an alcohol (*trans*-verbenol), whereas the koala can apparently carry out both oxidations

to produce the hydroxyacid metabolites and subsequently the lactones (Southwell et al., 1980). Thus, it may be that terpene oxidation is more extensive in the koala which, unlike the brushtail possum, is entirely dependent on a *Eucalyptus* leaf diet.

The only other published data on oxidative metabolism of terpenes in marsupials are also from the brushtail possum: *p*-cymene was reported to be oxidized to *p*-cresol and cumic acid; and 1,8-cineole was oxidized at the 9-methyl group to the corresponding alcohol and carboxylic acid (Flynn and Southwell, 1979; Southwell et al., 1980). Recently Carman and Klika (1992) have shown that the hydroxycineole and cineolic acid metabolites of 1,8-cineole are excreted by the brushtail possum as partially racemic mixtures, with a sex difference in the enantiomeric ratio. The authors have made an interesting proposal that the racemate may act as a pheromone in this species.

The present study has examined the metabolic fate of the terpene component of *E. radiata* in the common ringtail possum (*Pseudocheirus peregrinus*).

METHODS AND MATERIALS

Animals and Urine Collection. The study was approved by the Animal Experimentation Ethics Committee of the University of Tasmania and conforms with the Australian Code of Practice for the care and use of animals for scientific purposes. Three common ringtail possums were maintained on an artificial diet (fruit and cereals), then offered *E. radiata* foliage ad libitum for 10 days. All foliage was collected from a single *E. radiata* tree at one cutting. Food intake was measured daily. All urine produced was collected from galvanized iron trays into plastic bottles sitting in flasks of liquid nitrogen and thereafter stored at $-20°C$ or below. Urine collection began on the last day of the artificial diet (day 0) and continued for the 10 days of the *Eucalyptus* diet.

In a separate experiment, possums fed the artificial diet were gavaged with doses of individual terpenes (*p*-cymene, α-phellandrene) at 0915–0930 hr and urine collected as above for 24 hr. The terpenes were obtained commercially and were redistilled before use. All were $>90\%$ pure by GC.

Analysis of Eucalyptus Oil. The oil was obtained by steam distillation of a sample of the *E. radiata* leaf and analyzed by gas chromatography–mass spectrometry (GC-MS). The instrument used was a Hewlett-Packard 5890 gas chromatograph and 5970 series mass-selective detector with 59970A Chemstation for programming and data processing (Hewlett-Packard Australia Ltd., Melbourne, Australia). *Eucalyptus* oil was dissolved in hexane (5 μl/ml) and a 1-μl aliquot injected on to a 50-m fused silica capillary column (0.3 mm ID) coated with a polyethylene glycol bonded phase (BP 20; Scientific Glass Engineering Pty. Ltd., Melbourne, Australia). Operating conditions were: helium flow 2 ml/

min; injection split ratio 10:1; oven temperature 50°C for 1 min, then increasing by 6°C/min to a final temperature of 220°C; injector 260°C; open–split interface 240°C. Accurate mass and ammonia CI data were obtained on a Kratos Concept IH mass spectrometer.

Analysis of Urinary Metabolites. Glucuronic acid (free and conjugated) in urine was measured colorimetrically by the method of Fishman and Green (1955). Urine samples were diluted 50- or 500-fold before analysis. Other metabolites were analyzed by gas chromatography of urine extracts, using the following general method. To 0.25 ml urine in a 10-ml tapered centrifuge tube was added the internal standard solution (0.5 mg 2,5-dimethylbenzoic acid dissolved in 0.25 ml aqueous base) and 0.20 ml distilled water. For hydrolysis of conjugates, 0.2 ml acetate buffer (1.1 M, pH 5.2) was added, then 0.050 ml extract of *Helix pomatia* (β-glucuronidase plus aryl sulfatase; Boehringer, Mannheim, Germany) and overnight incubation at 37°C. This step was omitted for analysis of unconjugated metabolites. The mixture was next acidified to pH 1 with 5 M hydrochloric acid and extracted with ethyl acetate (1 × 2 ml followed by 2 × 1 ml), using a vortex mixer and centrifugation to separate the phases. A 1-ml aliquot of the combined ethyl acetate extracts was placed in a clean tube and concentrated to about 0.2 ml at 30°C under a gentle stream of nitrogen. The acid metabolites were then methylated by the addition of 0.5 ml of ethereal diazomethane (Vogel, 1956). After standing in a stoppered tube in ice for 30 min, the excess diazomethane was evaporated and the sample concentrated to about 0.2 ml at 20–25°C under nitrogen before GC analysis.

Metabolites were identified by GC-MS using the instrument described above for oil analysis. Quantitative analyses were made on another Hewlett-Packard 5890 gas chromatograph with flame ionization detection (FID). For all GC analyses of metabolites a fused silica capillary column was used [25 m × 0.32 mm ID, coated with 0.52-μm cross-linked 5% phenylmethylsilicone (HP 5); Hewlett Packard, Melbourne, Australia]. Operating conditions for quantification were: carrier gas He 2 ml/min; injector 250°C; detector 300°C; oven program 60°C for 1 min, then increasing at 10°C/min to 140°C, 5°C/min to 250°C, 30°C/min to 290°C; 1 μl injected with split ratio 20:1. Similar conditions were used for GC-MS.

The efficiency of extraction of metabolites from urine was examined by separately analyzing each of the three ethyl acetate extracts and two additional extractions, and comparing the amounts of metabolites found in each.

To facilitate metabolite identification, in some experiments acidic and phenolic metabolites were separated from neutrals before methylation and GC analysis. A 3-ml aliquot of the ethyl acetate extract was extracted with 2 × 3 ml aqueous sodium bicarbonate (5%) to remove carboxylic acids, then with 2 × 3 ml aqueous sodium hydroxide (5%) to remove phenolics, leaving neutral metabolites in the ethyl acetate. The acidic and phenolic extracts were then brought

to pH 1 with 5 M HCl and extracted with ethyl acetate and methylated as usual before GC analysis.

In other experiments, the effectiveness of enzymatic hydrolysis of conjugated metabolites was compared with acid hydrolysis of the same urine samples. Urine samples for acid hydrolysis were prepared without the acetate buffer and enzyme, as for analysis of unconjugated metabolites, except that sufficient 5 M HCl was added to bring the pH to 1. The sample was then either left overnight at 26–28°C, or placed in a boiling water bath for 2 hr.

Calibration Curves. Reference compounds were not available for most of the urinary metabolites, so an indirect method was devised to estimate their amounts in urine. The method is based on the observation that the molar FID response to a substance is proportional to the number of carbon atoms present (Jorgensen et al., 1990). GC-MS analyses showed that the terpene metabolites typically retained the 10-carbon skeleton and gained two, three, or four oxygen atoms to form hydroxyacids or mono- or dicarboxylic acids. Methylation added another carbon atom for each carboxylic acid group. Accordingly, GC calibration curves were prepared for a series of mono- and dicarboxylic acids added to blank ringtail possum urine (obtained from animals not eating *Eucalyptus* leaf). The acids used were hydrocinnamic, *n*-decanoic, camphoric, phenylsuccinic, 3,4-dimethoxyphenylacetic, 3-(3,4-dimethoxyphenyl)-propionic, 3-hydroxybenzoic, and hippuric. These acids were chosen because they had 10–13 carbons after methylation or, for the last two, had been found in the urine. Analysis was by GC-FID, using 2,5-dimethylbenzoic acid as internal standard, as described above for urinary terpene metabolites. The acids were dissolved together in methanol and different volumes added to urine to give eight concentrations ranging from 0 to 4.5 mg/ml. The relationship between the slopes of the calibration curves and the number of carbons in each compound was used to estimate the slope of the calibration curve for metabolites that were not available as pure substances. For some terpenoid metabolites for which the molecular formula, and therefore the number of carbon atoms, was not known, the slope for C_{12} (corresponding to a methylated dicarboxylic acid metabolite) was used.

RESULTS

Consumption of Terpenes. Steam distillation of the *E. radiata* leaf yielded 3.3% oil by wet weight (the leaf contained 59% water). Analysis of the oil showed that eight major components accounted for 85% of terpenes in the oil: α-phellandrene, 15.8%; β-phellandrene, 5.8%; *p*-cymene, 5.3%; *trans-p*-menth-2-en-1-ol, 16.9%; terpinen-4-ol, 3.5%; *cis-p*-menth-2-en-1-ol, 15.8%; *cis*-piperitol, 6.6%; and *trans*-piperitol, 15.6%. The mean daily consumption of

leaf is shown in Figure 1A. It can be seen that the consumption of leaf increased considerably during the experiment, from about 20 g wet wt on the first day of *E. radiata* diet to nearly 80 g on days 7–9.

Excretion of Glucuronic Acid. Total glucuronic acid excretion was low for ringtail possums fed the artificial diet, but increased rapidly when they were switched to *E. radiata* (Figure 1B). The proportion of urinary glucuronic acid that was conjugated varied from 0.49 (day 4) to 0.95 (day 0), with a mean of 0.64 (SE = 3.9, $N = 11$). There was a good correlation between mean food consumption and mean total glucuronic acid excreted ($R^2 = 0.862$, $P < 0.001$, $df = 9$).

Identification of Leaf-Derived Metabolites. GC-MS analyses showed that a large number of urinary metabolites were excreted by possums feeding on *E. radiata* leaf (Figure 2). Virtually none were present when the animals were fed the artificial diet (Figure 2 inset), indicating that all the metabolites were

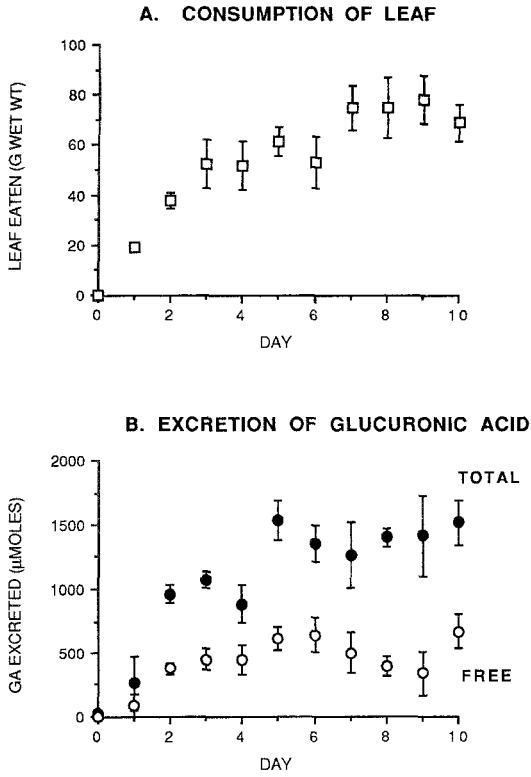

FIG. 1. (A) Consumption of leaf (g wet wt) per day. Mean (±SE) of three animals. (B) Excretion of glucuronic acid (μmoles) per day. Mean (±SE) of three animals.

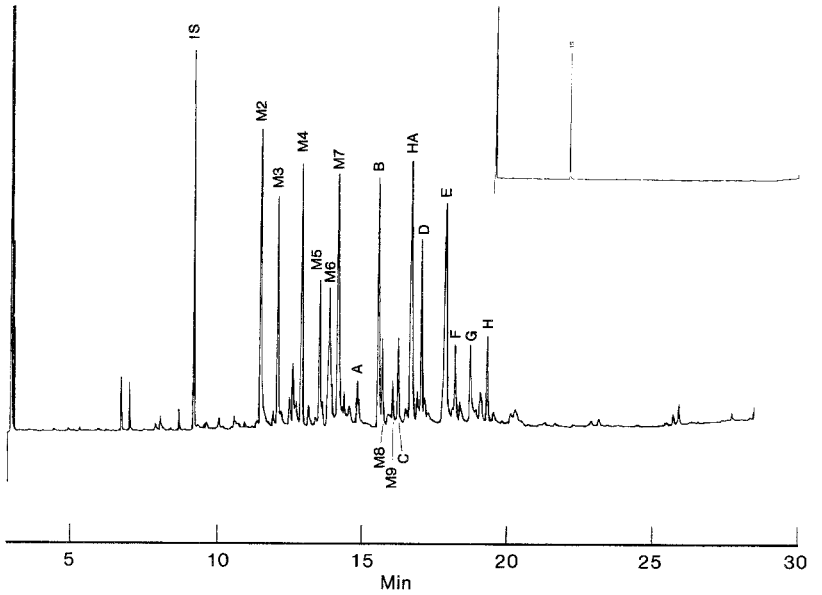

FIG. 2. GC-FID trace showing urinary metabolites (possum 1, day 5). Inset shows a GC-FID trace obtained under identical conditions using urine collected from the same animal on day 0, before the *Eucalyptus* diet. IS = internal standard, HA = hippuric acid.

leaf-derived. Analysis of the mass spectra showed the presence of hippuric acid (HA) and 3-hydroxybenzoic acid (M2), which were confirmed by comparison with the retention times and mass spectra of authentic materials (Table 1).

Other significant metabolites showed ions characteristic of the C_{10} terpenoid structure, as outlined below. Although authentic reference materials were not available, two known terpene metabolites (M5 and M8) were found and a number of other terpenoid metabolites (M3, M4, M6, M7, M9, D-F, H) were at least partially identified. For most metabolites, mass spectral analysis gave an accurate molecular ion (confirmed by low resolution CI-MS), which provided the molecular formulae (Table 1). Not all metabolites gave a molecular ion under electron impact conditions: for M6, B, and G only the CI molecular ion (MH^+) was found, and not even that was obtained for A and C.

The neutral fraction showed only two metabolites (M3 and M4), which had mass spectra similar to those of the two unsaturated lactones found by Southwell (1975) in the urine of koalas fed *E. puctata* leaf (Figure 3). All showed loss of methyl and the lactone ring to give ions at *m/z* 151 $[C_9H_{11}O_2]^+$ and *m/z* 93 $[C_7H_9]^+$, respectively. However, M3 and M4 differed in the relative abundances of ions, both from each other and from Southwell's lactones (which

TABLE 1 GC-MS DATA ON METABOLITES

Metabolite[a]	Empirical formula and calc. FW	M^+ found	Retention time (min)[b]	Significant EI ions (*m/z*) and relative abundance (%)								
M1	$C_{10}H_{11}O_3N$	193	16.65	193	161	134	105	77	51			
	193			8	8	17	100	44	18			
M2	$C_8H_{10}O$	152	11.44	152	123	121	93	65	43			
	152			45	11	100	44	28	21			
M3	$C_{10}H_{14}O_2$	166.1010	12.00	166	151	107	93	77	55			
	166.0994			22	100	28	58	27	41			
M4	$C_{10}H_{14}O_2$	166.1002	12.84	166	151	107	93	79	55			
	166.0994			28	93	40	100	59	50			
M5	$C_{11}H_{14}O_3$	194.0954	13.45	194	179	137	77	59	43			
	194.0943			1	100	24	16	30	94			
M6	$C_{11}H_{16}O_3$	197[c]	13.78		178	105	91	88	79	57		
	196				7	15	95	100	75	49		
M7	$C_{11}H_{18}O_3$	198.1279	14.09	198	183	166	155	139	128	96	69	59
	198.1256			13	17	24	17	15	11	100	33	21
M8	$C_{12}H_{14}O_4$	222.0898	15.61	222	191	163	131	103	77	59		
	222.0892			29	15	100	15	16	14	18		
M9	$C_{12}H_{16}O_4$	224.1054	15.96	224	193	165	137	105	91	88	77	59
	224.1048			31	11	15	45	100	24	20	25	28
A			14.77	163	152	137	93	91	59			
				6	15	17	77	48	100			
B		195[c]	15.47	194	164	163	137	105	88	77	59	
				7	39	55	58	71	100	56	56	
C			16.16	151	119	107	105	91	88	59		
				98	77	27	23	90	100	50		

D	$C_{13}H_{18}O_4$[d]	238.1204	16.96	238	223	151	137	119	91	88	59
	238.1205			2	3	47	32	73	100	26	26
E	$C_{11}H_{18}O_4$	214.1211	17.80	214	196	181	137	127	79	59	
	214.1205			1	5	10	64	100	45	62	
F	$C_{11}H_{14}O_4$	210.0881	18.10	210	195	179	151	105	91	79	59
	210.0888			59	100	53	39	32	54	52	41
G		273[c]	18.66		182	123	77	69	59	55	
					60	100	5	10	8	8	
H	$C_{11}H_{14}O_4$	210.0873	19.22	210	195	179	151	107	91	79	59
	210.0888			68	100	18	31	48	82	77	66

[a] Acids as their methyl ester.
[b] GC-FID conditions.
[c] MH^+ by CI-MS.
[d] One extra CH_2 probably from methylation of a double bond.

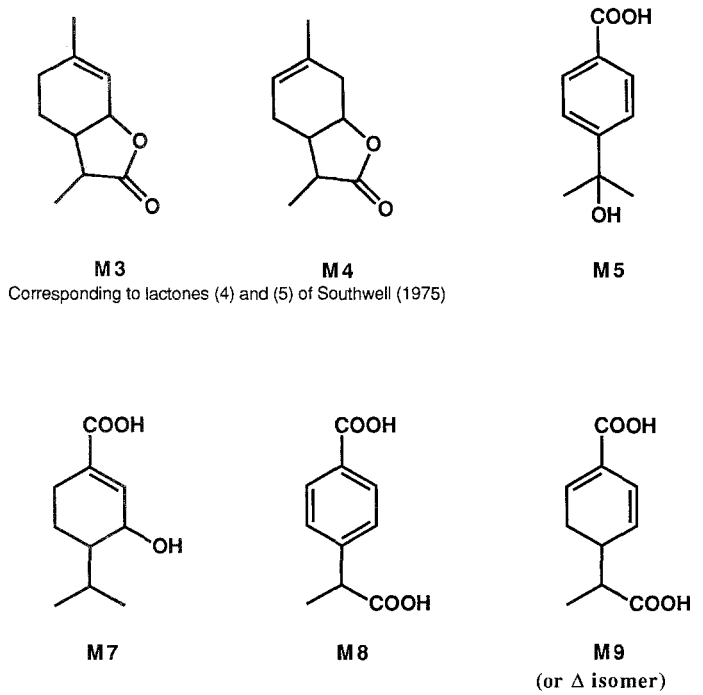

FIG. 3. Terpene metabolites found in urine. M3 and M4 probably differ from the lactones shown in the position of the double bond and in their stereochemistry. The position of the double bond in M7 is uncertain. See Results for details.

had identical mass spectra). M3 and M4 were partially purified by thin-layer chromatography, and NMR spectra indicated that M3 gave the same shifts as Southwell's lactone (4), while M4 corresponded to lactone (5). It is possible that M3 and M4 differ in the position of the double bond and in stereochemistry, as there are three chiral centers that could give rise to a number of possible diastereomers.

All other metabolites appeared in the carboxylic acid fraction and were analyzed as their methyl esters. Metabolites M5 and M8 have been reported previously as metabolites of *p*-cymene in the rat and guinea pig (Walde et al., 1983) and were also detected (by GC-MS) after administration of pure α-phellandrene or *p*-cymene to possums. Their mass spectra agreed with those found by Walde et al. (1983) and were consistent with the structures shown in Figure 3. Accurate mass data on M5 identified the following fragments: *m/z* 179 $C_{10}H_{11}O_3$ $[M\text{-}CH_3]^+$, *m/z* 137 $C_8H_9O_2$ $[M\text{-}C_3H_5O]^+$, and *m/z* 77 C_6H_5 (the ring structure). Major fragmentations for M8 were to *m/z* 191 $[M\text{-}CH_3O]^+$, *m/z*

163 $[M\text{-}COOCH_3]^+$, *m/z* 77 C_6H_5 (the ring), and *m/z* 59 $C_3H_3O_2$ (the carboxymethyl group).

Based on their molecular formulae, metabolites M6 and M7 are likely to be unsaturated hydroxy acids. The structure of M7 (Figure 3) is proposed to account for ions *m/z* 183 $C_{10}H_{15}O_3$ $[M\text{-}CH_3]^+$, *m/z* 166 $C_{10}H_{14}O_2$ $[M\text{-}CH_3OH]^+$, *m/z* 155 $C_8H_{11}O_3$ $[M\text{-}C_3H_7]^+$, *m/z* 139 $C_9H_{15}O$ $[M\text{-}COOCH_3]^+$, and *m/z* 128 $[C_6H_8O_3]^+$ formed by the retro-Diels-Alder elimination of C_5H_{10}, followed by the loss of CH_3OH to form an unsaturated lactone $C_5H_4O_2$ at *m/z* 96.

The mass spectrum of M9 was similar to that of M8 except that the molecular ion and several others were heavier by two hydrogens (Table 1). The same metabolite was found in urine of a possum administered α-phellandrene (a terpene with two double bonds), and M9 has been assigned a corresponding structure. The mass spectrum showed ions at *m/z* 193 $[M\text{-}OCH_3]^+$, *m/z* 165 $[M\text{-}COOCH_3]^+$, *m/z* 137 $[M\text{-}C_4H_7O_2]^+$, and *m/z* 105 $[M\text{-}COOCH_3\text{-}COOCH_3]^+$, and the formation of phenyl (*m/z* 77 C_6H_5) and carboxymethyl (*m/z* 59 $C_2H_3O_2$) ions. The ion at *m/z* 88 $C_4H_8O_2$ was also found for M6, B, C, and D and may arise from a McLafferty rearrangement of a hydrogen in the cyclohexane ring to the carbonyl on the isopropyl side chain.

Metabolites A–C could not be characterized from their mass spectra, but the ions were those expected for acidic terpene metabolites (as outlined above): *m/z* 59 ($C_2H_3O_2$), *m/z* 77 (C_6H_5), *m/z* 88 ($C_4H_8O_2$), *m/z* 91 (C_7H_9), and *m/z* 93 (C_7H_{11}). There is less evidence that metabolite G is terpenoid, but it is leaf-derived and has a similar molecular weight and so was provisionally grouped with the others.

Quantification of Other Leaf-Derived Metabolites. For each reference compound, an excellent linear relationship was found between peak area ratio (compound/internal standard) and amount added to urine ($R^2 \geq 0.999$) and the intercept was close to zero. There was a reasonably good correlation between the slopes of the individual calibration curves and the number of carbon atoms in each compound, from eight to twelve for the methylated acids ($R^2 = 0.729$, $P < 0.05$, $df = 6$). The equation of the line of fit was slope ($\times 10^2$) $= -1.55 + 0.85$ (no. carbon atoms). When the amounts of 3-hydroxybenzoic acid and hippuric acid excreted by possum 1 on days 0–10 were calculated using both the actual calibration curve for each compound and that estimated from the general slope versus carbon number relationship, there was no significant difference (paired *t* test: for M2, $P = 0.001$, $df = 7$; for HA, $P = 0.002$, $df = 8$). The unknown terpenoid metabolites A–C and G were assumed to have 12 carbons (i.e., to be methylated dicarboxylic acids) for the purpose of estimating their concentrations in urine.

Recovery experiments showed that very little of each metabolite remained after the third extraction with ethyl acetate. Three extractions removed 95–100% of most metabolites, except for metabolite C for which 93% was recovered.

Comparison of different methods of hydrolysis showed that enzymatic hydrolysis yielded as much or more of each free metabolite as either of the acid hydrolyses.

Time Course of Excretion of Leaf-Derived Metabolites. The daily excretion of hippuric acid (HA) and total 3-hydroxybenzoic acid (M2) is shown in Figure 4. The mean excretion of each increased with mean food consumption (for HA, $R^2 = 0.805$, $df = 9$, $P < 0.01$; for M2, $R^2 = 0.765$, $df = 9$, $P < 0.01$). The excretion of the terpenoid metabolites M3–M9 (after hydrolysis of conjugates) is shown in Figure 5. This also increased with daily food consumption ($R^2 = 0.826$, $df = 9$, $P < 0.01$). The major terpenoid metabolite was the hydroxyacid M7, followed by approximately equal amounts of M3, M4, M5, and M6, and smaller amounts of M8 and M9.

The excretion of the unknown metabolites A–H also increased over the first few days of the *Eucalyptus* diet, and then stabilized in amounts ranging from approximately 100 to 1000 μmol/day. The mean daily excretion of metabolites A–H was highly correlated with dietary leaf consumption ($R^2 = 0.775$, $P < 0.01$, $df = 9$). The A–H excretion data are not shown separately but are included with M3–M9 as total terpenoid metabolites in Figure 6.

Conjugated Metabolites. Only a minor proportion of the leaf metabolites was excreted as conjugates. Figure 6 shows this for the total terpenoid metabolites (i.e., M3–M9 plus A–H), where the amounts found after hydrolysis were only slightly greater than those found in unhydrolyzed urine ($t = 2.262$, $P = 0.047$, $df = 10$). The same pattern applied to the individual terpenoid metabolites and to 3-hydroxybenzoic acid (M2), which, while lacking the terpene skeleton, can also be conjugated with glucuronic acid.

By the second day of the terpene diet, the excretion of total conjugatable metabolites (Figures 4 and 6) far exceeded the total urinary glucuronic acid

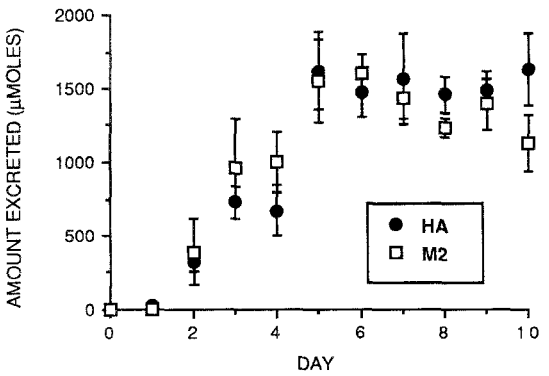

FIG. 4. Daily excretion of hippuric acid (HA) and total (free plus conjugated) 3-hydroxybenzoic acid (M2). Data are the mean (±SE) of three animals.

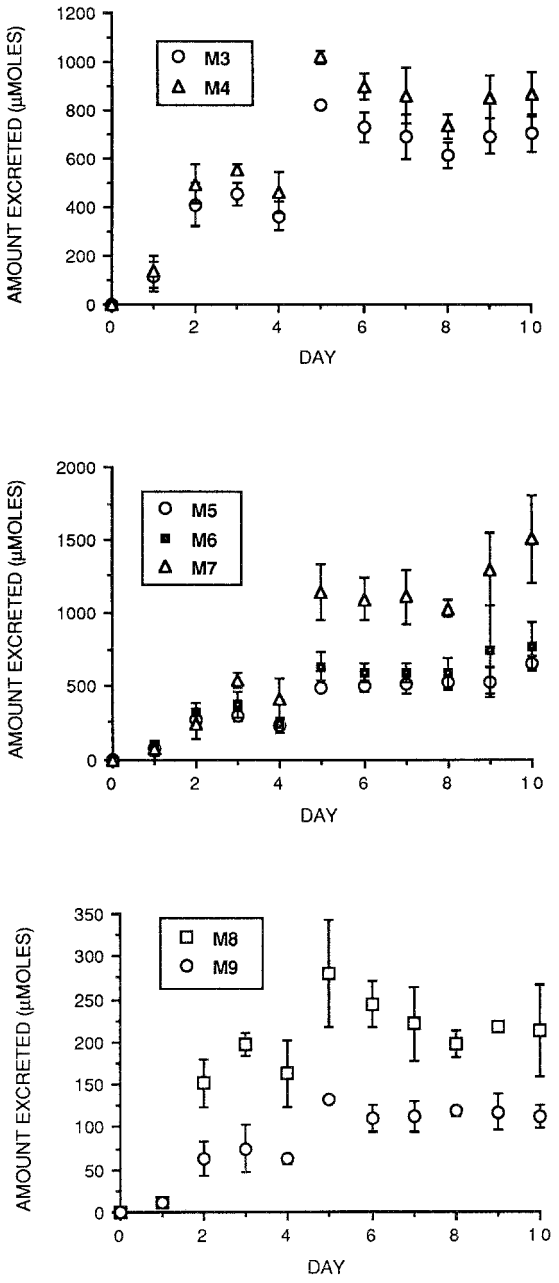

FIG. 5. Daily excretion of metabolites M3–M9. The total amount of each metabolite (free plus conjugated) is shown, as the mean (±SE) of three animals.

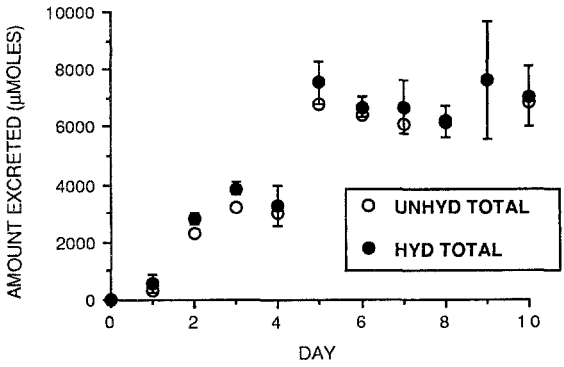

FIG. 6. Daily excretion of total of all terpene metabolites (M3–M9 plus A–H). UNHYD = before hydrolysis, HYD = after hydrolysis. Data are the mean (±SE) of three animals, except that the SE values have been omitted from the unhydrolyzed data for clarity.

(Figure 1B), supporting the observation that the metabolites are excreted mostly in the free form (Figure 6). Hippuric acid is the glycine conjugate of benzoic acid, and a GC-MS search was made for other possible glycine conjugates of carboxylic acids (especially M2), but none was found.

The proportion of total metabolites excreted as glucuronides was greatest (60%) on the first day of the *Eucalyptus* diet and declined thereafter (to 15–30%), although the total excretion of glucuronic acid peaked on day 5 (Figure 1B).

Accounting for Terpenes Consumed. The principal dietary terpenes had formula weights of 154 (the menthenols and piperitols, $C_{10}H_{18}O$), 134 (*p*-cymene, $C_{10}H_{14}$), and 136 (the phellandrenes, $C_{10}H_{16}$). In order to estimate the recovery of dietary terpenes as urinary metabolites, they were assigned a formula weight of 150 and the daily intake of *Eucalyptus* oil (in grams) was expressed as micromoles terpenes per day. This rose from a mean of 3.8 (SE 0.2) μmoles on the first day to a maximum of 15.6 (SE 1.9) μmol on day 9. The excretion of total terpene-derived metabolites (M3–M9 and A–H) is shown as a percentage of the dietary consumption of terpenes in Figure 7. Both M2 and hippuric acid were excluded as they lack the 10-carbon terpene skeleton. The proportion of dietary terpenes accounted for in the urine increased to an approximate steady state of one half after five days.

DISCUSSION

Eucalyptus radiata foliage was used in these experiments because it is an important natural diet item of common ringtail possums, and it is known to be rich in terpenes (Foley et al., 1987), a finding confirmed in this study. Although

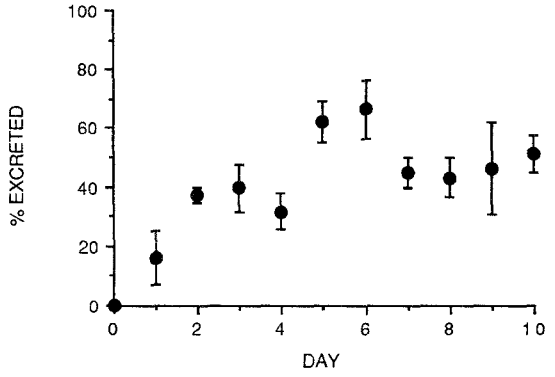

FIG. 7. Percentage of dietary terpenes found as urinary metabolites each day. Data are the mean (±SE) of three animals.

Boland et al. (1991) found cineole and piperitone to be major components of *E. radiata*, Foley et al. (1987) reported a different (and variable) composition. There may be up to six chemotypes in this species (Boland et al., 1991).

When offered the *E. radiata* leaf diet, the possums consumed small amounts at first, increasing their intake over several days, as has been reported previously (Foley, 1992). Glucuronic acid excretion increased with leaf consumption, to a maximum steady-state level of about 1.5 mmol/day. Dash (1988) reported a similar excretion rate of glucuronic acid in brushtail possums fed *E. melliodora* leaf. The free glucuronic acid is most likely the result of glucuronide conjugates being hydrolyzed in the bladder or in the urine after collection. Marsh (1969) found evidence of considerable β-glucuronidase activity in the urine of the brushtail possum. There was negligible glucuronic acid excreted by possums on the non-*Eucalyptus* diet, indicating that it is excreted as part of the process of eliminating *Eucalyptus* metabolites as glucuronide conjugates.

Hippuric acid (HA) has been found in many species that have dietary sources of benzoic acid (Bridges et al., 1970). It is the major metabolite of benzoic acid in the brushtail possum and six other marsupials, although small amounts were also glucuronidated (Awaluddin and McLean, 1985). Benzoyl glucuronide was not specifically tested for in the present study, and small amounts may have escaped detection. 3-Hydroxybenzoic acid (M2) might also have been expected to be conjugated with glycine, as its 2-isomer (salicylic acid) is extensively metabolized by this pathway in humans (Levy, 1965). However, no glycine conjugates were found for this or any of the other carboxylic acid metabolites. Salicylic acid is excreted largely unchanged by the rabbit (Williams, 1959b) and horse (Marsh et al., 1981), although both species quantitatively convert benzoic acid to its glycine conjugate. Separate enzymes may catalyze

these two reactions (Marsh et al., 1981). Only a small fraction of M2 was excreted in conjugated form each day (the mean ranged from 3 to 14%), except on day 2 when 53% was excreted as the conjugate (of a low total of 389 μmol).

Metabolites with a 10-carbon skeleton are more clearly derived from terpenes, and six were structurally identified and another five assigned a molecular formula (Table 1 and Figure 3). The pattern of formation of these metabolites involved oxidation of methyl groups to the corresponding carboxylic acids, and oxidation of other carbons to secondary or tertiary alcohols. Several metabolites were hydroxyacids (M5–M7) or their lactone derivatives (M3 and M4), the latter presumably formed in urine or during the work-up of samples. Being lipophilic, the lactones would be expected to be only slowly excreted by mammalian kidneys.

Early work on monoterpene metabolism in sheep and rabbits found oxidation to monoalcohols and monocarboxylic acids (Williams, 1959a). The rabbit oxidized *p*-cymene and other monoterpenes predominantly at a single carbon, producing the corresponding alcohol or monocarboxylic acid (Ishida et al., 1977, 1979, 1981, 1989; Asakawa et al., 1988). The guinea pig and rat similarly formed monoalcohol or monoacid metabolites of *p*-cymene, while the rat also produced some hydroxyacids (including M5) and the dicarboxylic acid M8 (16% dose) (Walde et al., 1983). Other major metabolites were cumic acid (19% dose in the rat) and its glycine conjugate (31% in guinea pig), neither of which was found in the ringtail possum. Menthol was found to be oxidized in rats successively by C-8 hydroxylation and C-1 carboxylation (Madyastha and Srivatsan, 1988).

In comparing these findings with previous reports, the extent of oxygenation of terpenes appears to be greater in ringtail possums than eutherians or even brushtail possums. Given that ringtail possums are more dependent on a *Eucalyptus* leaf diet than brushtail possums (Cork and Foley, 1991), it would not be surprising for this species to develop better systems for detoxification of terpenes.

Only a small proportion of metabolites were found as conjugates, and it is unlikely that there was significant conjugation that escaped detection. Sulfate conjugates would have been hydrolyzed by the enzyme mixture used, and in any case appear to be not formed in the possum (Roy, 1963). Acyl glucuronides are known to undergo rearrangements to isomers that are resistant to enzymatic hydrolysis (Faed, 1984), but this possibility was excluded by the demonstration that acid hydrolysis produced no more free metabolites than the enzymatic method.

The relatively low ceiling for glucuronide excretion is puzzling and may be a mechanism to conserve energy, by minimizing the loss of carbohydrate. For example, Cork (1986) calculated that excretion of glucuronic acid in koalas represented some 20% of the fasting glucose production. However, *Eucalyptus*

leaves contain significant amounts of ascorbate that could also serve as a precursor for glucuronic acid (Dash, 1988), and so the magnitude of the energy saving is not clear. It is also striking that benzoic acid was the only acid conjugated with glycine, although eutherians use this pathway extensively for elimination of carboxylic acids, and in this case the strategy may be to conserve nitrogen. Although *Eucalyptus* leaf is a poor source of nitrogen (Cork, 1986; Foley and Hume, 1987), ringtails possess effective systems for recycling and conserving nitrogen and again the potential saving is hard to estimate.

Ringtails fed *E. radiata* foliage excrete an acid urine (pH 5.7) that is rich in ammonium ion, and the speed of acid disposal may limit the rate at which animals can consume leaf (Foley, 1992). If this is true, the low level of conjugation could be disadvantageous because the renal clearance of glucuronide and other conjugates is generally much faster than that of the unconjugated metabolites (Caldwell, 1982; Moller and Sheikh, 1983), which presents the ringtail possum with the potential problem of accumulation of the carboxylic acid precursors. The ringtail possum may have a particularly efficient system to secrete organic acids across the renal tubular epithelium, and perhaps the extensive oxidation of the terpenes facilitates the renal clearance of unconjugated metabolites. Although a study of renal transport using cortical slices from the brushtail possum concluded that overall it was not significantly different from eutherians, it may be significant that penicillin G did not interact with the acid uptake system in the possum as it does in eutherians (Miller and Morris, 1982). There may also be quantitative differences, and these should be investigated.

After a few days of the leaf diet, over half of the ingested terpenes could be accounted for as urinary metabolites indicating that, despite the considerable uncertainties in estimating the levels of unknown terpenoid metabolites, the metabolite excretion data are approximately correct. The gradual increase in consumption of leaf over several days may reflect the possum's need to increase its capacity to detoxify the terpenes. Some terpenes are able to induce the synthesis of hepatic drug-metabolizing enzymes (Madyastha and Srivatsan, 1988), and substrates for renal tubular secretion can induce this transport system (Moller and Sheikh, 1983). A requirement for such adaptation may explain the slow increase in leaf consumption by possums offered the *Eucalyptus* diet. The interactions between pathways of metabolism of allelochemicals, nutrient requirements, and animal feeding have been little considered in previous studies of the interactions between mammals and woody plants. Clearly, such studies can help to explain the different effects of plant chemical defences on closely related herbivores.

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OVIPOSITION STIMULANTS AND DETERRENTS REGULATING DIFFERENTIAL ACCEPTANCE OF *Iberis amara* BY *Pieris rapae* AND *P. napi oleracea*

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Abstract—*Iberis amara* (Cruciferae) contains both stimulants and deterrents that are involved in regulating oviposition by *Pieris rapae* and *P. napi oleracea*. The most active deterrents to *P. rapae* isolated from butanol extracts of the plant were found to be 2-O-β-D-glucosyl cucurbitacin I and 2-O-β-D-glucosyl cucurbitacin E. However, *P. napi oleracea* was behaviorally insensitive to these compounds and was only weakly deterred by other individual fractions of the butanol extract. Stimulant activity of the postbutanol water extract of *I. amara* was associated with glucosinolates. The most abundant of these was identified as sinigrin, and a relatively minor component was shown to be glucoiberin. The isolated sinigrin was more stimulatory to *P. rapae* than was the glucoiberin-containing fraction, but *P. napi oleracea* was stimulated as strongly by the glucoiberin fraction, even though the concentration of this compound was much lower. The contrasting responses of the two *Pieris* species to the deterrents and stimulants in *I. amara* can explain the differential acceptance of the plant by these butterflies.

Key Words—*Pieris rapae*, *Pieris napi oleracea*, Lepidoptera, Pieridae, *Iberis amara*, oviposition, stimulants, deterrents, glucosinolates, glucoiberin, sinigrin, cucurbitacins.

INTRODUCTION

The ability of phytophagous insects to find and recognize their host plants depends on the processing of appropriate qualitative and quantitative sensory information by the central nervous system (Miller and Strickler, 1984). Plant

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chemistry plays a key role in the evaluation of potential hosts and may determine the host range of each species (Rodman and Chew, 1980; Renwick, 1988; Jaenike, 1990). This concept was emphasized recently when oviposition responses to a range of plant species by *Pieris rapae* and *P. napi oleracea*, which have overlapping but distinct host ranges (Richards, 1940; Chew, 1977a,b), were compared under controlled conditions (Huang and Renwick, 1993). In that study, *Iberis amara* plants were preferred over cabbage plants by *P. napi oleracea*, but were almost completely avoided by *P. rapae* when given the same choice. The butanol extract of *I. amara* was deterrent and the post-butanol water extract was stimulatory to both species. These results demonstrated the cooccurrence of deterrents and stimulants in this plant and provided further proof that final acceptance or rejection of a plant by the butterflies is mediated by a balance of sensory inputs from positive and negative chemical stimuli in the plant (Renwick and Radke, 1987). However, these stimulants and deterrents in *I. amara* were not identified.

The host ranges of *Pieris* butterflies have long been linked to the presence of glucosinolates in crucifers and certain plants from a few other families (Verschaffelt, 1911). The indole glucosinolate, glucobrassicin, at the surface of cabbage leaves can account for stimulation of oviposition by *P. rapae* (Renwick et al., 1992) and *P. brassicae* (van Loon et al., 1992). Furthermore, Traynier and Truscott (1991) found that glucobrassicin was much more effective than sinigrin in the associative learning behavior of *P. rapae*. However, *P. napi oleracea* appears to be more sensitive to aliphatic glucosinolates, such as the glucoiberin and glucocheirolin present in *Erysimum cheiranthoides*, than to glucobrassicin in cabbage. These aliphatic members of the group are only weakly stimulatory to *P. rapae* (Huang et al., 1993). It is therefore suggested that although glucosinolates may act as insect oviposition (reviewed by Schoonhoven, 1972) or feeding (Hovanitz and Chang, 1963; Bartlet and Williams, 1991) stimulants, and more than 70 glucosinolates are known (Fenwick et al., 1983), different insect species may have evolved different sensitivities to these compounds, and, therefore, different glucosinolates may play different roles in host selection by herbivorous insects. Furthermore, chemical deterrents may frequently cooccur with stimulants in a potential host plant, as in the case of cardenolides and glucosinolates, respectively, in *E. cheiranthoides*. Specific glucosinolates and cardenolides in this plant act as oviposition stimulants and deterrents to *P. rapae* and *P. napi oleracea* (Renwick et al., 1989; Sachdev-Gupta et al., 1990; Huang et al., 1993). More information is needed to understand better the role of such chemicals in the host selection mechanism of phytophagous insects.

The purpose of this study was to isolate and identify the oviposition stimulants and deterrents for *P. rapae* and *P. napi oleracea* from *I. amara*, to determine the relative importance of these semiochemicals in host-plant recog-

nition, and to explain chemically the differential acceptance of the plant by the two insect species.

METHODS AND MATERIALS

Insects and Plants. P. rapae and *P. napi oleracea* butterflies for behavioral assays were obtained from colonies started from field-collected insects each summer and maintained in the laboratory at ca. 22°C under fluorescent lights providing a photoperiod of 16:8 hr light–dark. Oviposition occurred in the greenhouse, with supplementary lighting, at ca. 25°C. *P. rapae* larvae were reared on cabbage (*Brassica oleracea* L. var. Golden Acre) and *P. napi oleracea* on *Conringia orientalis* plants. Pupae were separated by sex (Richards, 1940) and kept in screen cylinders until eclosion. *Iberis amara* (candytuft) seeds were obtained from Page Seed Co., Greene, New York. *Iberis amara* and cabbage plants (4–6 weeks old) for extraction were grown in an air-conditioned greenhouse at ca. 25°C. Supplemental light was provided by 400-W multivapor high-intensity discharge lamps.

Extraction of Plant Materials. Fresh foliage was extracted in boiling ethanol for 5 min, cooled, homogenized, and filtered. The ethanolic extract was evaporated to dryness under reduced pressure and lipids were removed with *n*-hexane. The defatted residue was dissolved in water and the aqueous extract partitioned three times with 1-butanol. The butanol extract and the postbutanol water extract were concentrated under reduced pressure at ca. 50°C and stored in the refrigerator.

Fractionation of Active Compounds by Open-Column Chromatography. The butanol and the postbutanol water extracts were previously found to contain oviposition deterrent(s) and stimulant(s), respectively, to both *P. rapae* and *P. napi oleracea*. The active materials were therefore subjected to preliminary separation by open-column chromatography using 45 × 2-cm reversed-phase columns packed with 30 g 55- to 105-μm preparative C_{18} (Millipore Corporation, Milford, Massachusetts 01757). About 200 gram leaf equivalents (GLE) of the extracts were loaded onto each column. For separation of the deterrent (butanol extract), the column was sequentially eluted with 75 ml each of 0.05% acetic acid; 5%, 10%, 25%, and 100% acetonitrile in 0.05% acetic acid; and five fractions (75 ml each) were collected. For separation of the stimulants (postbutanol water extract), 25 fractions (15 ml each) were collected by sequentially eluting the column with 0.5% potassium sulfate (150 ml); water (150 ml); and 25% (15 ml), 33% (15 ml), 50% (15 ml), and 100% (30 ml) methanol in water. The collected fractions were used in bioassays to test for deterrent or stimulatory activities.

HPLC of Desulfoglucosinolates. Desulfated samples were prepared to test

for the presence of glucosinolates, according to the method of Minchinton et al. (1982). A column was packed in a 12.5-cm Pasteur pipet with 200 mg DEAE Sephadex A-25 in 0.5 mol pyridine–acetate buffer. The column was conditioned with the buffer (6 ml) followed by water (6 ml). After loading the samples (5–10 GLE in 0.5–1.0 ml water), the column was eluted with water (10 ml or until the eluate was colorless), and 1 ml 0.25% aqueous solution of sulfatase (Sigma Chemical Co.) was applied. The column was kept at room temperature overnight and then eluted with 2 ml water. The desulfated products were filtered and analyzed by HPLC on a reversed-phase C_{18} column (25 × 0.46 cm) using a solvent gradient program as follows: 0% CH_3CN in water at 0 min, 10% at 35 min, 20% at 60 min, and 100% at 70 min. The flow rate was maintained at 1 ml/min. A diode array detector (Hewlett Packard model 1040A) was used to monitor the eluate at 219 nm.

Thin-Layer Chromatography. Thin-layer chromatography (TLC) on 5 × 10-cm, 0.25-mm-thick, Whatman K6 silica gel plates was employed to separate or identify the active compounds. Three solvent systems were used: (1) ethyl acetate–methanol–acetic acid–water (4 : 1 : 1 : 0.5); (2) butanol–acetic acid–water (4 : 1 : 1); and (3) ethyl acetate–methanol–water (8 : 2 : 0.8). The plates were dried with a hair dryer immediately after development. Spots were visualized by spraying with 1% ceric sulfate solution followed by heating at 110°C for ca. 15 min.

Isolation and Purification of Deterrents. The most active fraction from the open-column chromatography was separated by HPLC using a water–acetonitrile gradient. A semipreparative reversed-phase C_{18} column (50 cm × 8 mm) was used and the flow rate was maintained at 3.3 ml/min. The solvent ratio increased linearly from water to 25% CH_3CN at 2 min, and to 30% CH_3CN at 20 min, where the composition was held constant until 30 min. The composition was then changed to 35% CH_3CN at 45 min, and then to 100% CH_3CN at 50 min. The eluate was monitored at 219 nm. The most active compounds in HPLC fractions c and f were finally purified by HPLC with the same column and the same flow rate using the following gradients (% MeOH in H_2O):

Time (min)	Fraction c	Fraction f
0	40	50
2	50	60
10	60	80
20	70	90
30	100	100

The purified compounds as well as the minor remaining components were tested separately for deterrent activity.

Bioassays. Oviposition bioassays were conducted in screen cages (48 × 48 × 48 cm) in a greenhouse as described by Renwick and Radke (1988). Eight pairs of newly emerged butterflies were transferred to each cage in the greenhouse. Each cage was supplied with a vial of 10% sucrose solution containing yellow food coloring and a cotton wick to facilitate feeding. During the preoviposition period, a cabbage plant was placed in each cage. When more than 50 eggs in one day were observed, the plant was removed and the butterflies were used for testing the next day. Treated and control plants were placed in opposite corners of the cage. Positions of plants were alternated in each cage to control for possible position effects. However, the plants were randomly arranged in a circle when three or more test plants were included in each cage, as when the activities of fractions from a column were compared. Bioassays were started at 0930 hr and the eggs laid were counted at 1530 hr. When a comparison between the two *Pieris* species was necessary, both species were tested at the same time to minimize the possible effects of differences between plant batches, intensity of sunlight, and other factors on oviposition behavior. Treated plants were sprayed with samples dissolved in a methanol–water combination (70 or 90% methanol depending on solubility of the samples). For stimulant assays, control plants were sprayed with solvent alone or with cabbage postbutanol water extract (as a standard of stimulant). In deterrent assays, control plants were sprayed with solvent alone. The solutions were applied in a fine mist with a chromatographic sprayer to both upper and lower leaf surfaces. The concentrations applied were expressed as gram leaf equivalents (GLE) of the original plant foliage, and 5 GLE was used for each replication. Deterrent activity was monitored by applying test solution on cabbage plants grown individually in 10-cm cord pots. Stimulatory effects were tested using neutral (stimulants were not present) bean (*Phaseolis vulgaris* var. Sieva) plants as the oviposition substrate. The bean plants used for bioassays were presented as single plants at the two-leaf stage in plastic pots (6.25 × 6.25 cm).

Design and Analysis. A randomized complete block design was used in the bioassays. A replication consisted of one cage with eight pairs of butterflies, and four to eight replications were performed for each bioassay. When cabbage postbutanol water extracts were used as controls and when comparisons between bioassays were necessary, the relative stimulatory activities were presented as an oviposition stimulant index (OSI), where

$$\text{OSI} = 100\ (\text{treated} - \text{control})/(\text{treated} + \text{control})$$

Deterrent activities were compared by calculating an oviposition deterrent index (ODI), i.e.,

$$\text{ODI} = 100\ (\text{control} - \text{treated})/(\text{control} + \text{treated})$$

For assays with only one treated plant and one control plant, the proportion of

eggs laid on the treated plant to that on both treated and control plants was calculated, and the data were subjected to arcsine square root transformation and analyzed using a one-sample t test, under the null hypothesis that the total number of eggs was distributed evenly over treated and control plants. A Waller-Duncan K-ratio t test was used to assess significance of differences among treatments when three or more test plants were included in one bioassay.

Identification. Identification of the stimulants and deterrents was accomplished by comparing their UV spectra, hR_fs on TLC, and retention times on HPLC with those of the standard or known compounds. UV spectra of isolated compounds were obtained from the diode array detector in water–acetonitrile. The major glucosinolate **(2)** in the plant was collected as its desulfated product **(2a)** by HPLC on the semipreparative reversed-phase C_{18} column (50 cm × 8 mm) using a water–acetonitrile gradient as follows: 0% CH_3CN at 0 min, 1% CH_3CN at 15 min, 6% CH_3CN at 20 min and 100% CH_3CN at 25 min. The structure of the isolated desulfoglucosinolate **(2a)** was confirmed by ^{13}C NMR spectroscopy. The spectrum of the sample dissolved in MeOH-d_4 was recorded on a Bruker AM 300 NMR spectrometer at 75 MHz. The solvent was used as an internal reference.

RESULTS

Deterrents. The butanol extract of *I. amara* was strongly deterrent to both ovipositing *P. rapae* and *P. napi oleracea* (Huang and Renwick, 1993). After this extract was fractionated by open-column chromatography, the fractions differed in their deterrence (Figure 1). Both *Pieris* species were significantly deterred by fraction 5. However, the deterrence was much stronger for *P. rapae* than for *P. napi oleracea*, based on the relative numbers of eggs laid on the treated and control plants. Significantly fewer eggs were laid by *P. rapae* on the plants treated with fractions 1, 2, 3, or 4 than on the control plants, but the deterrence of each of these fractions was much weaker than that of fraction 5. Fractions 1, 2, 3, and 4 were not deterrent to *P. napi oleracea* when compared with the control.

HPLC of fraction 5 from the open-column chromatography revealed two major peaks and many minor peaks (Figure 2). Preliminary oviposition bioassays showed that both HPLC fractions II and III (Figure 2) were strongly deterrent to *P. rapae* but not to *P. napi oleracea* (Table 1). Fraction I was deterrent to both *Pieris* species, but the ODIs were relatively small. Fraction IV had no deterrent effect on either species. In fact, more eggs were laid by *P. napi oleracea* on plants treated with this fraction than on control plants, so that an ODI of −21.2 was obtained. This indicated the possible presence of stimulant(s) in the fraction. After fractions I, II, and III were further separated into smaller

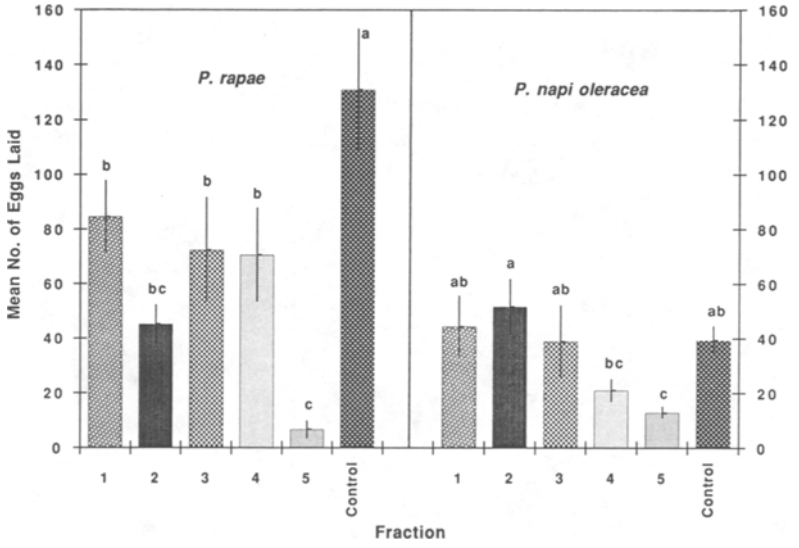

FIG. 1. Oviposition (deterrent assay) by *P. rapae* and *P. napi oleracea* on cabbage plants treated with different open-column fractions from the butanol extract of *I. amara* or with 90% MeOH in H_2O (control). Five gram leaf equivalents were used for each replication, replicated four times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$).

fractions (Figure 2), the highest deterrent activity on *P. rapae* was found in fractions c and f (Table 1, ODIs = 58.8 and 77.9, respectively). Significantly fewer eggs were laid by *P. rapae* on plants treated with fraction b, d, or g than on control plants, but the ODIs were smaller than those for fraction c or f. Fractions a and e were not significantly deterrent to *P. rapae* ($P > 0.05$). Fractions a, b, f, and g were not deterrent to *P. napi oleracea*.

Purification of each active compound in fractions c and f in Figure 2 was accomplished by HPLC using a methanol–water gradient. The hR_fs of these purified compounds on TLC in solvent system 3 were 74.2 and 78.0, respectively. The retention times of the same compounds on HPLC (Figure 2) were 20.23 min and 37.85 min. These features, as well as their UV spectra (maximum absorption at about 240 nm), were identical to those of the two glycosides identified by Sachdev-Gupta et al. (1993a) from a similar extract of the same plant species as 2-O-β-D-glucosyl cucurbitacin I and 2-O-β-D-glucosyl cucurbitacin E. We therefore conclude that the two major oviposition deterrents to *P. rapae* are these same compounds.

Significantly fewer eggs were laid by *P. rapae* on cabbage plants treated

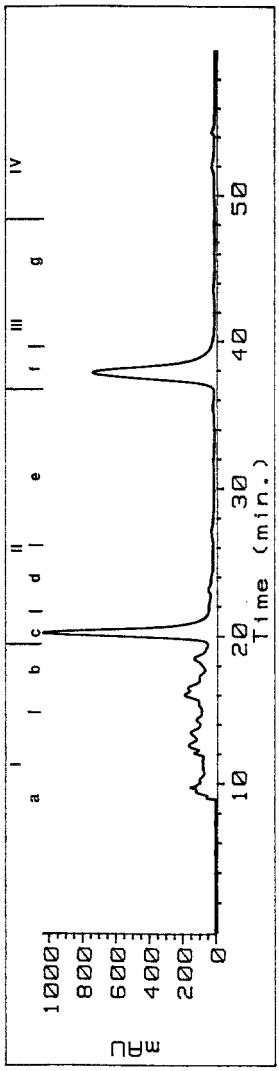

FIG. 2. HPLC separation of the open-column fraction 5 from the butanol extract of *I. amara*. UV monitoring at 219 nm.

TABLE 1. OVIPOSITION BY *Pieris rapae* AND *P. napi oleracea* ON CABBAGE PLANTS TREATED WITH HPLC FRACTIONS OF ACTIVE OPEN-COLUMN FRACTION FROM *Iberis amara* BUTANOL EXTRACT

HPLC fractions[a]	Replications[b]	*P. rapae*			*P. napi oleracea*		
		Eggs[c]	$P < 0.05$[d]	ODI[e]	Eggs[c]	$P < .05$[d]	ODI[e]
I	4	88	*	52.7	111	*	26.4
Control		263			186		
II	8	22	*	85.7	123		2.4
Control		253			124		
III	8	45	*	80.4	120		22.5
Control		342			176		
IV	4	189		22.0	119	*	−21.2
Control		270			81		
a	4	138		2.8	125		−2.29
Control		139			117		
b	4	110	*	37.3	66		25.2
Control		229			109		
c	8	91	*	58.8			
Control		330					
d	4	134	*	14.1			
Control		174					
e	4	185		−5.5			
Control		206					
f	8	47	*	77.9	141		4.8
Control		395			158		
g	4	206	*	27.1	139		5.0
Control		362			146		

[a] Fractions are shown in Figure 2. Control = 90% MeOH.
[b] A replication consists of one bioassay cage with eight pairs of butterflies.
[c] Average number of eggs laid per replication.
[d] One-sample *t* test for comparison between numbers of eggs laid on treatment and control. Significant difference is indicated with an asterisk ($P < 0.05$).
[e] ODI = 100 (control − treated)/(control + treated).

with either 2-O-β-D-glucosyl cucurbitacin I or 2-O-β-D-glucosyl cucurbitacin E than on the control plants (Figure 3). The minor components obtained from fractions c and f were not significantly deterrent to *P. rapae* (from fraction c: $P > 0.05$, ODI = 8.32; from fraction f: $P > 0.05$, ODI = 7.53). No deterrent effects of either the isolated 2-O-β-D-glucosyl cucurbitacin I or 2-O-β-D-glucosyl cucurbitacin E on *P. napi oleracea* were observed (Figure 3).

Stimulants. Two major glucosinolates were detected by HPLC of the desulfated products of the postbutanol water extract of *I. amara*. These desulfoglucosinolates were referred to as **1a** (retention time 10.67 min) and **2a** (14.08

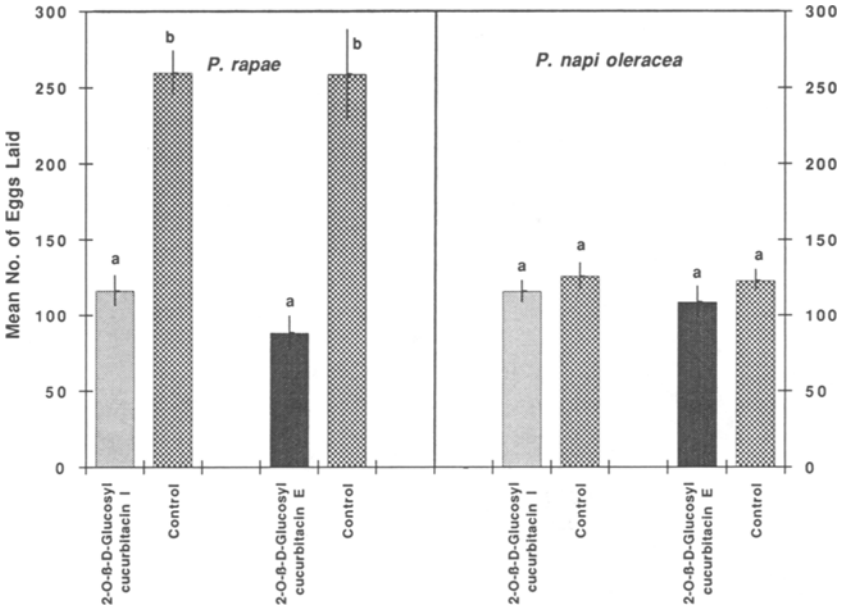

FIG. 3. Oviposition (deterrent assay) by *P. rapae* and *P. napi oleracea* on cabbage plants treated with 2-O-β-D-glucosyl cucurbitacin I and 2-O-β-D-glucosyl cucurbitacin E isolated from *I. amara* or with 90% MeOH in H_2O (control). Five gram leaf equivalents were used for each replication, replicated eight times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that eggs were distributed evenly over control and treated plants.

min), i.e., the desulfated products of glucosinolates **1,** and **2,** respectively. The concentration of **2a,** based on the peak areas of the HPLC chromatograms, was about 28 times higher than that of **1a.** HPLC of the desulfated samples of each of the 25 fractions from the open-column chromatography of the postbutanol water extract showed that the two glucosinolates were limited to fractions 5–11. No glucosinolates were detected in any of the other fractions. When a combination of fractions 5–11 was compared with four combinations of other fractions (Figure 4), significantly more eggs were laid by both *Pieris* species on bean plants treated with fraction 5–11 than on plants treated with other combinations or solvent (control). The numbers of eggs laid by the two insect species on plants treated with fractions 1–4, 12–15, 16–20, or 21–25 were not significantly different from those on control plants. The results indicated that the most significant stimulants were restricted to fractions 5–11. When the combination of fractions 5–11 was compared with an equivalent cabbage postbutanol water

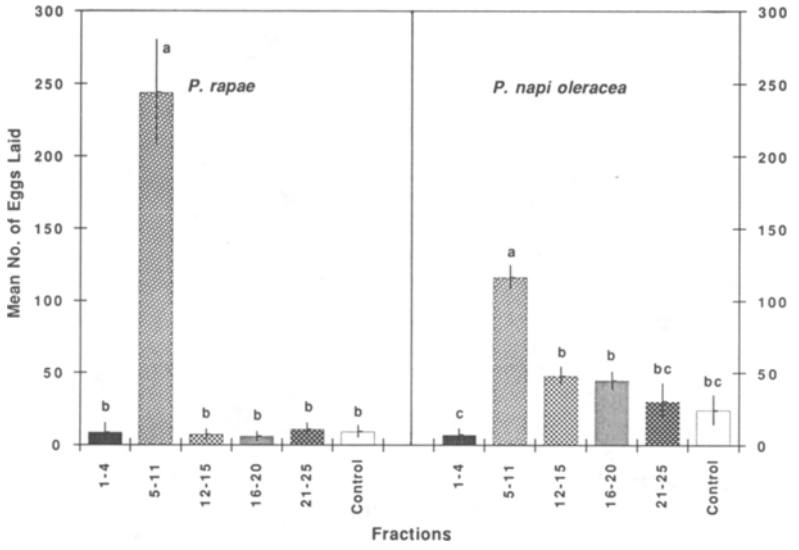

FIG. 4. Oviposition (stimulant assay) by *P. rapae* and *P. napi oleracea* on bean plants treated with different combinations of open-column fractions from the postbutanol water extract of *I. amara* or with 80% MeOH in H_2O (control). Five gram leaf equivalents were used for each replication, replicated four times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$).

extract (containing oviposition stimulants to both insect species), significantly more eggs were laid by both *Pieris* species on plants treated with the fractions than on plants treated with the cabbage extract (Figure 5). The OSIs for *P. rapae* and *P. napi oleracea* were 11.96 and 60.60, respectively. The results suggested that the activity of fractions 5–11 could account for the stimulatory effects of the postbutanol aqueous extract of *I. amara* foliage.

HPLC of desulfoglucosinolates of fractions 8–11 showed a single peak **(2a),** and TLC of these fractions in solvent system 1 also revealed only one visible spot. It was therefore concluded that the major compound in fractions 8–11 was the glucosinolate **2** and that the other glucosinolate **(1)** was restricted to fractions 5–7. TLC of fractions 5–7 showed several spots, including the one present in fractions 8–11 and others that were most abundant in the inactive fractions 3 and 4. After fractions 5–7 were reloaded onto the same C_{18} column and eluted with the same solvent system, more of compound **2** was obtained in fractions 8–11 and more of the inactive compounds were removed from fractions 5–7 into fractions 3 and 4. The same chromatographic procedure was repeated to achieve more complete separation. Fractions 8–11 from each run, which

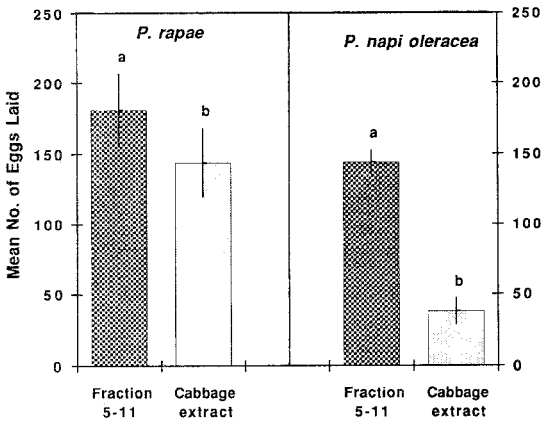

FIG. 5. Oviposition (stimulant assay) by *P. rapae* and *P. napi oleracea* on a choice of bean plants treated with open-column fractions 5–11 from the postbutanol water extract of *I. amara* or with the postbutanol water extract of cabbage (control). Five gram leaf equivalents were used for each replication, replicated eight times. A replication consists of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that eggs were distributed evenly over control and treated plants.

contained only glucosinolate **2,** were then combined. The purity of **2** in this sample was verified by TLC of the intact glucosinolate or by HPLC of the desulfoglucosinolates. Similarly, the fractions 5–7 from each run containing glucosinolate **1** were combined. HPLC of the desulfated products of this sample revealed a small portion (ca. 3%) of **2a** as well as **1a.**

Many more eggs were laid by *P. rapae* on plants treated with the isolated glucosinolate **2** than on the control plants (Figure 6). Significantly fewer eggs were laid in response to glucosinolate **1** even though some stimulation compared with the control was still obtained. In the case of *P. napi oleracea* (Figure 6), glucosinolates **1** and **2** had almost equal stimulatory activity.

The UV spectra of **1a** and **2a,** with the maximum absorption at ca. 230 nm, indicated that both glucosinolates were aliphatic. Their UV spectra, as well as retention times on HPLC were identical to those of the desulfated products of authentic glucoiberin (3-methylsulfinylpropyl glucosinolate) and sinigrin (allyl glucosinolate), respectively. TLC of isolated **1** and **2** or their desulfated products (**1a** and **2a**) in solvent systems 1 and 2 revealed the same hR_fs as for glucoiberin and sinigrin or their desulfated products (Table 2). ^{13}C NMR chemical shifts of **2a** at 153.32, 135.19, 117.63, 82.75, 81.92, 79.49, 74.28, 71.26, 62.67, and 37.52 confirmed its identity as desulfosinigrin (Sachdev-Gupta et al., 1992).

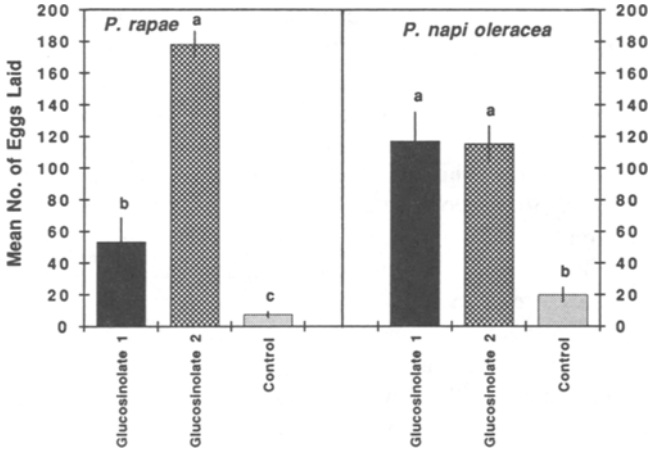

FIG. 6. Oviposition (stimulant assay) by *P. rapae* and *P. napi oleracea* in response to a choice of bean plants treated with glucosinolates **1** and **2** from the postbutanol water extract of *I. amara*. Five gram leaf equivalents were used for each replication. Control = 80% MeOH in H_2O. Replicated eight times. A replication consists of one bioassay cage with eight pairs of butterflies. Means ($\pm$SE) with the same letters in each series are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$).

TABLE 2. TLC OF ISOLATED GLUCOSINOLATES, THEIR DESULFATED PRODUCTS, AND RELEVANT AUTHENTIC COMPOUNDS

Compounds	hR_f in	
	Solvent system 1	Solvent system 2
1 or glucoiberin	4.5	11.0
2 or sinigrin	26.6	30.0
1a or desulfated glucoiberin	24.0	33.8
2a or desulfated sinigrin	70.1	70.3

From these results we conclude that the primary oviposition stimulants to *P. rapae* and *P. napi oleracea* in *I. amara* are sinigrin and glucoiberin.

Previous studies on another crucifer species (Huang et al., 1993) showed that glucoiberin was stimulatory to both *Pieris* species, although *P. napi oleracea* was much more sensitive to this stimulant than was *P. rapae*. In this study, commercial sinigrin was tested in comparison with cabbage extract and at 1 mg/plant was no more stimulatory to *P. rapae* than the cabbage extract (4 GLE/plant) (one-sample *t* test, $P > 0.05$, OSI = -5.80). However, *P. napi oleracea*

preferred the sinigrin-treated plant over the cabbage extract-treated plant (one-sample t test, $P < 0.05$, OSI = 25.10).

DISCUSSION

The results of this study indicate that the isolated 2-O-β-D-glucosyl cucurbitacin I and 2-O-β-D-glucosyl cucurbitacin E can account for most of the oviposition deterrent activity of *I. amara* extracts to *P. rapae*. These compounds were recently identified from *I. amara* during an investigation of the refusal of *P. rapae* larvae to feed on the plant. The cucurbitacin E glucoside was found to deter larval feeding, but the cucurbitacin I glucoside was inactive as a feeding deterrent (Sachdev-Gupta et al., 1993a).

Cucurbitacins are well known constituents of the Cucurbitaceae and act as potent feeding stimulants for adapted *Diabrotica* species (Metcalf et al., 1982; Ferguson et al., 1983; Deheer and Tallamy, 1991). However, the presence of cucurbitacins in plants is thought to function as a defense against herbivory in general, and *Iberis* is one of the few cruciferous genera that produce this class of compounds. Previous studies have shown that cucurbitacins E and I from foliage of *I. amara* inhibit feeding by the flea beetle, *Phyllotreta nemorum* (Nielsen, 1978). This author also reported the presence of minor quantities of glycosides in extracts, but these were not identified. Usher and Feeny (1983) tested cucurbitacins for possible toxicity to *Pieris rapae* larvae by adding the compounds to artificial diet, but no deleterious effect was detected. Most such studies of cucurbitacins have dealt with the aglycones. However, our results clearly show that the glycosides of these triterpenoids can have significant biological activity. To our knowledge, this is the first report of cucurbitacins, in this case glycosides, affecting oviposition behavior.

Although these cucurbitacin glycosides may largely explain the avoidance of *I. amara* by ovipositing *P. rapae*, other compounds appear to add to the deterrent activity of extracts. Fraction 5 from the open column separation of butanol extracts was clearly the most deterrent to *P. rapae*, but some reduction in the numbers of eggs laid in response to fractions 1, 2, 3, and 4 was also obtained when compared with the controls (Figure 1). Similarly, after HPLC of fraction 5, some activity was detected in fractions b, d, and g as well as in c and f, which contained the two cucurbitacin glycosides (Table 1). The additive effect of individual constituents was particularly noticeable in the case of *P. napi oleracea*, which was not deterred by the isolated cucurbitacin glycosides. Limited deterrent activity obtained from open column fractions (Figure 1) was much weaker than that of the original butanol extracts (Huang and Renwick, 1993; ODI = 67.7). After HPLC of fraction 5, only the first fraction resulting from this separation was deterrent to *P. napi oleracea* (ODI = 26.4, Figure 2, Table 1).

The oviposition stimulant activity of aqueous extracts of *I. amara* to both *P. rapae* and *P. napi oleracea* is clearly associated with specific glucosinolates. However, the two *Pieris* species respond differently to the individual glucosinolates present in the plant. The sinigrin and glucoiberin identified from stimulatory extracts appear to account for almost all the activity. However, *P. napi oleracea* was more strongly stimulated by the low concentration of glucoiberin, whereas *P. rapae* was more responsive to the high concentration of sinigrin. Previous work on *Erysimum cheiranthoides* also indicated that *P. napi oleracea* was much more strongly stimulated by glucoiberin in that plant than was *P. rapae* (Huang and Renwick, 1993).

Glucosinolate analyses of various plant parts of *I. amara* have been conducted previously by several authors. Glucoiberin was reported as the most abundant glucosinolate in seeds of this plant (Daxenbichler et al., 1991) and has also been found in roots and foliage (reviewed by Kjaer, 1960). Cole (1976) detected only 4-methylthioalkyl glucosinolates in the whole plant. However, our study has identified sinigrin as the major glucosinolate, with a relatively minor quantity of glucoiberin in foliage samples. We have compared the glucosinolate composition of *I. amara* from various sources, by HPLC of the desulfoglucosinolates. Seeds obtained from Agway (Ithaca, New York), the Page Seed Company, Greene, New York, and from Denmark (a gift from Dr. J.K. Nielsen) were used to grow plants for analysis, and sinigrin was found to be a major constituent of foliage in all cases.

Sinigrin has been widely tested as a behavior-modifying compound in plant–insect interactions. This glucosinolate is a feeding stimulant for a number of crucifer specialists such as the flea beetles *Phyllotreta nemorum* (Nielsen, 1989) and *P. armoraciae* (Hagerup et al., 1990). Nielsen et al. (1989) found that sinigrin was the major glucosinolate in garlic mustard, *Alliaria petiolata*, and it stimulated feeding by the monophagous weevil, *Ceutorhynchus constrictus*. However, this activity was only expressed in the presence of unknown polar compounds from the plant. Furthermore, insects that are associated with glucosinolate-containing plants do not always react positively to sinigrin. Bartlet and Williams (1991) found that the addition of sinigrin to rejected plants did not make these acceptable to *Psylliodes chresocephala*.

The involvement of sinigrin in stimulating oviposition by *Pieris* butterflies appears to be quite variable. When pure sinigrin was compared with glucobrassicin as an oviposition stimulant for *P. rapae*, its activity was relatively low (Traynier and Truscott, 1991; Renwick et al., 1992). In bioassays using green cards as a substrate, Renwick and Radke (1983) demonstrated that sinigrin could stimulate oviposition by *P. rapae*, but the response was inconsistent, and the butterflies failed to discriminate between treated and control cards. In our present study, open column chromatography of *I. amara* extracts provided glucosinolate-containing fractions that were stimulatory to both *P. rapae* and *P. napi*

oleracea. Fractions 5–11 were more acceptable to the butterflies than an equivalent amount of cabbage extract, based on fresh weight of the foliage (Figure 5). However, the concentration of sinigrin in *I. amara*, based on HPLC of the desulfoglucosinolates, was about three times the concentration of glucobrassicin in cabbage. The isolated sinigrin was more stimulatory to *P. rapae* than was the sample containing the glucoiberin (Figure 6). Thus sinigrin appears to be largely responsible for stimulating the limited oviposition by *P. rapae* on *I. amara*. In a related study (Huang and Renwick, 1993), the intact plants and postbutanol water extracts of *Brassica juncea*, when compared with cabbage plants and cabbage extract, respectively, were much more stimulatory to ovipositing *P. rapae* and *P. napi oleracea*. As previously reported (Sachdev-Gupta et al., 1992), sinigrin was the most abundant glucosinolate in *B. juncea* (confirmed by HPLC of desulfoglucosinolates). Bioassays of open column fractions of *B. juncea* extracts showed that the sinigrin-containing fractions were most active in stimulating oviposition by *P. rapae*. The butterflies discriminated well between bean plants treated with the isolated sinigrin or with solvent alone. Thus the role of sinigrin in host selection by *P. rapae* in nature is still not clear and may depend on the presence or absence of other chemical cues provided by the plant.

Acceptance or rejection of potential host plants by insects is believed to depend on a balance of stimulants and deterrents present in the plants (Dethier, 1982; Miller and Strickler, 1984; Renwick and Radke, 1987). This comparative study demonstrates that differences in host ranges of related species may be explained by differences in their sensitivity to the chemical stimuli involved. Both *Pieris* species were stimulated by glucosinolates present in *I. amara*, but they responded quite differently to deterrents. The preference of *P. napi oleracea* for *I. amara* over cabbage may be explained by its lack of sensitivity to the deterrent cucurbitacin glycosides, whereas these compounds greatly inhibit oviposition by *P. rapae* in a choice situation.

In a related study, deterrents from *Erysimum cheiranthoides* responsible for blocking oviposition by *P. rapae* were identified as strophanthidin-based cardenolides (Renwick et al., 1989; Sachdev-Gupta et al., 1990). Other cardenolides present in the plant were inactive as oviposition deterrents, but proved to be potent feeding deterrents to the larvae (Sachdev-Gupta et al., 1993b). Similarly, in this investigation we have found that two cucurbitacin glucosides are highly effective oviposition deterrents to *P. rapae*, but only one of these has activity as a larval feeding deterrent. We can therefore conclude that different life stages of an insect may rely on different semiochemicals or combinations of semiochemicals for discrimination between potential host plants.

The differential acceptance of *I. amara* by *P. rapae* and *P. napi oleracea* is clearly attributable, at least in part, to their differential sensitivities to the positive and negative stimuli provided by the plant. A similar divergence in

preference has been noted for *E. cheiranthoides* (Huang and Renwick, 1993), which is completely rejected by *P. rapae*. In this case, different responses have also been linked to differences in sensitivity of the two species to the glucosinolates and cardenolides (Huang et al., 1993). Further studies are needed to establish structure–activity relationships for responses of the two species to stimulants and deterrents and to determine whether differences in sensitivity occur at the peripheral sensory level or in the CNS processing of the chemical information.

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EVIDENCE FOR MEDIATION OF TWO RELEASER PHEROMONES IN THE AGGREGATION BEHAVIOR OF THE GREGARIOUS DESERT LOCUST, *Schistocerca gregaria* (FORSKAL) (ORTHOPTERA: ACRIDIDAE)

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Abstract—The response of nymphal and adult gregarious phase desert locust, *Schistocerca gregaria*, to a choice of two columns of air, one permeated with airborne volatiles emanating from nymphs or adults and the other untreated, was investigated in a single-chamber bioassay arena. The nymphs, whether released individually or in groups, preferred to be within the precinct of the air column treated with airborne volatiles of the nymphs but were indifferent to volatiles of the adults. Conversely, older adults responded only to their own volatiles but not to those of the nymphs or young adults. The young adults were responsive only to volatiles of the older adults. Charcoal-trapped volatiles from the nymphs and the adults reproduced the effect of living locusts. These results indicate that there are two different aggregation pheromones in *S. gregaria*: a juvenile pheromone produced by nymphs and an adult pheromone specific to adults.

Key Words—Airborne volatiles, bioassay, aggregation pheromones, gregarious locusts, olfactometer, semiochemicals, Orthoptera, Acrididae, *Schistocerca gregaria*.

INTRODUCTION

An important feature of locusts is their ability to reversibly transform between two extreme phases, solitaria and gregaria (Uvarov, 1966). Individuals in the two phases differ in morphology, physiology, and behavior (Uvarov, 1966; Steedman, 1988). The most striking feature of gregarious-phase locusts is their

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tendency to aggregate. They clump together in dense groups, march in bands as wingless hoppers, or swarm over long distances as adults (Steedman, 1988). Although phase dynamics is predicated on locust density, a number of mediating factors have been implicated, including visual (Ellis and Pearce, 1962), tactile (Chauvin, 1941; Ellis, 1959, 1962) and chemical (Nolte, 1963; Gillett, 1968) stimuli. Moreover, dietary factors (Jackson et al., 1978) and previous phase history of the locust (Michel, 1980) have also been shown to influence phase characters.

The mediation of a gregarizing pheromone was first recognized by Nolte (1963) and later confirmed by Gillett (1968, 1975), following observations that isolated individuals of several locust species, including *Schistocerca gregaria*, when kept in the same room with crowded locusts, continued to retain the pigmentary, morphometric, and grouping traits of the gregaria. Associated with gregarization was an apparent increase in chiasma frequency in adult males (Nolte, 1968; Nolte et al., 1973). Using this as the principal bioassay to quantify effects of the pheromone, Nolte et al. (1970) traced the source of the pheromone to the hopper feces. Examination of the constituents of the steam distillate of the feces of crowded *Locusta migratoria* hoppers led to the identification of 5-ethylguaiacol (2-methoxy-5-ethylphenol) referred to as locustol, as the principal component of the gregarization pheromone (Nolte et al., 1973; Nolte, 1976).

However, there have been serious doubts about the role of 5-ethylguaiacol in locust gregarization (Whitman, 1990). Although it was apparently effective in enhancing chiasma frequencies, it was not as effective in inducing other gregarious morphological traits (Nolte et al., 1973; Nolte, 1976). In any case, the close-to-lethal doses used (Nolte, 1976) make uncertain the biological validity of the results. Moreover, Gillett (1983) failed to observe a significant effect of 5-ethylguaiacol on the grouping behavior of nymphal *S. gregaria*. Recently, Fuzeau-Braesch et al. (1988) analyzed airborne volatiles collected from the cages of gregarious *S. gregaria* and *L. migratoria* (which included feces) but were unable to detect 5-ethylguaiacol and suggested that it may well be an artifact of steam distillation, the isolation technique used by Nolte et al. (1973).

In their study, Fuzeau-Braesch et al. (1988) sought to identify compounds present in laboratory gregarious-phase populations and to investigate their releaser effects on the aggregation behavior of similarly reared locusts. Airborne collections of different stages of *S. gregaria* and *L. migratoria* showed the presence of varying amounts of four components, three of which were identified as phenol, guaiacol, and veratrole. Behavioral tests indicated that the compounds, alone or in mixtures, were unattractive to locusts. However, phenol, guaiacol, and the mixture of the three elicited significant clumping behavior in both species and were considered to act as "cohesion" pheromones.

While the approach employed by Fuzeau-Braesch et al. (1988) is sound, several criticisms may be leveled against their methodologies. First, their col-

lection of volatiles was clearly inefficient based as it was on the condensation of these in an ice bath (0°C); minor and more volatile components could have escaped detection. Second, the complete volatile compositions emanating from the locusts were not assayed and, therefore, no basis exists for evaluating the relative importance of the identified compounds in the pheromone blend. Third, it is difficult to understand the rationale for a five-choice olfactometer design (four compartments connected to a central compartment in the form of a cross) in essentially a two-choice bioassay. Indeed, examination of their data suggests a consequent loss of sensitivity of the assay. Moreover, individual gregarious locusts were not bioassayed, which could have established if the pheromone blend does indeed act only when visual and tactile stimuli are already operational as proposed by the authors.

We have initiated a comprehensive reinvestigation of the pheromone system mediating both the primer and releaser effects associated with locust gregarization, and in this paper we describe the aggregative responses of gregarious nymphal and adult *S. gregaria* to their airborne volatiles.

METHODS AND MATERIALS

Insects. Crowded desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) from the ICIPE colony originating from a stock obtained from The Desert Locust Control Organization for Eastern Africa (DLCO-EA) in Addis Ababa, Ethiopia, was used for the study. Insects (300–400) of both sexes were bred under crowded conditions in aluminum cages (50 × 50 × 50 cm). They were reared in a special room (4.5 × 4.5 m) that was well aerated by a duct system that maintained a negative pressure with a nycthemeral temperature of 30–35°C and a light cycle of 12 : 12 hr light–dark. Fresh sorghum shoots (Serena variety) and wheat bran were provided daily.

Collection of Volatiles. Volatiles were collected on traps using charcoal as adsorbent (80–100 mesh Chrompack). Before use, the charcoal was cleaned by Soxhlet extraction with dichloromethane (Merck) for 72 hr, followed by activation under nitrogen (20 ml/min) at 250°C. Volatiles were collected separately from 100 individuals each of third- and fifth-instar nymphs and of young (four to eight days after ecdysis) and older adults (16–20 days after ecdysis), contained in a 5-liter three-neck round-bottomed flask. Charcoal traps were prepared by packing between two glass wool plugs ca. 1.2 g of charcoal in 6-cm-long × 8-mm-ID glass tubes. Air filters were prepared similarly and each consisted of a 6-cm-long × 25-mm-ID glass tube containing 4.2 g of charcoal. The two side necks of the flask were fitted with the activated charcoal columns, with the central neck closed with a stopper. All the joints were sealed with Teflon tape. A vacuum pump (Cole-Parmer air-cadet) sucked air through the collection appa-

ratus at 300 ml/min. Clean air (passed through the other activated charcoal column) was continuously drawn over locusts, which were kept at a temperature of 30 ± 1°C for 24 hr. All the charcoal traps were eluted with 8 ml HPLC-grade dichloromethane (Aldrich Ltd.) and concentrated under a stream of nitrogen to 300 μl at 0°C. All the volatile concentrates were stored in the freezer at −15°C until use.

Olfactometer Design. Bioassays were conducted in a glass chamber (60 × 30 × 30 cm) that minimized adsorption and subsequent desorption of active chemicals and allowed proper cleaning between experiments (Figure 1). A removable wire gauze covered the top. An aluminum metal plate drilled with 2-mm-diam. holes, 1 cm apart, was fitted in the bottom of the chamber, each half of which was attached to a square pyramidal aluminum funnel (base length 28 cm). Each funnel was connected by Teflon tubing to a 2-liter round-bottomed flask. Air from a compressed air cylinder, purified by passing through a charcoal filter, was split into two streams each passing into one flask and then into one of the two sides of the arena at a flow rate of 120 ml/min/side. One flask acted as a source of locust volatiles. Test insects were introduced into the olfactometer through a small door on the front of the chamber between the funnels. The olfactometer was placed in an extraction hood fitted with two 60-cm diffused-light tubes (60 W) to provide uniform illumination and was kept in a laboratory whose temperature was maintained at 30 ± 1°C. Between experiments, the connector tubings and flowmeters were flushed with clean air and were also changed regularly to minimize contamination with volatiles.

Responses to Volatiles Emanating Directly from Live Insects. We studied the behavioral responses of *S. gregaria* to a choice of two sides of the arena, one permeated with clean air and another with air enriched with airborne volatiles of locusts. Enrichment with locust volatiles was effected by placing 10 insects in the flask linked to the appropriate side of the olfactometer. No visual contact was possible between test insects and those used as odor source.

Locusts were released into the olfactometer singly or in groups of 10. After 30 min the number of locusts in each section of the arena was measured (previous 2-hr observations had demonstrated no significant change in locust distribution with time). Using single individuals eliminated possible effects of visual and tactile stimuli provided by other locusts. Second-, third-, fourth-, and fifth-instar nymphs, young and older adults (all mixed sex) were used as test insects and as odor sources. Tests involving groups were replicated 10 times each, while those involving individual insects were replicated 100-times. In all tests, locusts were used only once and then discarded. Uncommitted insects found in the middle part of the olfactometer were treated as nonresponders. The aggregation index (AI) was calculated as $100\ (T - C)/N$ where T is the number of locusts found in the treated compartment, C is the number of locusts found in the control

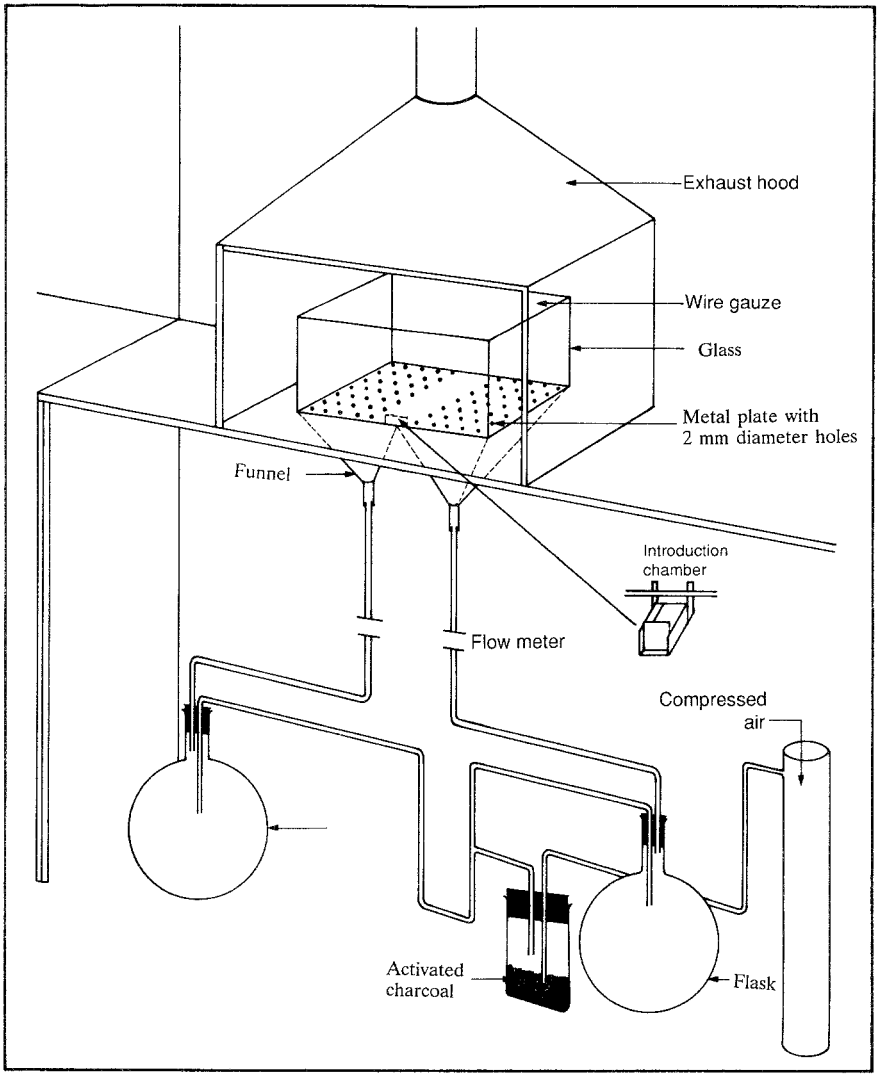

FIG. 1. Diagram of single-chamber choice olfactometer bioassay.

compartment, and N is the total number of locusts tested. Differences between treatments were tested using the chi-square test.

Response to Charcoal-Trapped Volatiles. Assays were carried out in the olfactometer to assess the responses of *S. gregaria* to the charcoal-trapped volatile extracts in two different sets of experiments described below. In both experiments, the concentrated extracts were made up to 500 μl with dichloro-

methane before use and transferred into 3.7-ml glass vials, each containing 2 ml of light paraffin oil (Merck) to ensure slow release of volatiles. Each vial was capped with a screw cap, which had a 1.5-mm vent. Doses were expressed as locust emission hours (LH) (1 LH = volatiles emitted by 1 locust for 1 hr). The test solution was held in one flask and the control (2 ml paraffin oil mixed with pure dichloromethane) in the other flask of the olfactometer. Test insects were released either singly or in groups.

In the first set of experiments, charcoal-trapped volatiles from third- and fifth-instar nymphs and older adults were tested at a single dose of 96 LH against control. Second-, third-, fourth-, and fifth-instar nymphs and older adults were released singly, as described earlier, into the olfactometer. A similar experiment was conducted on trapped volatiles from young adults (four to eight days after ecdysis) at a single dose of 96 LH, and the effects on aggregation of fourth- and fifth-instar nymphs, young and older adults released in groups of 10 in the olfactometer were determined.

In the second set of experiments trapped volatiles collected from fifth-instar nymphs and older adults were tested to determine dose effects. Doses of 24, 48, 96, and 240 LH were tested against controls. The effects of these treatments on aggregation of fifth-instar nymphs and older adults released in groups of 10 in the olfactometer were determined. Tests were replicated ten times.

Throughout the study, two blank controls were run periodically and we systematically switched sides of the chamber for the control and the treatment to minimize bias.

RESULTS

Distribution in absence of Odor. Preliminary observations were carried out to assess the distribution of locusts in the two sections of the olfactometer in the absence of an odor source. All stages of the locusts were randomly distributed with no preference for either of the sections. The number of locusts found in the two sections did not significantly vary with time up to 120 min. This indicated that there were no subtle factors affecting the distribution of insects in the choice olfactometer.

Responses to Airborne Volatiles. Tables 1 and 2 summarize the olfactometric distribution of locusts released singly and in groups of 10, respectively. No significant differences were observed between the two. Table 3 gives the distribution of locusts to trapped volatile extracts. The two sets of results show clearly that the nymphs responded only to nymphal volatiles but were indifferent to the adult volatiles. Conversely, the older adults responded only to their own volatiles but not to those of young adults and nymphs. Young adults responded only to volatiles of the older adults but were indifferent to their own and to

TABLE 1. BEHAVIORAL RESPONSES OF SINGLY RELEASED *S. gregaria* TO AIRBORNE VOLATILES OF LIVE NYMPHS AND ADULTS IN CHOICE OLFACTOMETER

Odor source[a]	Test insects[b]	Number in control	Number in stimulus	Aggregation index (%)[c]
2nd instar	2nd instar	9	87	78**
3rd instar	2nd instar	10	86	76**
4th instar	2nd instar	11	84	73**
5th instar	2nd instar	13	83	70**
Older adults	2nd instar	46	50	4 NS
2nd instar	3rd instar	9	89	80**
3rd instar	3rd instar	10	85	75**
4th instar	3rd instar	10	86	76**
5th instar	3rd instar	11	87	76**
Older adults	3rd instar	49	48	−1 NS
2nd instar	4th instar	10	85	75**
3rd instar	4th instar	10	87	77**
4th instar	4th instar	9	86	77**
5th instar	4th instar	8	88	80**
Young adults	4th instar	50	44	−6 NS
Older adults	4th instar	48	50	2 NS
2nd instar	5th instar	14	84	70**
3rd instar	5th instar	12	84	72**
4th instar	5th instar	12	83	71**
5th instar	5th instar	11	86	75**
Young adults	5th instar	47	48	1 NS
Older adults	5th instar	50	48	−2 NS
2nd instar	Young adults	51	49	−2 NS
3rd instar	Young adults	47	50	3 NS
4th instar	Young adults	48	49	1 NS
5th instar	Young adults	49	50	1 NS
Young adults	Young adults	51	45	−6 NS
Older adults	Young adults	14	84	70**
2nd instar	Older adults	48	50	2 NS
3rd instar	Older adults	48	51	3 NS
4th instar	Older adults	51	49	2 NS
5th instar	Older adults	49	49	0 NS
Young adults	Older adults	49	46	−3 NS
Older adults	Older adults	13	85	72**

[a] 10 locusts in a 2-liter flask. Air flow = 120 ml/min.
[b] 100 locusts tested per replicate.
[c] Difference from control (χ^2 test) indicated by: NS = not significant; ** $P < 0.01$.

TABLE 2. BEHAVIORAL RESPONSES OF *S. gregaria* RELEASED IN GROUPS OF 10 TO AIRBORNE VOLATILES OF LIVE NYMPHS AND ADULTS IN CHOICE OLFACTOMETER

Odor source[a]	Test insects[b]	Number in control	Number in stimulus	Aggregation index (%)[c]
2nd instar	2nd instar	8	88	79**
3rd instar	2nd instar	10	86	76**
4th instar	2nd instar	10	85	75**
5th instar	2nd instar	11	85	74**
Older adults	2nd instar	47	50	−3 NS
2nd instar	3rd instar	8	88	80**
3rd instar	3rd instar	11	86	75**
4th instar	3rd instar	11	84	73**
5th instar	3rd instar	10	87	77**
Older adults	3rd instar	49	50	1 NS
2nd instar	4th instar	11	85	74**
3rd instar	4th instar	10	87	77**
4th instar	4th instar	10	85	75**
5th instar	4th instar	9	88	79**
Older adults	4th instar	48	50	2 NS
2nd instar	5th instar	13	85	72**
3rd instar	5th instar	12	84	72**
4th instar	5th instar	10	85	75**
5th instar	5th instar	10	85	74**
Older adults	5th instar	50	49	1 NS
2nd instar	Older adults	49	47	−2 NS
3rd instar	Older adults	47	50	3 NS
4th instar	Older adults	48	48	0 NS
5th instar	Older adults	46	48	2 NS
Older adults	Older adults	13	84	71**

[a] 10 locusts in a 2-liter flask. Air flow = 120 ml/min.
[b] 100 locusts tested per replicate.
[c] Difference from control (χ^2 test) indicated by: NS = not significant; $^{**}P < 0.01$.

nymphal volatiles. There were no significant differences between the responses of nymphs of different stages to volatiles of one another.

The responses of fifth-instar nymphs and older adults to locust volatiles is dose-dependent (Table 4).

DISCUSSION

Our results show clearly that volatiles emitted by locusts stimulate grouping behavior in receptive individuals by retaining them within the precinct of atmosphere enriched with these volatiles. The insect's response increases in a dose-

TABLE 3. BEHAVIORAL RESPONSES OF *S. gregaria* NYMPHS AND ADULTS TO VOLATILE EXTRACT COLLECTED FROM THIRD-INSTAR NYMPS, FIFTH-INSTAR NYMPS, YOUNG ADULTS, AND OLDER ADULTS

Odor source[a]	Test insects[b]	No. in control	No. in extract	Aggregation index (%)[c]
3rd Instar extract	2nd instar	12	84	72**
	3rd instar	11	85	74**
	4th instar	13	84	71**
	5th instar	14	84	70**
	Young adults	50	48	−2 NS
	Older adults	48	50	2 NS
	2nd instar	11	85	74**
	3rd instar	13	84	71**
	4th instar	14	83	69**
	5th instar	14	82	68**
	Young adults	48	51	3 NS
	Older adults	45	49	4 NS
Young adult extract	4th instar	46	49	3 NS
	5th instar	47	50	3 NS
	Young adults	48	49	1 NS
	Older adults	50	46	−4 NS
Older adult extract	2nd instar	48	50	2 NS
	3rd instar	50	47	−3 NS
	4th instar	51	49	−2 NS
	5th instar	46	50	4 NS
	Young adults	15	83	68**
	Older adults	11	86	75**

[a] Trapped volatile extract at dose of 96 LH in 2 ml paraffin oil. Air flow = 120 ml/min.
[b] 100 locusts tested per replicate.
[c] Difference from the control (χ^2 test) indicated by: NS = not significant; $^{**}P < 0.01$.

dependent fashion and then levels off (Table 4). This implies that in natural gregarious populations, individuals would be drawn together until, on the average, the concentration of the pheromone reaches an optimal value. Significantly, the retentivity of the stimulus appears to be independent of whether the insect is alone or in a group (Tables 1 and 2). Thus, contrary to previous speculations (Gillett et al., 1976; Fuzeau-Braesch et al., 1988), visual and tactile stimuli are not prerequisites for the action of the releaser pheromone. Indeed, our results with individual insects suggest that pheromonal communication may be the principal mechanism modulating the aggregation behavior of the desert locust.

Our results also indicate the existence of two releaser pheromone systems in *S. gregaria*: a "juvenile aggregation pheromone" produced by nymphal stages and to which only the nymphs respond, and an "adult aggregation pheromone"

TABLE 4. BEHAVIORAL RESPONSES OF FIFTH-INSTAR NYMPHS AND OLDER ADULTS OF *S. gregaria* TO DIFFERENT DOSES OF THEIR RESPECTIVE VOLATILE EXTRACTS IN CHOICE OLFACTOMETER[a]

Dose in locust hours (LH)[b]	Aggregation index (%)[c]	
	5th instar	Older adults
24	30a	32a
48	50b	53b
96	74c	73c
240	73c	77c

[a] 100 locusts tested per replicate.
[b] Trapped volatile extract in 2 ml paraffin oil. Air flow = 120 ml/min.
[c] Values within the same column followed by different letters are significantly different at $P < 0.05$, chi-square test.

produced by older adults and specific to adults. Preliminary gas chromatographic examination of the volatiles has confirmed significant qualitative and quantitative differences in the compositions of nymphal and adult emissions. The significance of the evolution of two distinct sets of pheromones for the two stages of the insect is not clear but may be related to different behavioral ecologies of the two stages (Uvarov, 1966) and a concomitant need to separate them into different groupings.

The lack of response to volatiles of young adults by all stages of the gregarious desert locust, including young adults themselves (Tables 1–3), is noteworthy. Perhaps young adults are a transition stage where the production of the nymphal aggregation pheromone shuts down to give way to the biosynthesis of the adult aggregation pheromone. Interestingly, young adults were found to be responsive to volatiles of older adults. This is consistent with the fact that a swarming population of the desert locust is generally made up of different age groups including young adults (Uvarov, 1966). This association of young and older insects is important in facilitating synchronous maturation of members of a given locust generation, apparently modulated by a pheromone produced by mature males (Loher 1960; Norris 1962, 1964; Amerasinghe, 1978). Synchronous maturation is critical to the gregarious phase because it ensures simultaneous mating and gregarious oviposition (Popov, 1958; Stower et al., 1958; Norris, 1963), thus assuring the cohesiveness of the new hopper generation that emerges.

The chemical identity of the two sets of aggregation pheromones remains a question of special interest. We are now directing our attention to this. In addition, the longer-term primer effects of different locust volatiles on solitary locust individuals remain a subject for further research now under way.

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RESPONSE OF MALES TO FEMALE SEX PHEROMONE IN THE ORANGE WHEAT BLOSSOM MIDGE, *Sitodiplosis mosellana* (GÉHIN) (DIPTERA: CECIDOMYIIDAE)

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Abstract—Males of the orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin), were attracted by female but not by male extract in a Y-tube bioassay. In laboratory mating experiments, females exhibited typical calling behavior under all conditions tested. At 19°C in the dark, males exhibited a high frequency of wing vibration (a courtship behavior) and mating attempts, and 68% of females were mated. However, there was virtually no courtship or mating activity at 17°C and 23°C (0 and 11% mated, respectively); at 21°C, there was an intermediate level (43% mated). Light intensity of 1500 lux (as compared to darkness), or high relative humidity (96%, as compared to 70%) also inhibited mating activity. In trapping experiments in a wheat field, males but not females were caught in significantly greater numbers in traps baited with a solvent extract of virgin females, as compared with unbaited traps, at a trap height of 20 cm. At a trap height of 60 cm above ground, no males were caught. Males did not differentiate between traps baited with two calling females and a solvent extract of two virgin females, and the latter lost little activity over 48 hr under field conditions. There was a daily rhythm of male response to receptive females or female extract each evening between 1700 and 2200 hr CST. The sensitivity of males to environmental conditions and their consequent short daily period of response in the field are thought to be related to their high susceptibility to desiccation and lack of sources of food as adults.

Key Words—Pheromone, mating, midge, Diptera, Cecidomyiidae, trapping, weather, bioassay, *Sitodiplosis mosellana*, activity pattern.

INTRODUCTION

The orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin) (Diptera: Cecidomyiidae), was introduced into North America from Europe and was first

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reported in 1819 near Quebec City (Sanderson, 1915). Eggs of this insect are laid on wheat heads just prior to flowering, and the larvae feed on the developing kernels, causing them to shrivel. The mature larvae overwinter in cocoons in the soil. The adults emerge near the time that the wheat heads emerge (Barnes, 1956). Serious crop losses were first reported in the Canadian prairies in the 1980s (Barker, 1984; Olfert et al., 1985).

A number of cecidomyiid species produce female sex pheromones (Cartwright, 1922; Miller and Borden, 1984; Lee and Lee, 1985; Sain and Kalode, 1985; Williams and Martin, 1986). Pivnick and Labbé (1992) describe calling (behavior that accompanies the release of a sex pheromone) and mating behavior for *S. mosellana*. The female calls from within an hour of emergence and normally ceases upon mating. If mating does not take place, the female will continue calling until her death several days later. The frequency of calling is greatest at night and declines during the day.

A prerequisite for identification of the pheromone, or for its use in monitoring or mating disruption of the wheat midge in a pest management program, is the understanding of male response. This paper describes the response of males to females and female extracts in a laboratory bioassay, in field trapping experiments, and in laboratory mating experiments under a variety of controlled conditions.

METHODS AND MATERIALS AND RESULTS

Insects and Experimental Conditions

The top 5 cm of soil were collected from fields that had been planted to wheat the previous summer near Gronlid and Veregin, Saskatchewan, in April and September 1986 and September 1987. Soil was stored in plastic bags at 4°C until used. To obtain adults, 250 ml to 1 liter of soil was put in a plastic container with a clear lid, moistened, and placed at 21 ± 2°C, 70 ± 5% relative humidity, 16:8 hr light–dark photoperiod (1500 lux at the level of the cages). Emergence of adults generally took place within two to three weeks. Adults were removed hourly to minimize the likelihood of mating. All laboratory experiments were carried out under these conditions unless otherwise specified. Observations in the dark were carried out using illumination from either red, 7.5-W incandescent bulbs (ceramic-silicate red; Philips Electronics Ltd., Scarborough, Ontario, Canada), spaced 30 cm apart and 60 cm from the subject, creating a light intensity at the cages of 14 lux) or a 3-V flashlight, covered with tissue paper and a red Wratten filter (Kodak No. 29).

Preparation of Extracts

Virgin adults, no more than 4 hr old, were placed in dichloromethane, 10 μl per adult, at the start of the scotophase. After 24 hr, insects were removed and rinsed with another 10 μl of dichloromethane and the extracts combined. Because of evaporation during the procedure, the extract volume was ca. 13 μl per adult, referred to as 1 female equivalent (FE) or 1 male equivalent (ME). For laboratory bioassays and field trapping, extract was spotted onto 13-mm blotting paper disks, pinned to a rubber stopper for ease of handling, and held for 5 min before use to ensure evaporation of solvent. Blank baits, spotted with solvent only, were used as controls.

Laboratory Bioassay of Male Response to Male and Female Extract

Humidified, filtered air was passed through a glass Y-tube, with a trap in each arm, as described by Pivnick et al. (1990). Six to 20 males were placed in the base of each Y-tube (two were in use) for a single trial. One arm contained the test material, the other a blank. Each trial was run for the duration of the scotophase (the optimal period based on preliminary tests), after which the insects trapped in each arm were counted. The position of the baits was alternated between trials. Results were analyzed by paired t tests, with each trial treated as a single replicate.

Males responded positively to female extract. In seven trials with a total of 89 males, 34 males were caught in the arm containing extract, 14 in the control arm, and 41 were still in the body of the tube. Thus there was a significant preference for the female extract over the control ($t = 2.65$, $df = 6$, $P < 0.05$), although only 20% of males responded in the bioassay [(number choosing pheromone bait arm − number choosing control arm)/total number tested]. Males showed no response to male extract. Of 126 males tested in 11 trials, 49 were trapped in the arm containing the extract and 46 in the control arm ($t = 0.18$, $df = 10$, $P > 0.05$).

Influence of Environmental Conditions on Mating Behavior in Laboratory

Equal numbers of newly emerged males and females (8–10 of each) were placed in a rectangular clear plastic cage, 8 × 11 × 12 cm, in an environmental chamber to begin the experiment. Under standard conditions, the experiment was started at the onset of the scotophase (16:8 hr light–dark photoperiod), and at 19 ± 1°C, 70 ± 5% relative humidity. Some modifications were made to the standard conditions in each treatment. The midges were observed for 1 hr. In the first set of four conditions, only temperature was varied. In a second set, humidity, time of day (either at, or 4 hr before, the normal time of onset of scotophase at which the midge had been maintained), and lighting (lights left

on when the experiment was started at the beginning of the normal scotophase, or lights on or off when started 4 hr before the normal onset) were varied. An observer recorded midge activity into a tape recorder. As females moved very little, virtually all activity could be recorded. The number of times that males vibrated their wings (bouts of wing vibration), attempted to mount females (mating attempts), and the number of matings which took place were recorded. Other related behaviors were also noted. No attempt was made to remove males or females after mating. This was repeated four times under each set of conditions. In mating experiments, water was available to insects prior to, but not during, the experiments.

During observations under the eight different sets of conditions, we observed 484 bouts of wing vibration, 183 mating attempts, and 97 matings. The optimal temperature for mating was 19°C. At this temperature, males performed more bouts of wing vibration, made more mating attempts, and had more successful matings than at other temperatures (Table 1). In the second series of experi-

TABLE 1. EXPERIMENTS QUANTIFYING MATING ACTIVITY OF *Sitodiplosis mosellana* IN LABORATORY UNDER (A) DIFFERENT TEMPERATURES AND (B) DIFFERENT TIMES OF DAY, LIGHT, INTENSITIES, AND RELATIVE HUMIDITIES

Condition[a]	Pairs (N)	Bouts of wing vibration (mean ± SE)[b]	Mating attempts (mean ± SE)[c]	Percentage mating (total no. of matings)[c]
(A)				
17°C	38	1.5 ± 1.5 b	0.3 ± 0.3 b	0 (0)
19°C	37	26.5 ± 6.1 a	14.0 ± 6.6 a	68 (25)
21°C	37	12.0 ± 7.1 ab	3.0 ± 1.7 b	43 (16)
23°C	38	1.8 ± 1.8 b	0.3 ± 0.3 b	11 (4)
(B)				
4 HBS, light	37	17.0 ± 8.1 ab	6.0 ± 2.9 b	22 (8) b
4 HBS	38	56.5 ± 27.0 a	21.8 ± 5.5 a	92 (35) a
light	34	1.5 ± 1.5 b	0 b	9 (3) b
96% RH	36	2.0 ± 1.7 b	0.5 ± 0.5 b	17 (6) b

[a] Conditions are 19°C, 70% relative humidity, darkness, and mating experiments started within the first hour of the normal onset of scotophase, unless otherwise stated; 4 HBS = started 4 hr before the normal onset of scotophase; light = lights remained on.

[b] $N = 4$ replicates of 8–10 pairs of midges observed for 1 hr on separate days. Numbers in columns followed by different letters within (A) and (B) are significantly different at $P = 0.05$ based on an LSD test.

[c] In (A), a 4 × 2 chi-square test was carried out on the effect of temperature on the proportion of female mating: $\chi^2 = 48.2$, $P < 0.0001$. In (*B*), chi-square tests were carried out between all possible pairs. [The latter statistical approach could not be used in (A) because of the 0% mating at 17°C.] Incidences of mating which are significantly different at $P = 0.05$ are followed by different letters.

ments, high relative humidity reduced mating. In experiments with "lights on," mating was reduced when compared to the same conditions in darkness, both 4 hr before, and during the first hour of, the normal scotophase. The level of mating activity was not significantly different at the two times of day tested, although it was consistently higher at the earlier time. This was shortly after most of the male midges had emerged for the day (Pivnick and Labbé, 1992).

In all the trials, at least 70% of females were seen calling at one time during the hour of observation. Females also spread their wings (indicating receptivity to mate) (Pivnick and Labbé, 1992) 115 times when approached by males that did not initiate courtship. This occurred under conditions where both much and little mating activity took place. On the other hand, males unsuccessfully attempted to mate 183 times (sum of all mating attempts in Table 1). Of these, females actively refused male advances only 34 times (by vibrating their wings or walking or flying away) and in most cases they subsequently mated, although we could not always tell if it was to the same male. (Individual females were readily followed during the hour as they rarely moved; this was not true of the males.) Of the remaining 149 unsuccessful mating attempts, females did not spread their wings making coupling more difficult, or males lost their grip and fell from the female, or males were for some other reason unable to couple. It was not possible to assign one of these reasons to each unsuccessful mating attempt, but all occurred frequently and did not appear related to particular mating conditions.

As males were not marked, it usually could not be determined how many individual males were responsible for courtship and mating activity within a given trial. However, in one trial at 21°C, one male mated with at least four of eight females mated within the hour. The four matings were considerably longer (9, 6, 8, and 20 min) than the mean mating duration measured previously of about 3 min (Pivnick and Labbé, 1992). The results from this one trial accounted for 33 of 48 bouts of wing vibrations, 8 of 12 mating attempts, and 8 of 16 matings at 21°C. This suggests that the temperature window for mating is even narrower than it appears in Table 1. Moreover, at the end of this hour, five of 10 females were observed calling although eight matings had taken place. In other trials at the end of the hour there was, at most, one more female calling than the number of females in excess of the number of matings which had taken place.

Males attempted to mate with males (not included in "mating attempts") 10 times and actually coupled with them an additional four times. Males attempted to mate with a mating couple 12 times; in one particular trial, five different males attempted to mate with one mating couple. In two additional instances, males appeared to "couple" with a mating pair. On one occasion, a male dislodged a male in copula and immediately began to copulate with the female (this was considered to be two matings).

In summary, the number of unsuccessful mating attempts and successful matings reflected the level of male courtship activity as quantified by the number of bouts of wing vibrations. The level of male courtship activity was highly variable between treatments, while female calling and receptivity to males changed little.

Development of Field Trapping Methodology

Tests were carried out in a 35-ha wheat field near Preeceville, Saskatchewan, in July 1986 and in a 2-ha plot of wheat near Veregin, Saskatchewan, in July 1987. Both sites had been planted to wheat the previous year. The experiments were set up in randomized complete blocks (each block or replicate was a line of traps, separated from the next line by at least 40 m), with traps spaced 10 m apart within replicates. Traps were set out for either 24 hr (starting at 1700 hr CST in 1986 and at 2200 hr in 1987), or from 1700 to 2200 hr only, as described for each experiment. The shorter time period was used where baits were more fragile (calling females), or where timing was more crucial (extract longevity experiment), as virtually all trap capture occurred during this period. Traps used were Pherocon 1CP wing traps (white, with sticky bottoms; Zoecon Corp., Palo Alto, California) and orange delta traps (sticky on all three interior surfaces; Pherotech, Inc., Vancouver, British Columbia, Canada). Baits were suspended in the middle of the traps.

Even though a few females were caught in most experiments, only male trap catches are reported except in the experiment described in the next two paragraphs. Trapping results were square-root transformed and analyzed using a two-way ANOVA and an LSD test. Time of sunrise and sunset were approximately 0500 and 2130 hr CST.

Trap Height and Male and Female Response. Pherocon 1CP traps were placed in the field at either 20 or 60 cm above the ground (the crop height was approximately 100 cm. Baits were 2 FE or blanks for each height and there were three traps for each combination. The traps were held in place by attaching the wire hanger underneath the trap and inserting the free end into the ground (at 20 cm), or traps were attached to wooden stakes (60 cm). Trapped insects were sexed and counted. The experiment was run for 24 hr on July 18–19, 1986.

Males clearly responded to the female extract, but only close to the ground. Significantly more males were caught in baited traps at 20 cm (25 males) than at 60 cm (0 males), or at either height with blank baits (0 in both cases) (Mann-Whitney $U = 0$, $P < 0.05$). Females did not respond to the female extract but a few were caught in traps at both heights (two and six were caught in baited traps at 60 and 20 cm, respectively; six and three in unbaited traps; $F = 0.2$, $P > 0.05$). Based on these results, all further tests were carried out at 20 cm

above ground. As it was clear that females did not respond to female extract, in subsequent experiments only male trap catches are reported.

Trap Type. The effectiveness of Pherocon 1CP traps baited with 2 FE was compared with the same traps and baits placed upside down so that the sticky floor was on the ceiling of the trap, the same traps with blank baits, and orange delta traps with 2 FE (three traps for each; 20 cm above ground). The experiment was run from July 17 to 19, 1986 (two 24-hr periods).

The conventionally oriented Pherocon 1CP trap caught significantly more males (25) than the delta (5), the upside-down Pherocon 1CP (3), or the unbaited Pherocon 1CP (0); the delta also caught significantly more than the unbaited trap ($F = 22.5$, $P < 0.001$). Consequently, the Pherocon 1CP was used in all subsequent experiments.

Calling Females versus Female Extract. Pherocon 1CP traps (20 cm above ground) were baited with two freshly eclosed virgin females held in a 5-ml glass vial with both ends screened, which was suspended by a piece of wire from the middle of each trap. These were compared with traps baited with 2 FE and to control traps (three traps per treatment) from 1700 to 2200 hr on July 16, 1987.

There were no significant differences among the number of males caught with 2 FE (37 males: 2, 14, and 21 in individual traps), with two calling females (59 males: 1, 10, and 48), or with the blank traps (4 males: 1, 1, and 2) ($F = 0.13$, $P > 0.05$). Variability between traps was high, accounting for the lack of significant differences between the baited trap captures and the low catch of the blanks, but the female extract appeared similar in attractiveness to live females.

Dose–Response. Pherocon 1CP traps (20 cm above ground) baited with 2 or 4 FE, were compared with unbaited traps for a 24-hr period on July 15–16, 1986 ($N = 3$). Traps baited with 0.5, 2, and 8 FE were compared with unbaited traps on July 14–15, 1987 (24 hr; $N = 3$).

Doses of female extract between 0.5 and 8 FE captured similar numbers of midges in the traps, although the numbers caught at the higher doses tended to be less variable (resulting in significant differences from the blanks in 1987; Table 2).

Longevity of Female Extract Attractiveness. Traps (Pherocon 1CP traps, 20 cm above ground) were deployed from 1700 to 2200 hr on July 18, 1986, and July 15–16, 1987. Baits (2 FE; extract made in advance as in other experiments; $N = 3$) were prepared (extract spotted on filter paper) at different times before the designated trapping periods and placed in the field in traps so that the baits had been under field trapping conditions for varying periods between 0 and 72 hr when the actual trapping began. Blank baits were prepared each day.

On all days during which baits were "aged," the weather was warm, with daily highs ranging from 26 to 29°C. The results indicate that while some loss

TABLE 2. RESPONSE OF *Sitodiplosis mosellana* MALES TO DIFFERENT QUANTITIES OF FEMALE EXTRACT IN TWO FIELD TRAPPING EXPERIMENTS

Bait	Mean number of (± SE) of males caught[a]	
	1986	1987
8 FE[b]		12.3 ± 0.9 a
4 FE	7.7 ± 3.3 a	
2 FE	5.7 ± 1.5 a	15.3 ± 5.2 a
0.5 FE		11.3 ± 5.5 ab
Blank	0.3 ± 0.3 a	1.3 ± 0.3 b

[a]Means followed by different letters within a column significantly different at $P = 0.05$ (two-way ANOVA and an LSD test on square-root transformed data).
[b]FE = female equivalents.

of attractiveness of the extracts occurred in the first 24 hr, the extracts were still attractive after 48 hr (Table 3). It was unclear whether any attraction remained after 72 hr.

Daily Rhythm and Effect of Weather on Response of Males to Female Extract in Field Trapping

Pherocon 1CP traps (20 cm above ground) were baited with 2 FE, and were emptied more frequently (at 0500 hr, and hourly from 1000 to 2200 hr) to determine the daily pattern of male response to the female extract. Trapping was carried out on July 17–19, 1986 (two 24-hr periods), and July 13–16, 1987 (three 24-hr periods). From July 17 to 18, 1986, there were 12 replicates; on the other days, there were three traps per treatment. Light intensity (measured with a Lambda Instruments LI-185 photometer), air temperature, and relative humidity were measured hourly from 1000 to 2200 hr, at trap height within the plant canopy. Maximum wind speed over 1 min was also measured with a Turbometer wind indicator (Davis Instruments, Hayward, California).

Males responded to female extract over a short period of time each evening, primarily between 1800 and 2000 hr CST, as air temperature and light level dropped (below 26–23°C and 10,000–2000 lux), and relative humidity increased (Table 4). When temperature fell below 14–17°C, activity ceased. Trap capture and accompanying environmental conditions are presented in more detail for July 14 and 15, 1987 (Figure 1), the two days on which the most males were caught.

In this experiment, over five 24-hr periods, 246 males were collected. All but five were collected between 1700 and 2200 hr. Of these five, four were

TABLE 3. LONGEVITY OF *Sitodiplosis mosellana* FEMALE EXTRACT IN FIELD TRAPPING EXPERIMENTS IN WHEAT FIELDS IN SASKATCHEWAN

Time (hr) extract was in field prior to trapping	Mean number (±SE) of male midge caught[a]		
	July 18, 1986	July 15, 1987	July 16, 1987
0	8.3 ± 4.4 a		12.3 ± 5.5 a
20	10.0 ± 3.2 a		
22	2.0 ± 0.6 b		
24	2.3 ± 1.2 b	15.3 ± 5.2 a	4.7 ± 0.9 ab
28	2.3 ± 1.5 b		
32	1.3 ± 0.7 b		
35	0.7 ± 0.3 b		
48		14.7 ± 4.7 a	5.7 ± 4.2 ab
72			2.3 ± 1.2 ab
Blank	0 b	1.3 ± 0.3 b	1.3 ± 0.9 b

[a]Trapping was carried out from 1700 to 2200 hr. Means in columns followed by different letters differed significantly at $P = 0.05$ (two-way ANOVA followed by a protected LSD test on square-root transformed data).

TABLE 4. DAILY PATTERN OF CAPTURE OF *Sitodiplosis mosellana* MALES IN TRAPS BAITED WITH FEMALE EXTRACT IN RELATION TO WEATHER

Date	Hours (CST) when >10% of daily total were caught per hour	Total caught	Temperature (°C)	Light (lux)	Relative humidity (%)
July 17, 1986	1800–2000	53	26–18	5500–300	81–100
July 18, 1986	1800–2000	25	23–16	2000–500	83–90
July 14, 1987	1700–1900	104	23–21	10,000–700	61–63
July 15 1987	1700–2000	46	26–20	7000–350	67–83
July 16, 1987	1700–2100	18	24–14	10,000–25	56–81

collected between 1500 and 1700 hr, and one between 2200 hr and 0500 hr the following morning. Wind gusts never exceeded 10 km/hr when midges were caught.

DISCUSSION

This study provides unequivocal evidence of a female sex pheromone that is used by males to locate sexually receptive females. A sex pheromone has now been identified as (2*S*)-(*E*)-10-tridecen-2-yl acetate for one cecidomyiid,

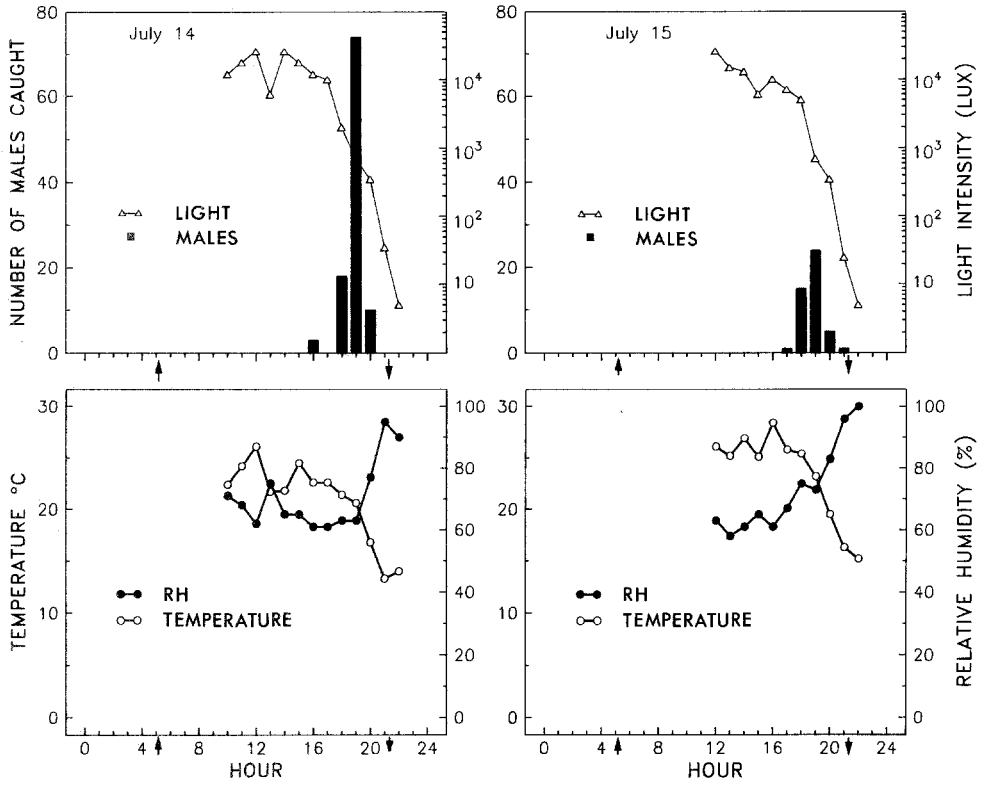

FIG. 1. Daily pattern of male *Sitodiplosis mosellana* trap capture using female extract as bait, and light intensity, temperature, and relative humidity on July 14 and 15, 1987. Bars represent the total number of males caught over the previous hour. Arrows represent sunrise (up) and sunset (down).

the Hessian fly, *Mayetiola destructor* (Say) (Foster et al., 1991). Wheat midge males are not attracted to other males, and the few observed mating attempts between males were likely due to the presence of female sex pheromone. Both males and females are capable of mating within an hour of emergence. Under field conditions, males fly low to the ground to locate calling virgin females. Females, whether mated or not, also stay low within the plant canopy, except during oviposition under favorable weather in the evening (Pivnick, unpublished data; Borkent, 1989). Other midge species have been observed to mate on or near the ground shortly after emergence (McColloch, 1923; Prasad, 1969; Miller and Borden, 1984).

The low percent response in the laboratory Y-tube bioassay was not surprising considering the sensitivity of male sexual activity to environmental con-

ditions. The temperature (21°C), the timing (at the onset of the scotophase), and possibly other factors in the bioassay were not optimal. Results in the present study also suggest that suboptimal conditions in other cecidomyiid bioassays (Lee and Lee, 1985; Williams and Martin, 1986) and in rearing cecidomyiids, including *S. mosellana* (Basedow and Schutte, 1973), may be the reasons for poor male response and poor mating success. On the other hand, some bioassays have demonstrated strong responses (McKay and Hatchett, 1984; Miller and Borden, 1984).

The trapping experiments demonstrated that males responded best to the female extract if the trap was placed near the ground and that they tended to land on the inside bottom of the trap. The fact that males fly and stay close to the ground affords them protection from the wind, as well as giving them better access to newly emerged calling females, which stay close to the ground in the field (Pivnick, unpublished data). Similarly, more pea midge, *Contarinia pisi* Winn., males were caught if pheromone traps were at heights of less than 0.3 m (Wall et al., 1985).

The Pherocon 1CP trap caught more males than the delta-type trap and is therefore recommended for monitoring purposes. The trapping results also indicated that the extract, at least within the range of doses used, was as attractive as calling females. Females normally call a mean of 70% of the time during this time period under seminatural conditions (Pivnick and Labbé, 1992), so two females can be expected to offer reasonably continuous competition to an extract. Harris and Foster (1991) found little difference in response of Hessian fly males in a wind tunnel to a sex pheromone component over three orders of magnitude of concentration.

Female calling is little affected by weather but is most intense during the evening and night and diminishes during the day (Pivnick and Labbé, 1992). In the present mating experiments, female calling also did not appear to be affected by environmental conditions. On the other hand, males were attuned to abiotic factors, as evidenced by the daily pattern of response to female extract in traps and by male response and mating success in the mating experiments.

The daily pattern of male response to female extract was not tested under controlled laboratory conditions. However, Miller and Borden (1984) found that the male Douglas-fir cone gall midge responds to female extract for only ca. 3 hr in the morning under controlled conditions. This coincided with the period of most intense calling by females in that species. On the other hand, Hessian flies mate only in the morning, but in this species the short period of mating is attributed to a short period of calling by females and not by a limited period of male response (McKay and Hatchett, 1984).

The sensitivity of males to environmental conditions is likely related to their small size (0.14 ± 0.02 mg fresh weight, mean $\pm$ SE, $N = 14$ newly eclosed males from field-collected larval cocoons) and limited access to water

and nutrients as adults. In the field, males avidly drink dew from leaf surfaces in late evening; no other feeding behavior has been observed (Pivnick, unpublished data). The longevity of virgin males in cages under the standard laboratory conditions used in these experiments (where active flight takes place) was 4.3 $\pm$ 0.6 days (mean $\pm$ SE; $N = 12$) with access to water and less than 24 hr in all cases ($N = 12$) without water. Their daily pattern of response likely allows conservation of limited water and energy stores.

In comparison with mate searching by males, female calling is energetically low in cost because they remain immobile (Pivnick and Labbé, 1992). Females also have limited time as calling becomes erratic by the fourth night, and virgins live only five to seven days (Pivnick and Labbé, 1992). Mated females have a similar life-span to virgins and lay most eggs on the second to fourth nights (Pivnick, unpublished data). They have no apparent source of adult nutrition which would suggest that any delay in mating would mean a loss in egg-laying potential. A further time limitation is imposed on ovipositing females by their sensitivity to weather (Oakley, 1981) making many evenings unsuitable for egg-laying. Thus, males appear to optimize their limited time in mate searching by restricting this behavior to conditions that are most favorable. Females, on the other hand, maximize their calling time, possibly reflecting the different ecological limitations of the two sexes on their respective reproductive potentials.

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ALLELOPATHIC COMPOUNDS IN LEAVES OF *Gliricidia sepium* (JACQ.) KUNTH EX WALP. AND ITS EFFECT ON *Sorghum vulgare* L.

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Abstract—Allelochemicals from *Gliricidia sepium* were extracted, identified, and quantified using HPLC. Fifteen toxic compounds, namely gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, β-resorcyclic acid, vanillic acid, syringic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid, ferulic acid, sinapinic acid (*trans* and *cis* forms), coumarin, and myricetin were identified and quantified. These compounds from the plant extracts were tested on the seeds of the crop plant, *Sorghum vulgare*. Rate of germination of the seeds and root elongation were found to be inhibited by the various compounds of the extract. Different quantities of *Gliricidia* leaf mulch, viz., 400, 800, and 1200 g/m^2 applied to the *Sorghum* grown fields, were found to effectively control weeds. Mulching improved the total yield of *Sorghum*. Leaf manuring and mulching showed better crop yield when applied up to 800 g of *Gliricidia* leaf/m^2. Crop yield was better in mulch-applied fields when compared to the manure-applied ones.

Key Words—Allelochemicals, *Gliricidia sepium*, *Sorghum vulgare*, HPLC, aglycone, phenolic acids, weed control, root biomass, shoot biomass, inflorescence biomass, grain yield, weed biomass.

INTRODUCTION

Gliricidia sepium leaves are widely used for mulching purposes in agricultural practice (Wilson et al., 1986). The performance of leaf mulch of *Gliricidia sepium* is more effective in weed control than the leaf mulch of *Leucaena leucocephala* (Budelman, 1988). *Gliricidia sepium* leaf mulch improved the yield

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of maize and promoted the soil fertility in degraded alfisol soil, and at the successive crop seasons, application of *Gliricidia* prunings as mulch did not affect the yield (Atta-krah and Sumberg, 1987).

Using a paper chromatography technique, Griffiths (1962) identified nine phenolic acids and a yellow fluorescing flavonol in the young and senescent leaves of *Gliricidia sepium*, which were allelopathic in nature (Rice, 1984). Protocatechuic acid was tentatively identified in *Gliricidia sepium* by Inostrosa and Fournier (1982). They studied the allelopathic effects of *Gliricidia sepium* on seed germination of a weed (*Bidens pilosa*) and tomato and found inhibition of germination.

Since *Gliricidia sepium* leaves are used as mulch and manure in dry-land agricultural practices, an attempt was made to identify and quantify the various allelochemicals present in the leaves of *Gliricidia sepium*. The biological activity of these allelochemicals present in the leaf extracts was tested on the seeds of the crop plant *Sorghum vulgare* (variety MSH 37). The impact of *Gliricidia* leaf mulch and manure on the yield of the crop plant *Sorghum vulgare* and weed control was tested under field conditions.

METHODS AND MATERIALS

Leaves of *Gliricidia* were collected from the Biomass Research Center of Madurai Kamaraj University, Madurai. Leaf extract was prepared according to the procedure of Singh et al. (1989). A crude extract was prepared by soaking 100 g of dried leaf material in 1000 ml of distilled water for 48 hr. Extract was freed from debris by centrifugation and filtration. Lipids and aglycones were removed by fractionation of crude extract with hexane (fraction A) and diethyl ether (fraction B), respectively. The remaining aqueous fraction was subjected to strong alkaline hydrolysis under nitrogen. The alkaline hydrolysate was acidified and fractionated with diethyl ether (fraction C). All extractions with solvents (100 ml) were repeated three times. The extracted fractions A, B, and C were subjected to bioassay with *Sorghum vulgare* seeds.

Bioassay

Three sets of Petri plates with filter papers were wetted evenly with fractions of A, B, and C separately (concentrations equivalent to 500 μl of fraction per dish). Excess of pure extraction solvent was added to the Petri dishes to ensure the uniform dispersal of compounds, and the dishes were allowed to dry completely. A fourth set, which received only the pure solvent used for fractionation, was used as control. Twenty seeds of *Sorghum vulgare* were placed in each Petri plate. The filter papers were saturated with double-distilled water. The assay was carried out by counting the seed germination for three days.

Identification and Quantification of Allelochemicals

Based on the bioassay results, identification of allelopathic compounds was done in the inhibitory fractions using a high-performance liquid chromatograph (LKB 2158, Uvicord SD HPLC equipped with LKB 2150 HPLC pump, LKB 2154 valve injector and LKB 2210 recorder). The solvent diethyl ether in the fractions was evaporated separately, and the residues were dissolved in the same volume of the mobile-phase liquid used in the HPLC. The columns (LKB 2113 Lichrosorb RP 18, 4 × 250 mm, 5 μM column) were eluted isocratically with 40% methanol in 1% acetic acid at a flow rate of 2 ml/min and monitored with a UV detector at 254 nm for detecting phenolic acids in fraction C (Cheng and Rimer, 1989). Acetonitrile and water were used as the mobile phase for aglycone in fraction B; the UV detection range was set at 280 nm (Singh et al., 1989). Authentic standards were used to identify the allelopathic compounds by comparing their retention time. Identified samples were spiked with authentic standards to reconfirm the identity.

Field Experiments. In southern India, *Sorghum vulgare* is cultivated in the semiarid zone. In Madurai (09'52°N, 74'10°E) the crop season falls between July and October. The field experiments in the present study were carried out between July and September 1991.

The trial area was divided into small experimental units of 2 × 3 m. A completely randomized block design of four treatments with 10 replicates was adopted. Irrigation channels (12 in. wide) divided the plots and acted as the buffer zone. The trial plots were saturated with water before transplantation. Eighteen-day-old uniformly grown *Sorghum* seedlings (variety MSH 37) were transplanted in trial plots in four rows with 45-cm intervals between the rows, and 15-cm intervals between the plants in the rows. Seven days after transplantation, a thorough weeding was done in the trial plots. The dry matter of *Gliricidia* leaves mulch (rachis included) was applied at the rate of 400 g (T1), 800 g (T2), and 1200 g (T3)/m^2. Mulching was excluded in the control plots. The plots were irrigated once in 10 days.

Weed sampling and crop plant samples were taken once in 20 days after the mulch layer was applied. A single weed sample per plot was taken using a 50 × 50-cm square iron quadrat. Weed frequency, weed density, weed abundance, and total weed biomass were calculated (Phillips, 1959). Five randomly selected sorghum plants from each plot for every treatment were uprooted, dried, and taken for analysis. Total root biomass (RB), shoot biomass (SB), inflorescence biomass (IB), and grain yield (GY) per plant were calculated for treated plants and compared with control plants.

The above experimental design was adopted for *Gliricidia* leaf-manuring experiments. Leaf manure were applied 20 days before *Sorghum* seedling transplantation at the rate of 400 g (t1), 800 g (t2), and 1200 g (t3)/m^2, and thor-

oughly mixed with the trial plot soil. Thorough weeding was done at 15 days and 45 days after the transplantation of *Sorghum* plants in the manure fields. Weed sampling was excluded in the manure fields. Plant sampling was done as that of mulch trials.

Statistical Analysis

Pair comparisons were done by using least significant difference analysis (LSD program, SPSS/PC IBM computer).

RESULTS

Bioassay of extracts B and C on the germination index of *Sorghum* seeds showed marked differences (Table 1). Extracts B and C (500 μl/Petri plate) showed inhibition of the root growth in *Sorghum vulgare*, of which extract C was comparatively more inhibitory than extract B. Extract A did not show any marked effect on the germination or elongation of root compared with control. Phenolic acids and aglycone in fractions B and C were identified and quantified in terms of percentage present in 10 μl of fraction. Since extract A did not have any effect on the germination of *Sorghum* seedlings, it was excluded in the present investigation. Fifteen allelochemicals were identified and quantified (Table 2), of which 13 compounds were phenolic acids. The flavonol identified in the leaves of *Gliricidia sepium* was myricetin. Coumarin and myricetin are the aglycones present in fraction B with trace amounts of other phenolics identified in fraction C. Some unknown peaks are also noted in the fractions B and C.

Effect of Leaf Mulch in Sorghum *Total Biomass and Weed Control*

The yields of control, T1, T2, and T3 plants were calculated at regular intervals and the results are shown in Table 3.

Root Biomass. The RB in T1 and T2 plants did not show any significant

TABLE 1. EFFECT OF VARIOUS OF LEAF EXTRACTS OF *Gliricidia sepium* ON GROWTH AND GERMINATION IN *Sorghum vulgare*[a]

Treatment 500 μl/dish	Germination (%) after 72 hr	Radicle length (cm)
Control	100	5.54
Fraction A	98	5.70 NS
Fraction B	80	1.32**
Fraction C	62	2.49**

[a]Levels of significance (compared to control): NS = not significant, ** = significant at 1% level.

TABLE 2. MAJOR ALLELOCHEMICAL COMPONENTS IN FRACTIONS OF *Gliricidia sepium* LEAF EXTRACT[a]

Allelochemical	Fraction B[b]	Fraction C[b]	% contribution in the total allelochemical content of the leaf
1. Gallic acid	–	+	1.79
2. Protocatechuic acid	–	+	1.65
3. *p*-Hydroxybenzoic acid	+	+	2.23
4. Gentisic acid	–	+	1.85
5. β-Resorcyclic acid	–	+	1.48
6. Vanillic acid	–	–	1.25
7. Syringic acid	–	+	1.45
8. *p*-Coumaric acid	+	+	18.55
9. *m*-Coumaric acid	–	+	14.91
10. *o*-Coumaric acid	+	+	20.09
11. Ferulic acid	+	+	5.19
12. *trans*-Sinapinic acid	+	+	14.47
13. *cis*-Sinapinic acid	+	+	6.48
14. Myricetin	+	+	4.93
15. Coumarin	+	+	3.68

[a]Total allelochemicals present in leaves of gliricidia = 5.4 mg/g matured leaf.
[b]Not detectable, –; present, +.

reduction when compared with the control RB. Initially T3 plants showed a reduction in RB, but RB soon recovered in later harvests.

Shoot Biomass. No significant difference was noted in the SB between control and treatments (T1, T2, and T3).

Inflorescence Biomass and Grain Yield. There was a conspicuous increase in the IB and GY in T2 and T3 mulch-treated plants compared to control. In mulch treatments, the total biomass increased more in T2 than others (Table 3). The higher quantity of mulch in T3 did not improve the GY/plant compared with T2, which received a lower quantity of mulch. The GY per plant showed no significant difference between control and T1 plants.

Weed Biomass. All the mulch treatments controlled total WB (Tables 4 and 5). Frequency percentage, abundance, density, and percentage of contribution to total weed biomass are shown in Table 4. Seven species of weeds were identified in the trial plots. In the treated plots, except *Cyperus difformis* and *Isachne dispar*, all other existing weed species (*Dactylotenium aegyptenium*, *Paspalidium flavidum*, *Amaranthus Spinosa*, *Boerhavia diffusa*, and *Phyllanthus nirurii*) are poor in percentage frequency and density compared to control. *Cyperus difformis* survived in all the mulch-treated plots. *Cyperus difformis* was more frequent (i.e., percentage frequency 100) in all four harvests in control,

TABLE 3. EFFECT OF MANURE AND MULCH ON BIOMASS OF *Sorghum vulgare*[a]

Treatment	Root (g)				Shoot (g)				Inflorescence (g)			Grain yield plant (g)
	I	II	III	IV	I	II	III	IV	I	II	III	
Control	0.28	2.03	2.39	2.41	1.32	16.6	43.5	47.5	0	2.21	7.58	7.92
4 tons (mulch)	0.18**	1.48*	2.35NS	2.43NS	1.74**	16.8NS	44.5*	48.6*	0	1.64NS	8.66**	7.99NS
8 tons (mulch)	0.21**	1.69NS	2.44NS	2.39NS	1.77**	17.2NS	44.6*	49.9**	0	2.95*	15.10**	11.87**
12 tons (mulch)	0.14**	1.62NS	1.89*	2.36NS	2.34**	16.7NS	44.0*	48.2*	0	2.68NS	10.23**	10.04**
Control	0.27	1.95	2.44	2.52	1.84	17.9	44.1	48.3	0	2.71	7.04	7.42
4 tons (manure)	0.62**	2.17NS	2.07**	2.21*	3.30**	19.5**	44.0NS	47.6NS	0	3.67*	7.31NS	8.47*
8 tons (manure)	0.59**	1.67NS	1.87**	2.01*	3.29**	19.4**	45.5**	49.2NS	0	2.97*	8.91*	10.72**
12 tons (manure)	0.23*	1.11**	1.26**	1.88*	3.06**	12.4**	43.2*	45.2*	0	1.10*	7.09NS	8.21*

[a]Levels of significance (compared to control): NS = not significant, * = significant at 5% level, ** = significant at 1% level. I, first harvest; II, second harvest; III, third harvest; IV, fourth harvest.

TABLE 4. EFFECT OF *Gliricidia* MULCHING ON WEED DISTRIBUTION[a]

	Abundance				Frequency (%)				Density				Contribution (%) to total weed biomass			
Treatment	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
Cyperus difformis																
Control	7.3	19.3	26.5	24.2	100	100	100	100	7.3	19.3	26.5	28.2	88	83	68	40
T1	6.2	15.3	17.9	26.2	100	100	100	100	6.3	15.3	17.9	16.5	100	88	83	40
T2	4.3	5.2	11.6	13.3	100	100	100	100	4.3	5.2	11.6	12.4	100	68	71	74
T3	1.0	2.5	2.4	2.5	20	60	50	90	0.2	1.5	1.2	0.9	100	100	100	100
Isachne dispar																
Control	8.0	8.2	8.6	8.6	100	100	100	100	8.0	8.2	8.6	9.2	10	12	9	34
T1	0.0	3.2	7.6	7.2	0	90	90	100	0.0	2.9	6.9	11.2	0	6	8	30
T2	0.0	2.4	3.4	3.0	0	70	90	100	0.0	2.2	2.4	2.4	0	30	21	15
T3	0.0	0.0	0.0	0.0	0	0	0	0	0.0	0.0	0.0	0.0	0	0	0	0
Dactylotenium aegyptium																
Control	7.3	19.3	26.5	6.0	30	100	100	100	0.4	2.4	6.6	6.2	0.5	2.7	10.6	12
T1	6.2	15.3	17.9	6.8	0	90	90	100	0.0	2.9	6.9	8.1	0.0	5.3	5.5	19
T2	4.3	5.2	11.6	2.8	0	0	0	100	0.0	1.0	6.3	0.4	0.0	0.0	0.0	3
T3	1.0	2.5	2.4	0.0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
Paspalidium flavidum																
Control	0	0	0	0	0	0	7	0	0.0	0.0	1.0	0.0	0	0	7	0
T1	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0	0	0	0
T2	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0	0	0	0
T3	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0	0	0	0

TABLE 4. CONTINUED

Treatment	Abundance				Frequency (%)				Density				Contribution (%) to total weed biomass			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
Amaranthus spinosa																
Control	1.0	1.0	1.0	1.3	20	20	30	60	0.2	0.2	0.3	0.3	0.4	1.0	1.7	7
T1	0.0	1.0	1.5	1.8	0	10	20	40	0.0	0.1	0.1	0.1	0.0	0.4	0.0	4
T2	0.0	1.0	1.5	1.0	0	10	20	20	0.0	0.1	0.0	1.0	0.0	0.8	0.0	3
T3	0.0	0.0	0.0	0.0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0
Boerhavia diffusa																
Control	1.0	1.0	1.0	1.0	10	30	20	60	0.1	0.3	0.2	0.7	0.9	0.3	2.2	5.6
T1	0.0	1.0	1.5	1.3	0	10	20	70	0.0	0.1	0.3	0.9	0.0	0.3	3.0	6.4
T2	0.0	1.0	1.5	1.0	0	10	20	10	0.0	0.1	0.3	0.2	0.0	0.8	7.3	3.7
T3	0.0	0.0	0.0	0.0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Phyllanthus nirurii																
Control	1	1	2	1	10	10	30	10	0.1	0.1	0.2	0.1	0.2	1.1	1.5	1.4
T1	0	0	1	1	0	0	20	30	0.0	0.0	0.1	0.1	0.0	0.0	0.5	0.6
T2	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3
T3	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

TABLE 5. WEED BIOMASS IN *Gliricidia* MULCH-APPLIED TRIAL PLOTS HARVESTED AT 20-DAY INTERVALS[a]

Treatment	Harvest I (g/0.5 m^2)	Harvest II (g/0.5 m^2)	Harvest III (g/0.5 m^2)	Harvest IV (g/0.5 m^2)
Control	5.56	10.74	22.45	34.72
T1	3.33*	8.54*	17.92**	39.92*
T2	1.80**	4.04**	5.01**	12.03**
T3	0.08**	0.50*	0.54**	1.20**

[a]Levels of significance (compared to control): NS = not significant, * = significant at 5% level, ** = significant at 1% level.

T1, and T2 plots; however, in T3 the frequency percentage of *Cyperus difformis* gradually increased (Table 4) from the first harvest to the fourth harvest. Other species of weeds slowly emerged in successive stages of the mulch decomposition (Table 5). There is a proportional relationship between mulch quantity and weed control. Weed control was found to be highest in T3, where the amount of mulch was 1200 g/m^2.

Effect of Leaf Manure on Sorghum

Root Biomass. Total biomass of *Sorghum* was estimated at successive intervals in the control and manure-applied plots, and the results are shown in Table 3. In the first harvest, there was a significant increase in RB of manure-applied plants; later the RB decreased significantly in all manure-treated plants. Heavy dose of leaf manure adversely affected the RB.

Shoot Biomass. SB increased significantly in first, second, and third harvests of all treated plants. In the final harvest there was no difference among the SB contents of control, t1, and t2, whereas the t3 showed a significant reduction in SB.

Inflorescence Biomass and Grain Yield. During second harvest, IB was increased in t1 and t2 plants, whereas there was a significant decrease in t3 plants. In the third harvest, only t2 showed a significant increase in IB compared to control. The GY significantly increased in all the manured plants and particularly in t2, which showed more GY than others.

DISCUSSION

Inhibitory effects of certain phenolic compounds on germination have been shown in *Sorghum* (Rasmussen and Einhellig, 1977; Einhellig et al., 1982). Phenolic compounds are known to be growth inhibitory and are allelopathic in

nature (Rice, 1984; Kuiters and Sarink, 1987; Weidenhamer et al., 1989). In the present study also there was an inhibition of seed germination and elongation of radicle in *Sorghum* due to the allelochemicals found in the leaves of *Gliricidia sepium* (Table 1).

Beneficial Effects

The performance of the mulching practice was better than manuring in the experimental plots. Mulch treatment of 800 g/m^2 was optimum for *Sorghum* under experimental conditions, and there was better performance under field trials. Obando (1987) has reported that *Gliricidia sepium* leaf mulches controlled certain weed species without affecting crops such as corn or beans. In the present study mulching controls weeds as well as nourishes the crop plant *Sorghum vulgare*.

Allelopathic Effects

The thick mulch layer of *Gliricidia* leaves applied in the plots retarded seed germination and establishment of the weeds by the potential allelopathic compounds. In the leaf-manure-treated plots, there was a reduction in the RB owing to the allelochemicals present in the rhizosphere of the sorghum plants of t1, t2, and t3. Heavy doses of leaf manure or mulch (above 800 g/m^2) did not promote the biomass yield as the optimal dose, possibly due to much accumulation of allelochemical, i.e., in the soil above optimal levels.

The farmers in the semiarid zones of Madurai District (Tamil Nadu, South India) apply only organic manure for most of the crops. *Gliricidia* leaf mulch could be a substitute for physical methods such as hand weeding, which are time-consuming and costlier. In dry land, crops are in need of moisture, and mulch conserves moisture. The traditional practices of dry-land agriculture in India provide the best base to develop an effective production system with minimum inputs. Haider and Martin (1975) reported that 42–98% of specifically labeled phenolic acids decomposed in 12 weeks in a green-field sandy loam topsoil obtained from a citrus grove. The soil samples were maintained in ideal conditions for microbial action, and therefore it is difficult to extrapolate to field conditions (Rice, 1979). *Gliricidia sepium* leaf mulch can be used as a cheap source of nourishment for sorghum grown in dry-land agroecosystems following the optimal application levels. It is necessary to test the effectiveness of *Gliricidia* leaf mulching on various other crop plants and weeds in the field condition.

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CHEMICAL ECOLOGY OF THE PALM WEEVIL *Rhynchophorus palmarum* (L.) (COLEOPTERA: CURCULIONIDAE): ATTRACTION TO HOST PLANTS AND TO A MALE-PRODUCED AGGREGATION PHEROMONE

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Abstract—Attraction to host plants by adult *Rhynchophorus palmarum* (L.) palm weevils was studied in the field and in the laboratory. Chemical analysis revealed the presence of ethanol and ethyl-acetate in stems of coco palms and in pineapple fruits and of pentane, hexanal, and isopentanol in coco stems. In the olfactometer, the first two compounds and isoamyl-acetate were attractive to the insects and the last three compounds, although not attractive by themselves, increased attractiveness when mixed with the first two compounds. Mixtures of these compounds, in proportions similar to the one occurring in attractive plant tissue, were as attractive as natural coconut tissue. In the field, the chemical compounds, either presented alone or as a mixture, did not attract the weevil. Males produce an aggregation pheromone when smelling ethyl-acetate. Rhynchophorol, 2(*E*)-6-methyl-2-hepten-4-ol, the known active component of the aggregation pheromone, attracts weevils in the olfactometer and in the field only if plant tissue, ethyl-acetate, or the above-mentioned odor mix are present. We propose that a complex mix of ethanol, ethyl-acetate, pentane, hexanal, isolamyl-acetate, and/or isopentanol serve as a short-range orientation cue to fresh wounds on the plant and that additional host odors, attracting weevils from a distance, have still to be

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discovered. Rhynchophorol can be considered to be a synergist, having an anemotactic action at a distance. We recommend the use of retention traps baited with rhynchophorol, ethyl-acetate, and sugar cane as an alternative control method for the pest.

Key Words—Palm weevil, *Rhynchophorus palmarum*, aggregation pheromone.

INTRODUCTION

The weevil *Rhynchophorus palmarum* uses plants of the family Palmae to oviposit, grow their larvae, and pupate (Wattanapongsiri, 1966). This insect is considered to be an important pest on coconut (*Cocos nucifera* L.) plantations and on the African oil palm (*Elaeis guineensis* Jacq.) in Venezuela, Mexico, Brazil, and the Caribbean (Barreto, 1984; Bedford et al., 1978; Dean and Velis, 1976; Ponte et al., 1971) due to direct damage to the plant and as a vector of the nematode *Bursaphelenchus cocophilus* (Cobb), which causes red-ring disease (Griffith, 1987; Genty, 1988).

Field observations showed that the weevil uses odor cues to orient to its host plants (Moura et al., 1989; Hernández et al., 1992). The traditional method of controlling this pest is the use of poisoned traps (Posada, 1988) baited with damaged tissue of coconut, pineapple, or banana fruits (Chavero, 1976; Dean and Velis, 1976; Morin et al., 1986; Vera and Orellana, 1988). The plant tissues show maximum attraction four to seven days after installation of the traps (Cerda et al., 1993; Hernández et al., 1992). Little is known about the specific chemicals used by the weevil to orient to the host. Ethanol is known to be produced by coconut, pineapple, and bananas (Macku and Jennings, 1987; Flath and Forrey, 1970) and has been shown to be attractive to palm weevil (Hagley, 1965). Recently, Rochat et al. (1991b) reported rhynchophorol, 2(*E*)-6-methyl-2-hepten-4-ol, as the aggregation pheromone produced by male weevils in the laboratory, which was confirmed using field experiments, as (*S*)-6-methyl-2(*E*)-hepten-4-ol by Oehlschlager et al. (1992, 1993).

Other odors known to be produced by plant material attractive to the weevil include: ethanol, present in sugar cane and pineapple; ethyl-acetate, present in pineapple, banana and sugar cane; isoamyl-acetate, present in pineapple and banana; and isopentanol, present in banana (Flath and Forrey, 1970; Macku and Jennings, 1987; Godshall et al., 1980).

We developed a retention trap for the weevil, which uses natural attractants to capture the pest, without the need of insecticides (Hernández et al., 1992) and designed an olfactometer for testing attractants in the laboratory (Cerda et al., 1993). The aim of this work was to further perfect a retention trap, using synthetic attractants as bait.

METHODS AND MATERIALS

Insects. Adult *R. palmarum* were collected at Dto. Acevedo, Edo Miranda, Venezuela, and were maintained in the laboratory at 23–27°C, 70–90% relative humidity, and a 12-hr photophase; they were fed sugar cane.

Equipment and Bioassay. Figure 1 illustrates the olfactometer, described in detail before (Cerda et al., 1993). It consisted of a device to fix the insect, which was glued to a wooden holder at the thorax. The insect was hanging on this holder and had a light foam ball (5 cm diam., 1.5 g) to walk on. When the insect started to fly, it released the ball and extended its wings. Compressed air, filtered with activated carbon and humidified before it reached the insect, was blown at its head through two ducts. Air flow was calibrated to 2.8 cm^3/sec just before flasks A and B and reached the insect at an angle of 45° to its longitudinal axis 5 cm from both sides of its head. Odor sources were introduced into the tubes using an Erlenmeyer flask through which air passed prior to reaching the animal. The animal walked by rotating the foam ball. By measuring the direction of rotation of the ball, we estimated the weevil's attempt to walk either to its left or to its right.

We measured the attractiveness to the weevil of each of the two air sources at either side of the insect by observing the insects during a 5-min period. The responses of the insects were classified in three categories: those with a definitive preference, walking or flying over 60% of the time heading in the direction of one of the air sources, those with no clear preference, walking alternatively in both directions, and those that showed no activity, i.e., remaining inactive during the observation period.

Bioassays were performed from 1000 to 1200 hr and 1600 to 1830 hr, times when adults were known to show maximum activity (Hernández et al., 1992).

Odors. Apical parts of *C. nucifera* palms without (healthy) or with red-ring disease (infested) were collected in the field and kept in the laboratory for fermentation under ambient temperature. For the bioassays, we used either 50 g of plant tissue (either pineapple fruit, banana fruit, or coconut stem) or 3 ml of pure synthetic reagents (Aldrich Chemicals, HPLC grade). The plant tissue, live adult weevils, or liquids placed in 5-ml vials were introduced into the Erlenmeyer flask over which air was blown prior to reaching the animal.

Serial Odors. In order to test the effect of various odors on the release rate of the aggregation pheromone by males, two Erlenmeyer flasks were connected in series and air was blown through them prior to reaching the hanging insect. In one flask, we placed five live males or females and in the other, 3 ml of pure odor. The relative position of the two flasks (i.e., the one over which air passed first) was critical and is indicated in Table 4 below.

Field Tests. The retention trap described by Hernández et al. (1992) was used. The trap, consisting of a plastic container allowing entrance but not exit

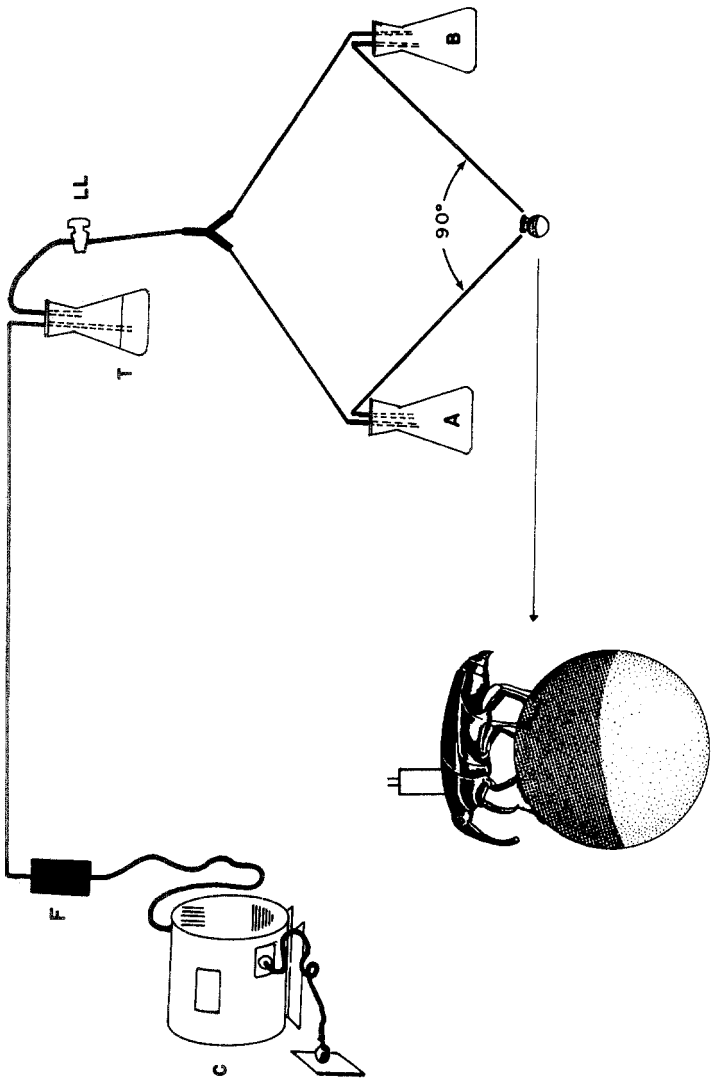

FIG. 1. Schematic representation of the olfactometer showing the Erlenmeyer flasks (A and B), a humidifier (T), a carbon filter (F), flowmeter (LL), and air pump (C). Additional flowmeters before and after flasks A and B are not shown. The weevil was fixed by the thorax and walked freely on the foam ball, dropping the ball when it started to fly.

of adult weevils was baited with two live adult weevils placed in a plastic box with holes and/or 50 ml of chemicals in 250-ml glass flasks. Traps were placed in the field in groups of three (Table 6 below) or two (Table 7 below) traps per location, approximately 50 m apart, and checked every three days. Locations were more than 3 km apart from one another. Tests were performed at Dto. Acevedo, Estado Miranda, Venezuela.

Rhynchophorol for field testing was formulated in ethanol at dilutions of 0.01%. We used 50 ml of this dilution to bait the traps, which took approximately 15 days to evaporate in the field, giving a release rate of the pheromone of 0.03 mg/day.

Statistical Analysis. Results from the olfactometer were analyzed statistically at three levels (Siegel, 1956): (1) Number of those responding to each of the two odor sources were compared with a binomial test. (2) To confirm a lack of any preferential response, the number of animals that responded in the bioassay with a clear preference to either source was compared to those not responding (inactive) plus those not showing any preference (no preference), with a binomial test. (3) Differential responses of males versus those of females were compared with Fisher's test using a 2 × 2 matrix constructed with the responses showing a clear preference to either source.

Chemical Identification. Plant tissues (coconut and pineapple) were analyzed using CH_2Cl_2 extracts and the headspace method, i.e., the tissue was left in a sealed glass container for 24 hr and a 1-ml gas sample was then extracted for analysis. Analysis was performed isothermically (30°C) with GC using a 25-m × 0.2-mm internal diameter methylsilicone HP-1 column, helium as carrier gas, and a mass spectrometer coupled to a gas chromatograph (GC-MS, HP 5890 series II with a MS 5971A). Identification of compounds was confirmed using the Wiley library for mass spectra and by coinjecting pure samples with natural odors in our GC system.

The temporal analysis of the relative proportions of each compound in the head space was quantified by comparing peak areas with that of known amounts of synthetic standards. A Perkin Elmer 3920-A GC with a 8-m × 2-mm-ID column, filled with diethylene-glycol-adipate on 80–100 mesh Chromosorb, was used. Temperature was run from 50°C to 120°C at a rate of 16°C/min.

RESULTS

Chemical Identification of Volatiles from Hosts. GC-MS of volatiles from infested and healthy coco plants revealed the presence of water, CO_2, ethyl-acetate, pentane, hexanal, isopentanol, and ethanol. Fruits of pineapple produced ethanol and ethyl-acetate. No other compound could be detected with our methods.

The temporal analysis of the relative proportions of these compounds in healthy and infested coconut tissue (Figure 2) showed that the proportion of ethanol vs. ethyl-acetate changed during decomposition of the material, reaching a proportion of 7:3 after three days of decomposition and remained relatively constant up to day 9. This timing coincides with the moment when plant tissue showed maximal attraction in the laboratory and in the field (Cerda et al., 1993; Hernández et al., 1992). Pentane concentrations did not vary significantly during decomposition of the plant tissue. Hexanal and isopentanol were present in trace amounts.

Attractants. Table 1 shows the responses of the weevil to pure air and to two simultaneous sources of ethanol (controls). In neither case was any preference evident. The results in Table 1 also show that all types of plant tissues produce attractive odors. Among the volatiles identified, ethanol was shown to be attractive (Table 2); in addition, isoamyl-acetate, reported to be attractive to the weevil (Hagley, 1965), and known to be present in pineapple and banana (Flath and Forrey, 1970; Macku and Jennings, 1987), was attractive in the

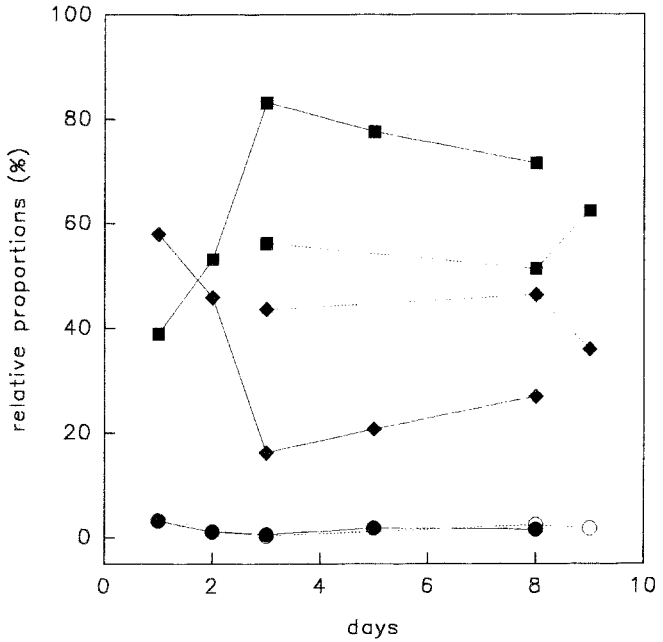

FIG. 2. Temporal variation of volatiles present in coco palm tissue. The relative proportion between ethanol (squares), ethyl-acetate (diamonds), and pentane (circles) is represented after various days of decomposition for healthy tissue (solid lines) and infested tissue (dotted lines).

TABLE 1. PERFORMANCE OF ADULT *R. palmarum* IN OLFACTOMETER MEASURING RESPONSE TO DIVERSE ODOR SOURCES

	Males	Females	Total[a]
Air	2	1	3
Air	0	1	1
No preference	3	4	7*
Inactive	5	4	9
Ethanol	3	2	5
Ethanol	3	3	6
No preference	12	10	22*
Inactive	2	4	6
Pineapple	6	8	14†
Air	4	1	5
No preference	2	3	5
Inactive	3	3	6
Banana	4	5	9†
Air	1	1	2
No preference	3	3	6
Inactive	2	1	3
Healthy *C. nucifera*	4	3	7†
Air	1	0	1
No preference	0	1	1
Inactive	0	1	1
Infested *C. nucifera*	3	3	6†
Air	0	0	0
No preference	2	1	3
Inactive	0	1	1

[a] Statistically significant values ($P < 0.05$) are indicated with: *binomial test between animals which responded with a preference to either source, and inactive animals plus animals showing no preference; and †binomial test between animals responding to each of the two odor sources.

olfactometer. Pure ethanol or isoamyl-acetate were as attractive or even more attractive than natural plant tissue (i.e., pineapple vs. isoamyl-acetate, Table 2).

Table 3 shows the attractiveness of some mixtures of these compounds (total volumes of 3 ml). We observed that ethanol and ethyl-acetate in a proportion of 70:30, as found in coco plants, was the most attractive two-compound mixture for the weevils. Weevils did not seem to distinguish between methanol and ethanol. Successive enrichment of the ethanol–ethyl-acetate mixture with traces of pentane, isoamyl-acetate, hexanal, and isopentanol increased the attractiveness of the mixtures, although differences between different complex mixtures of these substances were not always statistically significant. A mixture of 68% ethanol, 27% ethyl-acetate, and 5% pentane was statistically as attractive

TABLE 2. RESPONSE OF ADULT *R. palmarum* TO SYNTHETIC COMPOUNDS

Compounds	Males	Females	Total[a]
Ethanol	7	6	13†
Air	2	1	3
No preference	2	6	8
Inactive	4	2	6
Ethyl-acetate	3	1	4
Air	0	4	4
No preference	8	5	13*
Inactive	8	8	16
Isoamyl-acetate	5	5	10†
Air	0	0	0
No preference	0	0	0
Inactive	0	0	0
Pentane	3	1	4
Air	0	3	3
No preference	1	1	2
Inactive	1	0	1
Hexanal	2	1	3
Air	3	2	5
No preference	0	1	1
Inactive	0	1	1
Healthy *C. nucifera*	6	5	11
Ethanol	2	3	5
No preference	1	2	3
Inactive	1	0	1
Healthy *C. nucifera*	3	5	8
Isoamyl-acetate	4	5	9
No preference	3	2	5
Inactive	1	1	2
Banana	1	2	3
Isoamyl-acetate	3	3	6
No preference	1	0	1
Inactive	0	0	0
Pineapple	0	0	0
Isoamyl-acetate	3	4	7†
No preference	2	1	3
Inactive	1	0	1

[a] Statistically significant values ($p < 0.05$) are indicated as in Table 1.

as natural plant tissue. Isoamyl-acetate in pure form was the most attractive compound tested. None of these compounds or mixtures attracted a single weevil in the field (12 traps for two weeks, not shown).

Male-Produced Aggregation Pheromone. Table 4 shows that when tested

TABLE 3. RESPONSE OF ADULT *R. palmarum* TO DIVERSE MIXED ODOR SOURCES

Mixtures[a]	Males	Females	Total[b]
70% EtOH:30% EtAc	5	1	6
50% EtOH:50% EtAc	1	2	3
No preference	5	2	7
Inactive	4	1	5
70% EtOH:30% EtAc	3	4	7†
80% EtOH:20% EtAc	0	0	0
No preference	4	2	6
Inactive	3	4	7
70% MeOH:30% EtAc	2	1	3
70% EtOH:30% EtAc	1	2	3
No preference	2	0	2
Inactive	0	2	2
63% EtOh:17% EtAc:10% IsAc	3	6	9†
70% EtOH:30% EtAc	2	0	2
No preference	3	4	7
Inactive	0	0	0
35% EtOH:15% EtAc:50% IsAc	0	0	0
7% EtOH:3% EtAc:90% IsAc	3	3	6†
No preference	1	1	2
Inactive	1	1	2
68% EtOH:27% EtAc:5% Pent	5	7	12†
70% EtOH:30% EtAc	2	0	2
No preference	3	3	6
Inactive	0	0	0
66% EtOH:27% EtAc:5% Pent:1% IsAc:1% Ipen	3	2	5†
67% EtOH:27% EtAc:5% Pent:1% IsAc	0	0	0
No preference	1	3	4
Inactive	1	0	1
66% EtOH:26% EtAc:5% Pent:1% IsAc:1% Ipen: 1% Hxal	2	4	6
66% EtOH:27% EtAc:5% Pent:1% IsAc:1% Ipen	1	0	1
No preference	2	1	3
Inactive	0	0	0
Infested *C. nucifera*	2	2	4
68% EtOH:27% EtAc:5% Pent	1	0	1
No preference	7	6	13*
Inactive	0	2	2

[a] EtOH: ethanol; MeOH: Methanol; EtAc: ethyl-acetate; IsAc: isomyl-acetate; Pent: pentane; Ipen: isopentanol; Hxal: hexanal.

[b] Statistically significant values ($P < 0.05$) are indicated as in Table 1.

TABLE 4. RESPONSE OF ADULT *Rhynchophorus palmarum* TO LIVING WEEVILS[a]

	Males	Females	Total
5 M	9	4	13†
4 F	1	0	1
No preference	11	7	18
Inactive	5	13	18
IsAc + 5M	4	2	6
IsAc + 5 F	2	1	3
No preference	10	9	19
Inactive	0	2	2
EtOH + 5 M	3	3	6
EtOH + 5 F	3	1	4
No preference	9	6	15
Inactive	1	4	5
Pent + 5 M	2	3	5
Pent + 5 F	2	2	4
No preference	8	6	14
Inactive	3	3	6
EtAc + 5 M	9	13	22†
EtAc + 5 F	1	1	2
No preference	0	0	0
Inactive	4	0	4
5 M + EtAc	0	2	2
5 F + EtAc	1	0	1
No preference	0	11	11
Inactive	0	0	1
70% EtOH:30% EtAc + 5 M	7	5	12†
70% EtOH:30% EtAc + 5 F	2	0	2
No preference	5	5	10
Inactive	3	3	6
70% EtOH:30% EtAc + 5 M	2	6	8†
EtAc + 5 M	0	1	1
No preference	1	0	1
Inactive	0	0	0
EtAc + 5 M on cane)	7	4	11†
(5 M on cane) + EtAc	1	1	2
No preference	2	5	7
Inactive	0	0	0

[a] EtAc: ethyl acetate; EtOH: ethanol; IsAc: Isoamyl acetate; Rync: rhynchophorol; Pent: pentane; cane: sugar cane; M: males; F: females; + indicates that the first odor was placed prior to the other in a separate flask. Statistically significant values ($P < 0.05$) are indicated as in Table 1

in our olfactometer, males and females were only attracted to males. If vapor of ethyl-acetate or a mixture of ethyl-acetate and ethanol was blown over the males, they became very attractive to both males and females. No attraction could be observed when the air was first blown over the males in the Erlenmeyer flask and then over these chemicals, or if they were blown over females. In other words, not the presence of ethyl-acetate per se, but the odor of ethyl-acetate or the ethanol–ethyl-acetate mixture by the males in the Erlenmeyer made them attractive to the weevils. The results also show that males feeding on sugar cane were not as attractive as males smelling ethyl-acetate while feeding on sugar cane.

The racemic mixture of pure rhynchophorol elicited no attraction but was attractive when diluted to less than 0.1 ppm with ethanol (not shown) or with an ethanol, ethyl-acetate, and pentane mixture (Table 5).

Interestingly, all odors tested produced similar responses in male and female weevils (no significant differences using analysis 3 described in Methods and Materials).

TABLE 5. RESPONSE OF ADULT *Rhynchophorus palmarum* TO RHYNCHOPHOROL[a]

	Males	Females	Total
A Rync	2	1	3
Air	3	2	5
No preference	7	10	17*
Inactive	3	2	5
B Rync: 1%	2	4	6
Mix	5	1	6
No preference	1	1	2
Inactive	0	0	0
C Rync: 10^{-5} %	7	7	14†
Mix	2	1	3
No preference	8	9	18
Inactive	3	3	6
D Rync: 10^{-8} %	4	5	9†
Mix	1	1	2
No preference	10	6	16
Inactive	3	3	6
E Rync: 10^{-11} %	2	3	5†
Mix	0	0	0
No preference	2	2	4
Inactive	1	1	2

[a]Concentrations of Rync: pure racemic mixture in experiment A; and diluted in a 68% EtOH + 27% EtAc + 5% Pent mixture (Mix) in experiments B–E in proportions given as a % of the solution. Statistically significant values ($P < 0.05$) are indicated as in Table 1.

Field Tests. As field populations of flying adult weevils may vary from day to day, only comparisons between traps tested at the same sites and days were made. Tables 6 and 7 show the results of field tests comparing the relative efficiency of different baits using our retention trap. Experiment 1 (Table 6) showed that the presence of sugar cane and, to a lesser extent, that of ethyl-acetate increased the capture of weevils by traps baited with males. Rhynchophorol alone did not attract weevils to the trap (experiment 2, Table 6), even if

TABLE 6. FIELD CATCHES OF ADULT WEEVILS USING DIFFERENTLY BAITED TRAPS[a]

Trap with	Captured insects (mean per trap ± SD)			*P* (Wilcoxon test males/female)
	Total adults	Males	Females	
Experiment 1				
2 males + cane + EtAc	4.4 ± 4.7	1.8 ± 2.4	2.7 ± 2.7	0.001
2 males + cane	3.0 ± 4.2	1.3 ± 1.9	1.7 ± 2.6	0.107
2 males + EtAc	1.1 ± 1.8	0.4 ± 0.7	0.7 ± 1.2	0.037
ANOVA (df = 48)				
F (Friedman)	27.70	13.14	17.09	
P	<0.001	0.001	<0.001	
Experiment 2				
Cane + Rync	2.3 ± 3.2	1.6 ± 2.2	0.7 ± 1.1	0.01
Cane	0.1 ± 0.3	0.1 ± 0.3	0.0 ± 0.0	0.18
Rync	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.00
ANOVA (df = 54)				
F (Friedman)	10.33	8.58	5.33	
P	0.006	0.014	0.069	
Experiment 3				
Rync + cane	3.0 ± 3.4	1.1 ± 1.6	1.9 ± 2.1	0.025
Rync + EtAc	0.7 ± 1.2	0.2 ± 0.5	0.5 ± 1.0	0.114
Cane	0.2 ± 0.6	0.03 ± 0.1	0.1 ± 0.4	0.109
ANOVA (df = 30)				
F (Friedman)	20.07	9.05	15.52	
P	<0.001	0.011	<0.001	
Experiment 4				
Rync + Mix	0.4 ± 0.7	0.1 ± 0.3	0.3 ± 0.5	0.180
Rync + EtAc + EtOH	0.4 ± 0.5	0.0 ± 0.0	0.4 ± 0.5	0.018
Rync + EtAc	1.5 ± 1.3	0.3 ± 0.7	1.2 ± 0.9	0.043
ANOVA (df = 24)				
F (Friedman)	2.67	0.50	3.50	
P	0.264	0.799	0.174	

[a]Six catches for three-day periods for each trap are given. Mix represents a solution of 66% EtOH:27% EtAc:5% Pent:1% IsAc:1% Ipen. All three means in each column are statistically different among them as tested with Wilcoxon's matched pairs test ($P < 0.05$). Catches for three-day periods are given. Abbreviations as in Tables 4 and 5.

TABLE 7. FIELD CATCHES OF ADULT WEEVILS USING DIFFERENTLY BAITED TRAPS[a]

	Total adults	Males	Females
Experiment 1			
Cane + Rync + Mix	15.0 ± 8.2	7.5 ± 5.0	7.5 ± 3.9
Cane + 2 males + EtAc	3.7 ± 1.1	1.8 ± 0.9	1.8 ± 1.3
Friedman's F (df = 5)	3.0	3.0	3.0
P	0.083	0.083	0.083
Experiment 2			
Rync + cane + EtAc	35.6 ± 17.9	15.3 ± 6.9	20.3 ± 11.2
Rync + cane	11.5 ± 4.3	3.8 ± 2.1	7.7 ± 4.3
Friedman's F (df = 11)	6.00	6.0	2.7
P	0.014	0.014	0.102
Experiment 3			
Rync + Mix	2.0 ± 1.5	1.1 ± 1.0	0.9 ± 0.6
Rync + cane	14.3 ± 8.2	6.9 ± 4.5	7.4 ± 4.7
Friedman's F (df = 17)	9.0	9.0	9.0
P	0.003	0.003	0.003
Experiment 4			
Rync + EtAc	1.1 ± 0.9	0.6 ± 0.8	0.5 ± 0.5
Rync + Mix	1.1 ± 1.6	0.5 ± 0.8	0.6 ± 1.0
Friedman's F (df = 11)	0.17	0.67	0.0
P	0.683	0.414	1.00

[a] Six catches for three-day periods for each trap are given. Legend as in Table 6. No significant differences between the number of captured males and females was found using Wilcoxon's matched pairs test ($P > 0.05$).

presented at a release rate of 30 mg/day (not shown), but together with sugar cane and ethyl-acetate or with ethyl-acetate alone did attract insects of both sexes (experiment 3, Table 6). Ethyl-acetate or different mixtures of the attractive odors trapped insects when presented together with rhynchophorol without sugar cane (experiment 4, Table 6); complex odor mixtures did not seem to trap more insects than ethyl-acetate alone.

Experiment 1 (Table 7) compares traps baited with rhynchophorol, as developed by Oehlshlager et al. (1993) and improved with our odor mix, to traps baited with live males stimulated with ethyl-acetate. The results show that rhynchophorol together with sugar cane and our odor mix attracts more weevils than calling males. The most attractive trap was that baited with rhynchophorol, ethyl-acetate, and sugar cane (experiment 2, Table 7), as our mixture of volatile compounds without sugar cane was always less attractive than sugar cane alone if presented with rhynchophorol (experiment 3, Table 7). Again, the synergistic effect on rhynchophorol of a complex odor mixture seems statistically indistinguishable from that exercised by ethyl-acetate alone (experiment 4, Table 7).

Thus, although our odor mix of six compounds (ethanol, ethyl-acetate, pentane, isopentanol, isoamyl-acetate, and hexanal) seemed a little more attractive than less complex mixtures in the laboratory, traps baited with rhynchoporol, ethyl-acetate, and sugar cane were statistically indistinguishable in trapping efficiency from those baited with rhynchophorol, sugar cane, and our six-compound odor mix (not shown). The presence of sugar cane in baits always increased the trapping efficiency of our odor mix, indicating that some odors, present in sugar cane, were absent from our odor mix or that the relative concentrations of the various compounds in our mix were inappropriate.

In order to exclude the possibility that weevils were escaping at larger rates from traps without sugar cane (Oehlschlager et al., 1993), we repeated experiment 2 in Table 7 placing soapy water at the bottom of the trap, killing all trapped adults. Results were indistinguishable from those reported in Table 7 (not shown).

DISCUSSION

Adult *R. palmarum* weevils are attracted by a variety of plant tissues (pineapple, banana, coco), which justifies their use as baits in traps. Our results show that pineapple and coco plant tissue produce ethyl-acetate and ethanol and that adult weevils are attracted to these compounds in the olfactometer with maximal attraction to mixtures of these components at relative concentrations similar to those produced by decomposing natural host-plant tissue. This suggests an orientation function of these compounds, although field trials showed that these compounds alone were not sufficient to attract weevils. Thus, other host-plant odors, in addition to ethanol and ethyl-acetate, are used by adult weevils to locate their host plant.

We found that coco plant tissue also produces isopentanol, hexanal, and pentane. The presence of pentane was confirmed several times in coco but not in other tissues processed at the same time in the laboratory, suggesting that it was not a contaminant. These compounds, although not attractive by themselves, increase the attractiveness of an ethanol–ethyl-acetate mixture when added to it, suggesting that they synergize the attractive effect of these compounds. Weevils may use complex odor mixtures to orient to their host plants, and relative proportions of these compounds in the mixture may be important, although we were unable to test all combinations of the infinite variety of possible relative proportions. The various field tests of mixtures of the six compounds, in proportions similar to that decomposing coco plant tissue, did not attract weevils. This suggests that a complex odor mixture that includes other compounds, in addition to the one found here, are used by weevils to locate their host plants.

The contradictory results between laboratory experiments using an olfactometer and field assays using baited retention traps are probably due to the fact that the two methods measure different phenomena. The olfactometer measures short-range attraction of walking weevils to odors, whereas field traps basically measure attraction of flying weevils, where short-range attractants are only required to enter the trap. If long-range attractants are absent, weevils would be unable to find the trap in the first place. This suggests that ethanol, ethyl-acetate, and the synergist pentane, hexanal, isoamyl-acetate, and isopentanol among other unknown compounds, orient and attract weevils only at short distances. Other odors, such as rhynchophorol and unknown mixtures of host odors, attract and orient weevils at longer distances through anemotaxis. Circumstantial evidence that gives some weight to this hypothesis comes from field observations of flying weevils (Sánchez et al., 1993). The insects, when approaching a freshly cut palm tree fly anemotactically (and possibly also klinokinetically) 5–30 m from the source. At shorter distances, weevils fly in zigzags and semicircles around the odor source, suggesting that they are using klinokinetic and possibly also visual orientation mechanisms. The presence of high humidity and/or ethanol enhance entrance to the traps once the weevil has landed on it (Hernández et al., 1992). Thus, two orientation mechanisms could be used by palm weevils to locate the host plant: a long-range anemotactic orientation signal and a short-range klinokinetic orientation mechanism modulated by mixtures of ethyl-acetate, ethanol, pentane, and other undiscovered odors. Rhynchophorol seems to act as a long-range attractant, but naturally decomposing palm and pineapple tissue also produce a long-range attractant, which we have not yet identified. Rhynchophorol by itself seems to be repellent at high concentrations and could, in addition to its anemotactic effect described above, synergize other host odors and/or other components of the male aggregation pheromone.

Previous research suggested that males of the palm weevil may produce an aggregation pheromone when feeding on host plant tissue (Moura et al., 1989; Rochat et al., 1991a). Our results showed that males release the pheromone when smelling ethyl-acetate, which is produced by the host-plant tissue. Rhynchophorol, a major component of the pheromone (Rochat et al., 1991b; Oehlschlager et al., 1992) is attractive to palm weevils only if presented in diluted form, together with sugar cane, ethyl-acetate, or the ethyl-acetate, ethanol, pentane mixture. The highest attractiveness of rhynchophorol is reached when it is presented with sugar cane and ethyl-acetate, suggesting that the aggregation pheromone, in order to be active, needs synergistic odors from the host plant, one of them being ethyl-acetate. Very small amounts of the compound (i.e., concentrations of less than 10^{-11}%) are detected by the weevil.

Rochat et al. (1991b) and Oehlschlager et al. (1993) suggested that rhynchophorol alone attracts adult weevils. Oehlschlager et al. (1993) showed that traps baited with rhynchophorol improved the captures of weevils if they placed

sugar cane in the traps to prevent weevils from escaping. Here we showed that without sugar cane, traps baited with rhynchophorol are inactive. Rhynchophorol, in order to be active, needs the presence of ethyl-acetate or an odor mixture, although for maximal efficiency, other unknown compounds present in sugar cane are required.

Based on data in the literature and on our results, we propose the following: (1) Male weevils explore the field for new host plants, which they locate by a complex mix of odors, among them ethanol, ethyl-acetate, isoamyl-acetate, pentane, isopentanol, and hexanal. (2) Once a suitable host plant is found, which has to produce ethyl-acetate among other odors, the male weevil, stimulated by feeding and/or smelling the host-plant tissue, produces an aggregation pheromone containing, as an important component, rhynchophorol. (3) Males and females are attracted by a mix of host odors and aggregation pheromone to the newly discovered host plant. (4) Aggregated males and females copulate on the host plant and females start laying eggs (Sánchez et al., 1993). At this stage, plants seem to be most susceptible to being infested with red-ring disease. (5) A large number of larvae start feeding on the host plant, enhancing the decomposition of the plant tissue due to associated nematodes and other microorganisms, thus resulting in more decomposed tissue available for feeding. (6) Aggregation on the decomposing host plant continues for several months (Sánchez et al., 1993).

Traps baited with rhynchophorol, sugar cane, and ethyl-acetate may be used to trap adult weevils in the field to control infestations. This should reduce pest populations; although some exploring males probably will not be captured with these traps, most females should be attracted to it. Thus, we recommend the use of retention traps, such as proposed by Hernández et al. (1992) or Oehlschlager et al. (1993), baited with rhynchophorol, ethyl-acetate, and sugar cane, to control *Rhynchophorus* pests, but more research on host plant odors is needed to design an even more efficient control system in the future.

Our field traps sometimes captured significantly different proportions of females and males, suggesting that the sex proportion of field populations vary. Weevils of different sexes could also respond slightly different to semiochemicals, although a differential response by the sexes to odors was not suggested by our laboratory experiments.

Oehlschlager et al. (1992) showed that rhynchophorol released at a rate of 30 mg/day captured more insects in the traps than if released at a rate of 0.3 mg/day. We captured insects at a release rate of 0.03 mg/day of the pheromone. Clearly more work needs to be done to determine the optimum release rate of the pheromone in traps. Despite the incomplete knowledge of the complex semiochemical system used by *R. palmarum* to colonize their host plants and to aggregate, traps baited with rhynchophorol, ethyl-acetate, and sugar cane are a more efficient alternative for trapping the pest than those baited with plant

tissue (Hernández et al., 1992), with rhynchophorol alone (Oehlschlager et al., 1993), or with males and ethyl-acetate.

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IDENTIFICATION AND SYNTHESIS OF A KAIROMONE INDUCING OVIPOSITION BY PARASITOID *Aphytis melinus* FROM CALIFORNIA RED SCALE COVERS

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Abstract—The parasitoid wasp *Aphytis melinus* uses a kairomone from the cover of its scale host, California red scale (*Aonidiella aurantii*), as an oviposition stimulant. The kairomone was isolated from extracts of scale covers, and identified as *O*-caffeoyltyrosine by a combination of spectroscopic methods. The kairomone was synthesized, and the synthetic compound was determined to be as active as the chemical isolated from scale covers.

Key Words—Kairomone, oviposition stimulant, *O*-caffeoyltyrosine, *Aphytis melinus*, Hymenoptera, Encyrtidae, *Aonidiella aurantii*, Homoptera, Diaspididae.

INTRODUCTION

Many parasitic wasps in the genus *Aphytis* are effective biological control agents of several "armored" scale insect pests in the family Diaspididae (Rosen and DeBach, 1979). These armored scale insects secrete a hard cover of nonliving material external to their bodies. Covers are composed of waxy filaments and polar, nonwaxy material that cements these filaments together, plus the cast skins of previous instars. The proportions of waxy and polar constituents vary among species, as does the color and thickness of covers (Foldi, 1990). Scale covers are not attached to the scale body during the scale's growing stages and can be easily removed, and scales whose covers have been damaged or removed can repair or entirely replace their covers if desiccation does not occur (Baker, 1976).

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Aphytis wasp species utilize a combination of physical characteristics of the scale cover (size and shape) and kairomones from the scale cover and body in making oviposition decisions (Baker, 1976; Luck et al., 1982). Oviposition behavior is characterized by extensive examination of the scale cover with the antennae, followed by probing with the ovipositor, and finally, egg deposition.

Only one such oviposition-stimulating kairomone for an *Aphytis* species has been identified to date; the wasp *A. yanonensis* uses a mixture of wax esters from the cover of its scale host, *Unaspis yanonensis* (Kuwana), as an oviposition stimulant (Takahashi et al., 1990). However, Quednau and Hübsch (1964) demonstrated that California red scale, *Aonidiella aurantii* (Mask.), produces a kairomone that influences *Aphytis coheni* oviposition; water extracts of scale covers elicited typical preoviposition behavior from wasps. Luck and Uygun (1986) corroborated and extended this study by demonstrating that several *Aphytis* species (*A. coheni*, *A. melinus*, and *A. lingnanensis*) responded to water and alcohol extracts of California red scale covers. Our objective in the present study was to isolate, identify, and synthesize the kairomone(s) present in California red scale covers that stimulate oviposition behavior in the parasitoid *A. melinus*.

METHODS AND MATERIALS

Insect Rearing

California red scale were reared on detached lemons as previously described (Tashiro, 1966). Briefly, newly emerged scale crawlers were allowed to settle on fresh lemons, where they spent the remainder of their lives. Lemons with scale were placed in a constant-temperature room (26°C, 60–70% relative humidity, constant illumination). *A. melinus* parasitoids were reared on oleander scale, *Aspidiotus nerii*, (26°C, 40–60% relative humidity, 16:8 hr photoperiod) as previously described (Opp and Luck, 1986). Lemons with oleander scale were placed in a cage with *A. melinus* adults twice a week to maintain the colony. After three weeks, scale were examined under a microscope, and wasp pupae were collected manually from parasitized scales and individually transferred to 3-ml glass vials sealed with a cotton plug. Upon emergence, wasps were sexed (females have an ovipositor that is easily recognized) and allowed to mate. A drop of honey was placed in each vial as a food source. After mating, wasps were sexed again and females were held in isolation for 48 hr before being used in bioassays.

It had been previously demonstrated that *A. melinus* responded to crude scale cover extracts placed on filter paper disks the approximate size of scale covers (Luck and Uygun, 1986). Thus, measured quantities of test solutions were pipetted onto 2.0-mm-diameter filter paper disks (Whatman No. 2), with a control disk being treated with solvent. Treated disks were spaced equidistantly

around the circumference of a 2-cm circle inside a Petri dish bioassay arena (60 mm diam × 15 mm high), a gravid female was added, and her behavior was observed for 10 min with a 6× dissecting microscope. Wasps initially investigate scales or scale mimics by palpation with their antennae as they walk from one edge of the scale cover to the other. They then turn ~90° and repeat this behavior one or more times. This behavioral bout has been termed "drumming and turning" (Luck et al., 1982; Luck and Uygun, 1986). In addition, we observed that female wasps also would probe treated disks with their ovipositors. The number of drums and turns and the number of oviposition probes on each disk were tabulated. Bioassays were usually conducted between 1000 and 1500 hr at 24–26°C, and test arenas were illuminated by fiberoptic quartz lighting on either side of the test arena. The frequency distribution of the number of drums and turns and oviposition probes on treated and untreated disks were analyzed by goodness of fit (*G* test) (Sokal and Rohlf, 1981) to the null hypothesis that the distribution of observed behaviors was independent of treatment.

Synthetic and natural *O*-caffeoyltyrosine were also bioassayed simultaneously at several concentrations, with a control disk treated only with solvent. Ten replicate trials of 10 wasps were bioassayed, with fresh disks prepared for each trial. The resulting data were analyzed by ANOVA followed by a series of single degree-of-freedom contrasts.

Isolation, Identification, and Synthesis of the Kairomone

Proton NMR spectra were recorded on a GE-300 NMR spectrometer at 300 MHz, with chemical shifts reported relative to TMS. IR spectra were recorded as KBr pellets with a Perkin Elmer 283 infrared spectrometer. Mass spectra were recorded with VG Instruments (Danvers, Massachusetts) mass spectrometers. Electron impact (70 eV, direct insertion probe) and desorption electron impact (50 eV, sample desorbed off a heated platinum wire) mass spectra were run on a VG 7070E instrument. FAB mass spectra were recorded from a VG ZAB-2fHf instrument. Ultraviolet spectra were recorded with a Hewlett-Packard 8452A diode array spectrophotometer. HPLC was used to monitor the purity of synthetic intermediates and the natural *O*-caffeoyltyrosine, using a Perkin Elmer Series 410 HPLC pump and a PE LC235 diode array detector controlled by a PE LCI-100 computing integrator. A Beckman ODS column (4.6 × 25 cm, 5 μm particle size) was used for analytical separations, with gradients of water or 0.075% aqueous trifluoroacetic acid and acetonitrile. Chromatograms were generated by monitoring wavelengths of 220 and 325 nm.

For preliminary experiments with crude extracts of scale covers, late third-instar scale covers were manually removed from scale reared on lemons, homogenized in MeOH, and filtered. Water was added (25% v/v) to the methanolic extract, and the resulting solution was back-extracted three times with chloro-

form–hexane (3 : 1) to remove lipids. The aqueous extract was concentrated to a final ratio of one scale cover equivalent of kairomone per microliter.

In preliminary experiments, the aqueous methanolic extract was fractionated on C_{18} reverse-phase TLC plates (Whatman 5 × 20 cm, 200 μm thickness) in mixtures of acetonitrile and water. In subsequent experiments, the extract was fractionated by HPLC on an Ultremex 5 C_{18} analytical column (250 × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, California) with a Beckman C_{18} guard column (45 × 4.6 mm, 5 μm particle size, Beckman Instruments Inc., San Ramon, California), eluting with a gradient of 100% H_2O to 100% acetonitrile in 25 min (1.2 ml/min), monitoring the effluent at 240 nm, and collecting 5-min fractions. The active fraction was then refractionated with a slower flow rate (1 ml/min), collecting individual peaks. The activity was concentrated in a peak eluting at 25.6 min.

A portion of the active extract was submitted to standard acidic protein hydrolysis conditions; the active extract was added to 6 M HCl and heated at 110°C overnight in a sealed glass vial. The sample was then concentrated to dryness under a stream of N_2, reconstituted in acetonitrile, and a portion was bioassayed. A further portion of the hydrolyzed extract was subjected to amino acid analysis using precolumn *o*-phthalaldehyde (OPA) derivatization as previously described (Hare et al., 1990). The remainder of the hydrolyzed extract was derivatized according to Zumwalt et al. (1987) to form volatile *n*-butyl, *N*-trifluoroacetyl derivatives of amino acids in the hydrolysate, as follows: 200 μl of hydrolysate was evaporated under N_2 and 600 μl of *n*-butanol·3 N HCl was added. The mixture was sonicated for 10 min to ensure dissolution, heated (100°C) for 30 min, concentrated to dryness under N_2, and dried azeotropically with methylene chloride. The residue was treated with trifluoroacetic anhydride in methylene chloride (1 : 2) in a sealed vial at 150°C for 5 min. The sample was then concentrated under nitrogen again, taken up in acetone, and analyzed by coupled GC–MS (Hewlett Packard 5970 mass selective detector coupled to a H-P 5890 GC; 25 m × 0.2 mm Ultra-2 column, H-P, Avondale, Pennsylvania), temperature programming from 100 to 275°C.

A second portion of the active peak recovered from HPLC was analyzed by reverse-phase HPLC with diode array UV detection, eluting with gradients of 0.075% aqueous trifluoroacetic acid and acetonitrile (see above), both before and after diazomethylation. Diazomethylation was carried out by addition of an ethereal solution of diazomethane to a methanolic solution of the active fraction until the yellow color persisted. Excess diazomethane was quenched by addition of dilute acetic acid. The sample was then concentrated and analyzed by HPLC as described above.

Procedures for preparative isolation of the kairomone are presented in Figure 1. Twenty thousand late third-instar California red scale (25–30 days old) were aspirated off lemons over a period of one week. The scale were stored in

20,000 Scale Covers: Extract With Methanol

	Solvent Blank	Methanol Extract	*G*-statistic	df	*P*	# Wasps
Drums & Turns	2	20	72.66	6	<0.0001	6
Ovipositions	0	11	15.25	4	0.004	

Methanol Extract: Add Water (25%),back Extract With Hexane

	Hexane	Aqueous Methanol	*G*-statistic	df	*P*	# Wasps
Drums & Turns	0	72	30.50	5	0.0001	5
Ovipositions	0	5	6.031	3	0.074	

Aqueous Methanol Extract: Solid Phase Extraction (C_{18}) Elute with H_2O, ACN, MeOH

	H_2O	ACN	MeOH	*G*-statistic	df	*P*	#Wasps
Drums & Turns	1	14	5	19.13	8	0.014	8
Ovipositions	0	9	4	13.55	6	0.035	

ACN, MeOH Fractions Combined: Size Exclusion Chromatography With 0.1 M Ammonium Acetate

Fraction #	1	2	3	4	*G*-statistic	df	*P*	# Wasps
Drums & Turns	10	3	3	50	115.33	36	<0.0001	12
Ovipositions	0	0	0	23	63.77	18	<0.0001	

Fraction 4: C_{18} Reverse Phase HPLC in 0.075% Aqueous TFA in ACN

Fraction #	4-1	4-2	4-3	4-4	4-5	4-6	*G*-statistic	df	*P*	# Wasps
Drums & Turns	0	0	9	4	0	18	74.68	36	0.0002	6
Ovipositions	0	0	0	0	0	10	38.92	24	0.027	

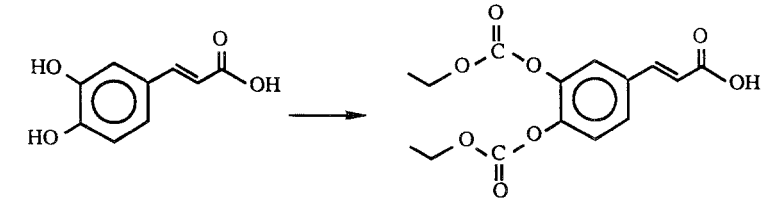
FIG. 1. Preparative extraction, isolation, and bioassay of *O*-caffeoyltyrosine from 20,000 covers of California red scale.

Optima grade MeOH (Fisher Scientific) at 4°C until all were collected. The combined methanolic slurry (~50 ml) was sonicated for 2 hr to disrupt the scale covers. The slurry was then filtered with suction, and the cake was rinsed several times with MeOH. The filtrate was made up to 160 ml with MeOH, and 40 ml of distilled water was added, resulting in some precipitation. The mixture was centrifuged at ~10,000*g* for 20 min, and the supernatant was decanted. The supernatant was extracted with hexane (3 × 200 ml) to remove lipids and traces of the wax from the lemons upon which the scale had been reared. All kairomonal activity remained in the aqueous layer. The yellow aqueous layer was concentrated to 20 ml at 55°C under partial vacuum. The residue was acidified with 1 ml acetic acid, then loaded onto two reverse-phase solid-phase extraction cartridges (300 mg C_{18} packing, Fisher Scientific, Cat. No. P453) that had been preconditioned with 5% aqueous acetic acid and MeOH (2 ml aq. acid, 2 ml MeOH, 6 ml aq. acid). The cartridges were rinsed with aq. acetic acid (4 ml) and water (3 ml), then eluted with 3 ml of acetonitrile followed by 3 ml of MeOH. Kairomonal activity was found only in the acetonitrile and MeOH fractions. The methanol fractions were concentrated, taken up in aq. acetic acid, and the solid-phase extraction step was repeated as described above. The com-

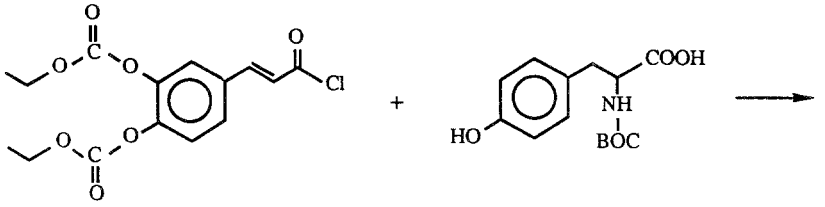
bined acetonitrile fractions were made up to 10 ml, and 1 ml was set aside as a reference standard. The remainder was concentrated to 2 ml, diluted with 8 ml of 0.1 M ammonium acetate buffer (pH 7.0), and loaded onto a size-exclusion chromatography column (Bio-Gel P4 polyacrylamide gel, exclusion limit 4000 D, 33 × 1 cm; Biorad, Richmond, California). The column was eluted with 0.1 M ammonium acetate buffer, monitoring the effluent by UV (280 nm). Four major UV-active fractions were eluted, with the fourth fraction containing most of the activity. This fraction was purified further by reverse-phase HPLC, using the Ultremex 5 C_{18} analytical column with isocratic elution (25% acetonitrile–75%, 0.075% trifluoracetic acid in water, flow rate 1.0 ml/min, 100 μl per injection), and monitoring the effluent at 280 nm. Individual peaks were collected as they eluted. The fractions were lyophilized and reconstituted in acetonitrile for bioassay. The major peak, eluting at 10.2 min, was biologically active. This fraction (1.2 mg) was concentrated under nitrogen and prepared for spectral analysis. ^{1}H NMR (CD_3OD): δ 7.72 (d, 1H, J = 16.0 Hz, olefin H β to carbonyl), 7.35 (d, 2H, J = 8.45 Hz, aromatic H, tyrosine), 7.14 (d, 2H, J = 8.45 Hz, aromatic H, tyrosine), 7.12 (d, 1H, J = 2.0 Hz, ortho H, caffeic acid), 7.01 (dd, 1H, J = 8.4, 2.0 Hz, ortho H, caffeic acid), 6.79 (d, 1H, J = 8.4 Hz, meta H, caffeic acid), 6.44 (d, 1H, 16.0 Hz, olefinic H α to carbonyl), 3.76 (dd, 1H, J = 9.1, 4.2 Hz, $\underline{C}HNH_2$), 3.34 (dd, 1H, J = 14.5, 4.2 Hz, benzylic H), 3.01 (dd, 1H, J = 14.6, 9.1 Hz, benzylic H). MS (desorption electron impact, 50 eV), *m/z* (rel. abundance): 326 (5, M-NH_3), 279 (2), 263 (3), 220 (16), 189 (70), 163 (13), 136 (27), 120 (15), 107 (100), 89 (12), 77 (20). HRFABMS, calcd. for $C_{18}H_{18}NO_6$ $(M + H)^+$: 344.1134; found: 344.1155. UV (from diode array HPLC detector) λ_{max}: 218 nm, 248 nm, shoulder 304 nm and 334 nm.

Synthesis of Kairomone (Scheme 1)

3,4-Diethoxycarbonyl-caffeic acid **(2)**. This compound was prepared by a minor modification of the method of Flammang et al. (1969). Thus, caffeic acid **1** (10 g, 55 mmol) was dissolved in 2 M NaOH (82.5 ml, 165 mmol) under N_2. The resulting yellow solution was cooled to ~ -5°C in an ice–salt bath, and ethyl chloroformate (11.6 ml, 120 mmol) was added dropwise, maintaining the temperature < -5°C. The mixture was stirred for 15 min, followed by the addition of a further 20.5 ml of NaOH solution and 3 ml of ethyl chloroformate, and stirring for 15 min. The mixture was then slowly acidified by dropwise addition of 6 M HCl, with vigorous stirring. The free acid precipitated as a pink gum. Water was added (15 ml) and stirring was continued until the precipitate broke up into small granules. The mixture was filtered, and the filter cake was rinsed thoroughly with water and compacted to express trapped water. The cake was broken up and air dried, then dissolved in benzene (250 ml), and the benzene



1 2



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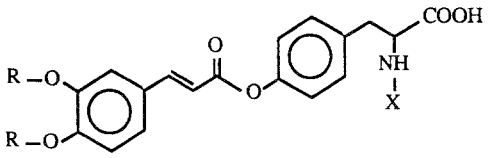

5: R = OCOEt, X = BOC

6: R = OCOEt, X = H

7: R = H, X = H

SCHEME 1.

solution was dried over Na_2SO_4. The solution was concentrated to 100 ml, warmed to dissolve any precipitated solids, and poured slowly into 300 ml of vigorously stirred hexane. The flocculent white precipitate was filtered off and air dried (13.99 g, 78.5%). ^1H NMR ($CDCl_3$): δ 7.73 (d, 1H, J = 16.0 Hz, CHCOOH), 7.49 (d, 1H, J = 1.9 Hz, isolated ortho aromatic H), 7.45 (dd,

1H, J = 8.4, 1.9 Hz, ortho aromatic H), 7.34 (d, 1H, J = 8.4 Hz, meta aromatic H), 6.42 (d, 1H, J = 16.0 Hz, olefinic H), 4.34 (2 overlapped quartets, 4H, J = 7.3 Hz, CH_2s), 1.39 (2 overlapped triplets, J = 7.3 Hz, methyls). IR λ_{max} (cm^{-1}): 3300–2400 (s, br), 1770 (s), 1680 (s), 1635 (m), 1250 (s, br), 1195 (m). MS (70 eV, direct insertion probe), *m/z* (rel. abundance): 324 (3, M^+), 307 (2), 280 (4), 252 (7), 236 (5), 208 (28), 180 (100), 163 (19), 134 (26), 89 (12), 78 (13). HRMS: Calcd. for $C_{15}H_{16}O_8$: 324.0845; found, 324.0826. UV (CH_2Cl_2) λ_{max} 305 (ϵ = 24,700).

3,4-Diethoxycarbonyl-caffeoyl chloride ***(3)***. Acid **2** (5.42 g, 16.7 mmol) was stirred with 15 ml of thionyl chloride at 50°C overnight. The mixture was concentrated with aspirator vacuum, and the solid brown residue was recrystallized from 200 ml of hot hexane, yielding 5.37 g (94%) of acid chloride **3** as cream-colored needles, mp 70–72°C (lit. 71°C; Flammang et al., 1969). ^1H NMR ($CDCl_3$): δ 7.79 (d, 1H, J = 15.8 Hz, olefinic H), 7.53 (d, 1H, J = 2.0 Hz, isolated ortho H), 7.48 (dd, 1H, J = 8.4, 2.0 Hz, ortho H), 7.38 (d, 1H, J = 8.4 Hz, meta H), 6.62 (d, 1H, 15.8 Hz, olefinic H), 4.35 (overlapped quartets, 4H, J = 7.3 Hz, CH_2s), 1.40 (overlapped triplets, 6H, J = 7.3 Hz, methyls). IR λ_{max} (cm^{-1}): 2998 (m), 1770 (s, br), 1680 (s), 1640 (s), 1250 (s, br). MS (70 eV, direct insertion probe), *m/z* (rel. abundance): 344 (0.6, M^+), 342 (1.5, M^+), 307 (6), 270 (4), 235 (5), 219 (6), 194 (19), 191 (42), 163 (100), 134 (25), 89 (13), 77 (13). HRMS, Calcd. for $C_{15}H_{15}O_7Cl$: 342.0506; found 342.0525.

Coupling of 3,4-diethoxycarbonyl-caffeoyl chloride and N-t-*BOC-(L)-Tyrosine. N-t*-BOC-(L)-tyrosine (1.41 g, 5 mmol; Sigma Chemical Co., St. Louis, Missouri) was dissolved in a mixture of 10 ml 1 M aq. NaOH and 20 ml acetone. The solution was cooled to −15°C, and acid chloride **3** (1.71 g, 5 mmol) in acetone (5 ml) was added dropwise over several minutes, with vigorous stirring. The cooling bath was removed, and the reaction was allowed to warm for 15 min. The mixture was then acidified with 1 M aq. HCl, precipitating the product as a sticky gum. The gum was washed twice with water, dissolved in acetone, filtered, and the filtrate was concentrated under partial vacuum. The viscous residue was pumped under vacuum (0.2 mm Hg) for several hours, yielding **5** as a yellowish, slightly sticky foam (2.84 g, quantitative). This was used without further purification. ^1H NMR ($CDCl_3$): δ 7.79 (d, 1H, J = 15.9 Hz, olefinic H), 7.52 (d, 1H, J = 1.8 Hz, isolated aromatic H), 7.48 (dd, 1H, J = 8.5, 1.8 Hz, aromatic H), 7.35 (d, 1H, J = 8.4 Hz, aromatic H), 7.24 (d, 2H, J = 8.4 Hz, tyr aromatic H), 7.13 (d, 2H, J = 8.4 Hz, tyr aromatic H), 6.58 (d, 1H, J = 15.9 Hz, olefinic H), 4.99 (br. d, 1H, NH), 4.61 (m, 1H, NH-CH), 4.35 (br. quartet, 4H, CH_2s), 3.17 (m, 2H, benzyl CH_2), 1.44 (br. s, 9H, t-BOC methyls), 1.39 (t, 6H, J = 6.9 Hz, methyls). IR λ_{max} (cm^{-1}): 3700–2400 (br), 2990 (m), 1774 (s), 1730 (s), 1644 (m), 1507 (m), 1382 (m), 1340–

1090 (s, br), 1050 (m), 979 (m). FABMS (nitrobenzyl alcohol matrix): *m/z* 532 (M + 1-C_4H_8). UV (CH_2Cl_2) λ_{max}: 282 (ϵ = 14,500).

O-*Caffeoyl-tyrosine (7).* The fully protected kairomone **5** (664 mg) was stirred in 6.0 ml of a 4:1 mixture of methylene chloride and trifluoroacetic acid at room temperature for 16 hr. The solvent was removed under vacuum, giving crude **6,** to which 15 ml of 97:3 MeOH–NH_4OH were added. The dicarbonate dissolved in several minutes, giving a clear bright yellow solution, which then began to precipitate after about 30 min. The reaction was complete by TLC (silica; *n*-butanol–water–AcOH, 4:1:1) after 1.5 hr. In particular, the dicarbonate and the deprotected kairomone had similar R_f values, but the free kairomone fluoresced under longwave UV, whereas the dicarbonate did not. The mixture was filtered with suction, rinsing the filter cake several times with small portions of ethanol. The amorphous solids were then pumped under vacuum to remove traces of solvent. The filtrate was concentrated to dryness under vacuum, then triturated with ethanol (2 × 5 ml), and a second crop of amorphous solid was recovered by filtration, for a total yield of 290 mg (77%). The ^1H NMR (CD_3OD), UV, and EI mass spectra (desorbtion probe, 50 eV), and the HPLC retention time on a reversed phase column matched those of the compound isolated from scale covers. IR λ_{max} (cm^{-1}): 3700–2400 (br), 1695 (m), 1600 (s), 1506 (m), 1444 (m), 1280 (s), 1198 (s), 1167 (s), 1144 (s), 980 (m). UV (MeOH), λ_{max} (extinction coefficient): 218 (17,200), 248 (8,900), 304 (10,700), 334 (14,600).

RESULTS

Luck and Uygun (1986) demonstrated that *A. melinus* females underwent stereotypical oviposition behavior (drumming with antennae and turning) in response to filter paper disks treated with aqueous or aqueous ethanolic extracts of California red scale covers. With only this information available, preliminary experiments were conducted to determine the properties of the kairomone before embarking on a large-scale isolation program. It was determined that an active extract could be prepared by extracting detached covers of California red scale with alcohol or aqueous alcohol mixtures. Upon back extraction of the aqueous extracts with hexane or chloroform, only the aqueous fraction elicited oviposition behavior. However, the kairomone could be extracted from aqueous solution by *n*-butanol, suggesting that the kairomone was of intermediate polarity.

The kairomone was mobile on C_{18} reverse-phase TLC plates developed with water-acetonitrile mixtures. Crude fractionation of extracts on reverse-phase HPLC, fractionating by time, showed that fractions eluting between 15–20 and 20–25 min were biologically active (drums and turns: $G_5 = 64.030$, $P < 0.001$; ovipositions: $G_5 = 88.001$, $P < 0.001$). More careful HPLC fractionation led to the isolation of a single major active peak.

To test whether the kairomone might be proteinaceous, the active HPLC peak was subjected to acidic hydrolysis, followed by amino acid analysis of the hydrolysate. Tyrosine was the only amino acid identified from the hydrolysate by reverse-phase HPLC of the OPA derivatives. The presence of tyrosine in the hydrolysate was confirmed by a second two-step derivatization followed by GC-MS analysis. However, it was evident that the kairomone was not tyrosine, as tyrosine was not present in the active fraction prior to hydrolysis (as determined by OPA derivatization and HPLC analysis), nor was it active in bioassays. However, subjecting the kairomone to the two-step derivatization conditions did result in small amounts of derivatized tyrosine, suggesting that the kairomone was partially degrading to tyrosine under the derivatization conditions.

In an attempt to determine which functional groups of the tyrosine fragment were bonded to the second as yet unidentified fragment of the kairomone, the active HPLC fraction was briefly treated with diazomethane; this treatment both destroyed the biological activity and made the active compound more lipophilic, as judged by increased retention time on reverse-phase HPLC. This suggested the presence of one or more free carboxyl and/or phenolic groups in the kairomone. The pH dependence of the UV spectrum was also checked, to determine the presence of free phenolic groups. However, it was found that treatment with dilute aqueous NaOH (pH 11) destroyed the kairomone, and furthermore, that the base treatment released free tyrosine, suggesting that the tyrosine was bonded to the second fragment through an ester linkage and that the amine function of the tyrosine was free. With this preliminary information in hand, we began a large-scale isolation sequence (Fig. 1).

Experiments described above had demonstrated that the kairomone contained a tyrosine fragment, that the tyrosine was bonded to the rest of the kairomone by a base-sensitive (probably ester) linkage, that the rest of the kairomone contained a chromophore with extended conjugation with a strong UV absorbtion at 334 nm, and that the molecular weight was less than 500 Da. The molecular weight of the isolated kairomone was determined to be 343 Da by FABMS, and exact mass measurements gave a molecular formula of $C_{18}H_{17}NO_6$.

The proton NMR (CD_3OD) was readily interpreted (Figure 2). Once the signals at δ 7.35, 7.14, 3.76, 3.34, and 3.01 due to the tyrosine proton resonances were discounted, there were five remaining resonances, each corresponding to a single proton. An isolated AX system (δ 7.72, 6.44; J = 16.0 Hz) was readily assigned to a *trans* 1,2-disubstituted olefin in an α,β unsaturated carbonyl system. The three remaining protons were aromatic, with a coupling pattern indicative of a 1,3,4-trisubstituted benzene. The chemical shifts of these protons, in conjunction with the positions and strengths of the UV absorbtion bands and the molecular formula, suggested a 3,4-dihydroxycinnamic acid (caffeic acid) structural unit, esterified to the tyrosine fragment. This gross structure was

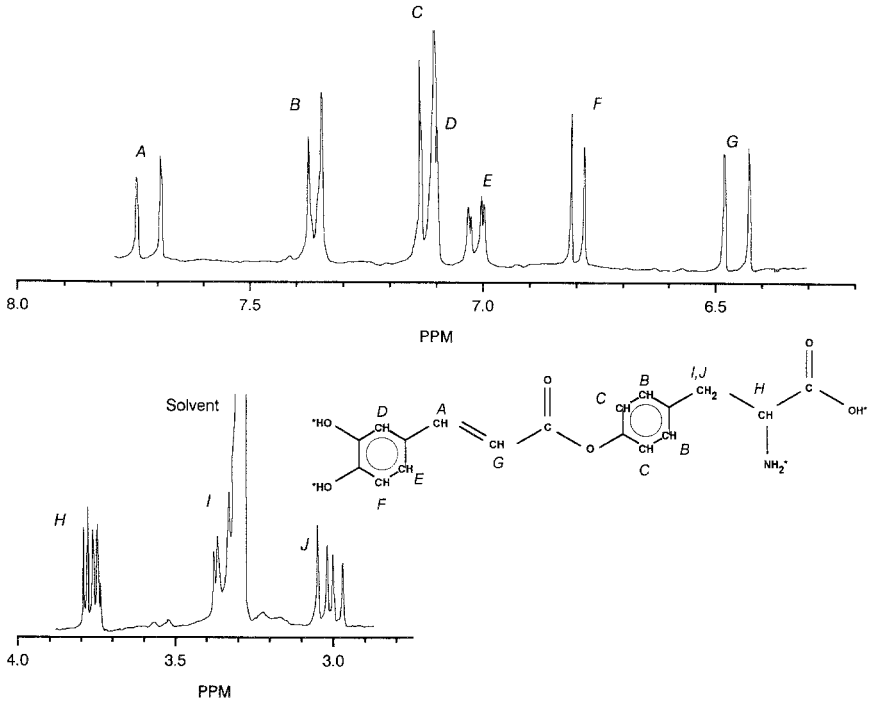

FIG. 2. ^{1}H NMR spectrum (in CD_3OD) and assignment of protons to the structure of *O*-caffeoyltyrosine. Protons marked with asterisks were lost due to exchange.

confirmed by mild base hydrolysis of the kairomone, followed by reverse-phase HPLC analysis; two major fragments were identified with retention times and UV spectra identical to tyrosine and caffeic acid. Of the three possible ester linkages to join the two fragments, the ester between the carboxyl of caffeic acid and the phenolic hydroxyl of tyrosine seemed most likely, due to the close correspondence between the UV spectra of the kairomone and caffeic acid; esterification of one of the caffeic hydroxyls would appreciably change the UV spectrum. This tentative structure was proven by synthesis (Scheme 1).

Thus, the hydroxyl groups of caffeic acid **1** were protected as ethylcarbonates. The acid **2** was then converted to the acid chloride **3** by treatment with thionyl chloride. Reaction of the acid chloride with commercially available *N-t*-butoxycarbonyl (L)-tyrosine **4** completed the carbon skeleton. Sequential removal of the *N*-BOC and carbonate protecting groups with trifluoroacetic acid and methanolic ammonium hydroxide, respectively, gave the kairomone **7**, identical in all respects to the compound isolated from California red scale covers.

The synthetic kairomone was bioassayed at 6 and 15 ng/disk, roughly

equivalent to 0.2 and 0.5 scale cover equivalents of kairomone per disk (Hare et al., 1993). Treated disks elicited significantly more drums and turns and oviposition probes than the control disk, especially at the higher concentration (drums and turns: $G_2 = 15.919$, $P < 0.001$; ovipositions: $G_2 = 27.674$, $P < 0.001$; Table 1).

Synthetic *O*-caffeoyltyrosine was also bioassayed versus natural *O*-caffeoyltyrosine isolated from scale covers, and versus a control (Figure 3). The proportion of drums and turns differed significantly among treatments ($F_{4,45} = 11.68$, $P < 0.0001$). Disks treated with either purified natural kairomone or synthetic kairomone were more attractive than the control disk ($F_{1,45} = 14.98$, $P < 0.0003$), and synthetic *O*-caffeoyltyrosine was more attractive than natural *O*-caffeoyltyrosine ($F_{1,45} = 5.18$, $P = 0.028$). Wasps showed a more intense behavioral response to the disks treated with 14.8 ng than with 5.9 ng of synthetic *O*-caffeoyltyrosine ($F_{1,45} = 24.72$, $P < 0.0001$); this difference was not

TABLE 1. NUMBER OF DRUMS AND TURNS AND OVIPOSITION PROBES IN RESPONSE TO SYNTHETIC KAIROMONE BY EACH OF 10 WASPS IN 10 MIN

Wasp	6 ng	15 ng	Control
Drums and turns			
1	1	3	0
2	2	0	0
3	2	0	0
4	6	6	1
5	0	0	0
6	0	0	0
7	0	10	0
8	0	0	0
9	0	0	0
10	0	0	1
Sum	11	19	2
Ovipositions			
1	0	1	0
2	0	0	0
3	0	0	0
4	1	4	0
5	0	0	0
6	0	0	0
7	0	10	0
8	0	0	0
9	0	0	0
10	0	0	0
Sum	1	15	0

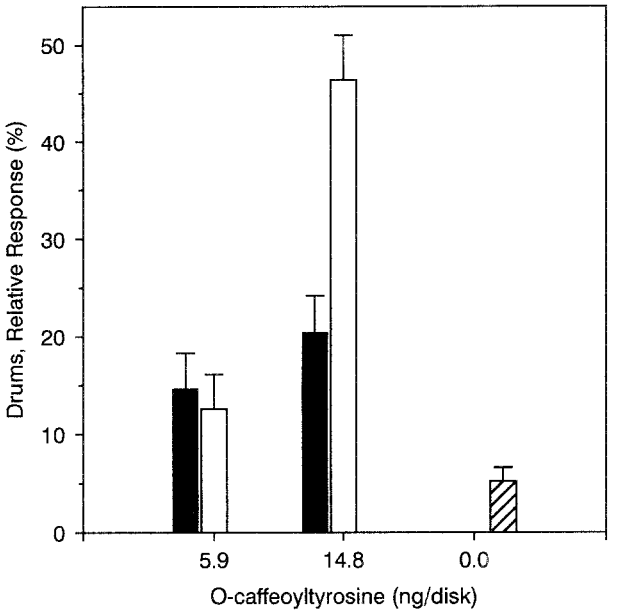

FIG. 3. Comparison of attractiveness of natural and synthetic *O*-caffeoyltyrosine to *Aphytis melinus*. Bars indicate the mean (±standard error; 10 trials with 10 wasps each) percentage of drums and turns observed on disks treated with natural (filled bars) or synthetic (open bars) *O*-caffeoyltyrosine. Cross-hatched bar indicates percentage of drums and turns observed on control disks treated only with solvent.

seen with natural kairomone ($F_{1,45} = 1.85$, $P = 0.18$). The reduced activity of the *O*-caffeoyltyrosine isolated from scale covers may have been due to its partial degradation during the five months between the time it was isolated and the time it was used in the bioassay. Nevertheless, these results clearly demonstrate the biological activity of synthetic *O*-caffeoyltyrosine. Too few oviposition probes were observed (16, all in one trial, all but one on the disk treated with 14.8 ng of synthetic *O*-caffeyoltyrosine) to justify statistical analysis.

DISCUSSION

Preliminary bioassays and experiments with crude extracts of *O*-caffeoyltyrosine were confounded by the chemical and biological properties of the kairomone. First, it was discovered that the kairomone was difficult to reconstitute after concentration to dryness, contributing to variability in the doses being tested in bioassays and the quantity of material actually available for other chemical analyses. Second, it was found that *A. melinus* responded to a remark-

ably narrow range of dosages of *O*-caffeoyltyrosine (Hare et al., 1993), consistent with the kairomone concentration serving to indicate a susceptible stage. The natural variability in the amount of *O*-caffeoyltyrosine in the scale covers, when combined with these two factors, initially resulted in difficulties in obtaining consistent and reproducible bioassays.

Responses of individual wasps were also highly variable, with some individuals displaying no interest in even crude scale cover extracts. However, replication of each test a number of times and analysis of the cumulative responses left no doubt that both synthetic and natural *O*-caffeoyltyrosine were behaviorally active, as untreated control disks gave virtually no response (Table 1, Figures 1 and 3), while treated disks were subjected to drumming and turning and oviposition probing by the majority of the test insects.

In order to standardize our bioassay conditions as much as possible, the original bioassay described by Luck and Uygun (1986) was simplified by removing the lemon fruit substrate and replacing it with a glass Petri dish. This was done to remove potential variation in response among trials due to variation among individual fruits (e.g., slightly different colors, odors, textures, etc.). While recognizing that the Petri dish arena presented wasps with an austere sensory environment, potentially depressing the wasp's behavioral activity overall, it was felt that the test environment should be as free as possible of uncontrolled environmental variation. With the kairomonal component now identified, it will be possible to examine the effect of substrate cues under controlled conditions to determine how they may interact with *O*-caffeoyltyrosine in the mediation of oviposition behavior by *A. melinus*.

In addition, investigation of the pattern of occurrence and concentration of *O*-caffeoyltyrosine and related compounds in hosts of *A. melinus*, and among hosts and nonhosts, may provide a better understanding of the process and evolution of host recognition by *Aphytis* and related species and suggest how such a process may be manipulated to improve the effectiveness of hymenopteran parasitoids for biological control of pest insects.

It is noteworthy that this kairomone component is completely unlike those involved in a similar scale-parasitoid interaction; the wasp *Aphytis yanonensis* utilizes a series of nonpolar lipophilic wax esters from the cover of its scale host, *Unaspis yanonensis*, as an oviposition-stimulating kairomone blend. However, the morphology of the *U. yanonensis* scale cover is quite different from that of *A. aurantii*, consisting of loose wax flakes, instead of the hard and relatively smooth cover of *A. aurantii*.

Esters of caffeic acid with alcohols, phenols, saccharides, and flavonoids are common phytochemical constituents (Herrmann, 1978). However, to our knowledge, this represents the first report of *O*-caffeoyltyrosine, although the analogous amide, *N*-caffeoyltyrosine, has been isolated from coffee beans (Clifford et al., 1989).

During the identification of the kairomonal component, we experienced problems in obtaining mass spectra of the purified compound by desorption electron impact (50 eV) or fast atom bombardment mass spectrometry; trace amounts of sodium and other ions led to poor and irreproducible mass spectra. The problem was eventually overcome by repurification of the kairomone with a solid-phase extraction cartridge, eluting the kairomone into a polyethylene vial immediately before mass spectral analysis.

The synthesis of the kairomone, although short, required some attention to detail. The acid chloride of caffeic acid had been previously reported [Freudenberg and Fischer, 1956; more recent reports indicate that treatment of caffeic acid with thionyl chloride actually results in the formation of a cyclic sulfite derivative, i.e., the reaction both protects the ortho hydroxyls and generates the acid chloride (Husson et al., 1983)]. However, subsequent reaction of this acid chloride with *N*-protected tyrosine led to complex reaction mixtures, so this route was not pursued. Van Heerden et al. (1980) have also reported erratic results with this acid chloride.

Instead, the *o*-dihydroxy system of caffeic acid was first protected by formation of the ethyl carbonate derivatives (Flammang et al., 1969) followed by conversion to the acid chloride. Coupling of the acid chloride and *N*-*t*-butoxycarbonyl tyrosine did not proceed well under standard conditions (THF, pyridine or triethylamine, with or without catalytic dimethylaminopyridine). However, addition of an acetone solution of the acid chloride to a cooled solution of *N*-BOC tyrosine in acetone and aqueous NaOH rapidly and cleanly yielded the coupled product. Removal of the BOC group, before or after removal of the carbonate groups, was readily accomplished with 20% trifluoroacetic acid in CH_2Cl_2 (room temperature, 16 hr). Removal of the carbonate protecting groups was more problematic. Refluxing in aqueous acetic acid (Haslam et al., 1961), treatment with 1 M aqueous NH_4OH (Fischer and Fischer, 1913), or treatment with cold aqueous sodium hydroxide in acetone (Flammang et al., 1969) all resulted in extensive hydrolysis of the ester linkage. Finally, it was found that treatment with methanolic ammonium hydroxide (97:3) at room temperature for 2 hr removed the carbonate groups with minimal ester cleavage (Husson et al., 1983).

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RESPONSE OF MALE CODLING MOTHS (*Cydia pomonella*) TO COMPONENTS OF CONSPECIFIC FEMALE SEX PHEROMONE GLANDS IN FLIGHT TUNNEL TESTS

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Abstract—In flight tunnel tests, the percentages of oriented upwind flights of male codling moths culminating in contacting a source of different compositions of female sex pheromone gland components were determined over a dosage range of 0.1–100,000 μg. The following compositions were tested: (1) (*E,E*)-8,10-dodecadien-1-ol of 99.7% isomeric purity; (2) 1 + dodecan-1-ol + tetradecan-1-ol; (3) 2 + decan-1-ol + (*E*)-9-dodecen-1-ol; and (4) an equilibrium mixture of 8,10-dodecadien-1-ol isomers (61% *EE*, 5% *ZZ*, 14% *ZE*, and 20% *EZ*). The ratios of the components in compositions 2 and 3 were chosen to produce vapor ratios equal to the natural ratios found in the female effluvium by Arn and coworkers. As the dose of composition 1 was increased from 0.1 to 10 μg, response increased from 0 to about 80% and then was approximately constant from 10 to 300 μg. Over the range 0.1–300 μg, the percentage of males contacting the septum was virtually the same as the percentage flying upwind. From 300 to 100,000 μg, the percentage of males flying upwind and contacting the source steadily decreased from about 80 to 0%. The male responses to compositions 2 and 3 were virtually identical to the response to 1. These results indicate, contrary to published reports, that dodecan-1-ol and tetradecan-1-ol in combination with 1 do not increase the responses of the behavioral modes determining degree of attractancy and disruption of sexual communication over that of 1 alone. These results also show that decan-1-ol and (*E*)-9-dodecen-1-ol do not enhance response in the five-component mixture. The response to composition 4 increased from 0% at a dose of 0.3 μg to 26% at a dose of 30μg and then decreased to 0% at a dose

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of 3000 μg. Thus, the inhibiting effect of the isomers on response was greater at the higher doses.

Key Words—Codling moth, *Cydia pomonella*, Lepidoptera, Tortricidae, sex pheromone, flight tunnel, mating disruption, (*E*,*E*)-8,10-dodecadien-1-ol, (*E*)-9-dodecen-1-ol, decan-1-ol, dodecan-1-ol, tetradecan-1-ol.

INTRODUCTION

Roelofs et al. (1971) identified the sex pheromone of the codling moth as a single component [(*E*,*E*)-8,10-dodecadien-1-ol, 1] by a then novel method based on gas chromatography–electroanntenography and correlations of electroanntenographic responses to model compounds. Subsequently, Beroza and et al. (1974) confirmed the presence of a dodecadien-1-ol in female extract by mass spectrometry, and McDonough and Moffitt (1974) confirmed the positions of the double bonds by ozonolysis.

Almost all insect pheromones are multicomponent (Wood et al., 1967; Roelofs, 1977; Silverstein, 1977), and consequently other workers have searched for secondary components of the codling moth pheromone. Einhorn et al. (1984) and Arn et al. (1985) identified several other pheromonelike compounds in extracts of female sex pheromone glands. Additionally, Arn et al. (1985) identified components in effluvium of calling females. The compounds identified in the effluvium and their relative amounts were: 1, 100; decanol, 2.78; dodecanol, 64.8; (*E*)-9-dodecen-1-ol, 14.8; tetradecanol, 9.26; hexadecanol, 18.5; octadecanol, 22.2; *ZE* isomer of 1, trace; *EZ* isomer of 1, 5.5. Both Einhorn et al. (1986) and Arn et al. (1985) reported that dodecanol combined with 1 was more active than 1 alone in flight tunnel tests, but that this combination did not produce greater trap catch in field tests than 1 alone. Using a male wing-flutter bioassay, Bartell and Bellas (1981) reported that 1 alone produced less response than an equal amount of 1 in extract of female sex pheromone glands. Subsequently, Bartell et al. (1988) reported that 1 combined with dodecanol and tetradecanol produced a response equal to gland extract, but that neither dodecanol nor tetradecanol alone enhanced 1. Rothschild et al. (1988) have patented a mating disruptant based on 1 plus dodecanol and tetradecanol as the preferred composition, which was claimed to be superior to 1 alone.

In another study of the codling moth sex pheromone system, Preiss and Priesner (1988) reported male responses in a flight tunnel to 1, to alcohols related to 1, and to binary combinations of 1 and the related alcohols. Most of the related alcohols produced some response, but none of the binary combinations produced stronger responses than 1 alone.

Our interest in this area arose as part of a program to attempt to develop a mating disruptant for codling moth more efficacious than presently available.

Charmillot (1990) showed that 1 can be an effective mating disruptant, but at high required minimum evaporation rates of 10–40 mg/ha-hr. Minks and Cardé (1988) pointed out that a replicate of the natural blend would be expected to be the most effective disruptant based on most of the proposed mechanisms for mating disruption. Thus, identification of behavioral activity in other sex pheromone gland components could provide an improved mating disruptant for the codling moth.

Evidence for the absence or presence of behavioral activity had not been provided for all identified components of the sex pheromone gland of female codling moths. This study was undertaken to add to our knowledge of possible behavioral activity of these components. Surprisingly, we found that our results did not support the published reports ascribing behavioral activity to dodecan-1-ol and tetradecan-1-ol.

METHODS AND MATERIALS

Insects. Pupae were obtained from a laboratory colony reared on artificial diet (Howell, 1972; Toba and Howell, 1991). Individual pupae were placed in 15 × 50-mm vials and maintained at 20°C and 68% relative humidity on a 10:14 hr dark–light cycle. In the flight tunnel tests, males were used three days posteclosion and 0.25–2.5 hr after the onset of scotophase.

Chemicals. Compound 1, decanol, dodecanol, and tetradecanol were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Compound 1 was purified by three low-temperature recrystallizations from pentane, and the isomeric purity was 99.7% *EE* by gas chromatographic analysis. (*E*)-9-Dodecen-1-ol was obtained from the now defunct Farchan Division of Story Chemical Corp. (Willoughby, Ohio). All chemicals were at least 98% pure by gas chromatographic analysis. The equilibrium mixture of 8,10-dodecadien-1-ol isomers was prepared by the procedure of Henrick et al. (1975) by heating 50 mg of 1 with 1 mg of thiophenol in a water bath (100°C) for 1.5 hr. Heptane (20 ml) was added and then removed with a rotary evaporator to codistill the thiophenol. The procedure with heptane was repeated once or twice until the odor of thiophenol could not be detected. Gas chromatographic analysis confirmed the absence of thiophenol and showed the isomeric content to be 61% *EE*, 20% *EZ*, 14% *ZE*, and 5% *ZZ*.

Flight Tunnel. A flight tunnel constructed as described by Miller and Roelofs (1978) was used. The flight compartment was 210 cm. The air speed directly downwind of the dispenser was 8.5 cm/sec, and the temperature was maintained at 22.5° ± 0.5°C. The tunnel was dimly lighted overhead by red lights controlled by a rheostat. The effluvium from the flight tunnel was evacuated directly into a fume hood.

Pheromone Dispensers. Gray elastomeric septa (formulation No. 1888, size No. 1, West Co., Phoenixville, Pennsylvania) were chosen as the controlled release device because they allow substantially less isomerization than natural rubber septa (Brown and McDonough, 1986). Prior to use, the septa were extracted in a Soxhlet apparatus in hexane for 24 hr and then in dichloromethane for another 24 hr. Test mixtures were applied to the cup of the septa in 200 μl of dichloromethane in doses ranging from 0.1 to 5000 μg of 1. As the septa became dry, an extra 200 μl of dichloromethane was added to each septum to ensure efficient penetration of the test compounds. The maximum dose that will efficiently penetrate into a septum is 10,000 μg. For this dose, 1 was added in two applications of 5000 μg in 200 μl. For test doses of 30,000 and 100,000 μg, 3 or 10 septa were used. The small ends of the septa were then inserted into a piece of 0.64-cm hardware cloth so that there was space between adjacent septa.

To determine the evaporation rates from these septa, gray and red natural rubber septa were each dosed with 3 mg each of 1 and dodecanol and hung under the lid of a Pherocon 1C trap (Trece Corp., Salinas, California) (bottom removed) in an apple orchard in 1991. At intervals over a 40-day period, septa were removed and analyzed for content of 1 and dodecanol ($N = 6$), and half-lives were calculated from these data. In red septa, the half-lives and correlation coefficients (R^2) were: 1, 11.4 days, 0.997; dodecanol, 7.7 days, 0.990. In gray septa, these values were: 1, 28.6 days, 0.968; dodecanol, 21.8 days, 0.858. The ratios of half-lives of 1 to dodecanol were 1.48 for red septa and 1.31 for gray septa. Under standard conditions of 20°C and 20 cm/sec air speed, the half-life of dodecanol in red septa is 17.5 days (McDonough, 1991), and, therefore, that of 1 under standard conditions is 25.9 days (17.5 × 1.48). The heat of vaporization, ΔH, for 1 in rubber septa is 17.6 kcal/mol (L.M. McDonough and L.I. Butler, unpublished). The ΔH value along with the Clausius-Clapeyron equation (McDonough et al., 1989) and the fact that $t_{1/2}$ is inversely proportional to air speed allowed the calculation of $t_{1/2}$ of 1 in red rubber septa (McDonough, 1991) at 22.5°C and 8.5 cm/sec air speed, the conditions of the flight tunnel, which gave a value of $t_{1/2} = 47.2$ days. Since the ratio of the $t_{1/2}$ of 1 in gray to 1 in red is 2.50, the $t_{1/2}$ of 1 in gray septa was 118 days (47.2 × 2.50). This value was used to calculate the evaporation rate, E, of 1 from the equation $E = (P\ln 2)/t_{1/2}$ (McDonough, 1991) where P is the dose of 1.

The dose of each pheromone component, P, in the multicomponent test mixtures was calculated from the equation, $P = Et_{1/2}/\ln 2$. To calculate the relative doses of each component in septa, the relative half-lives of the components in the gray septa were assumed to be the same as those in the red septa. The amount of each component in the vapor is directly proportional to the evaporation rate, E. Therefore, the data of Arn et al. (1985) on the composition

of the effluvium of calling female codling moths was used to obtain relative *E* values. The data are summarized in Table 1.

Test Protocol. Septa containing the test mixtures were held in place at the upwind end of the flight tunnel by a bent wire. At the downwind end of the tunnel, three male codling moths in a small covered dish were placed on a platform in the middle of the airstream. After 1 min, the cover was removed and the behavior was observed. If a male did not flutter its wings, it was considered nonviable. The number of nonviable males was generally 0–2 out of 27. Each group of nine was considered a replicate, and there were three replicates for each test point. If nonviable males were found, additional males would be tested to complete the 27. Upwind flights culminating in contact with the septum were recorded. Once a male contacted the septum, it was removed via a tube connected to a vacuum. Each test period was 10 min. Only one concentration was tested per day. Lower concentrations were tested before higher concentrations. After a test the hood and flight tunnel were allowed to operate for 3 hr so as to thoroughly purge traces of pheromone components.

RESULTS AND DISCUSSION

Male Response to 1 Alone. In the flight tunnel tests shown in Figure 1, the percentage of male codling moths making oriented upwind flights culminating in contacting the dispenser are plotted as a function of increasing logarithm of doses of 1. The responses increased from zero at a dose of 0.1 μg to a maximum

TABLE 1. DOSES OF COMPONENTS, $P_{(relative)}$ RELATIVE TO *E*8,*E*10–12:OH = 100, ADDED TO GRAY SEPTA FOR SERIES OF TEST COMPOSITIONS USED IN FLIGHT TUNNEL STUDIES[a]

Compound	Effluvium amount (ng)	$t_{1/2}$ (hr)	$P_{(ng)}$	$P_{(relative)}$
10:OH	0.15	56.1	12.1	0.25
*E*9–12:OH	0.80	381	440	9.08
12:OH	3.50	421	2126	43.9
*E*8,*E*10–12:OH	5.40	622	4846	100
14:OH	0.50	3154	2275	46.9

[a] $P_{(relative)}$ values, calculated from $P_{(ng)}$. $P_{(ng)}$ are the required doses in a septum in nanograms needed to produce evaporation rates, *E* (ng/hr), equal to the effluvium amounts (ng) and were calculated from the equation, $P_{(ng)} = E_{(ng/hr)}t_{1/2}(hr)/\ln 2$. The effluvium amount (ng) is that reported by Arn et al. (1985). The half-life of *E*8,*E*10–12:OH in hours was taken from this report; the other half-lives were obtained by dividing the values for the acetates (McDonough et al., 1989) by 2.1 (McDonough, 1991).

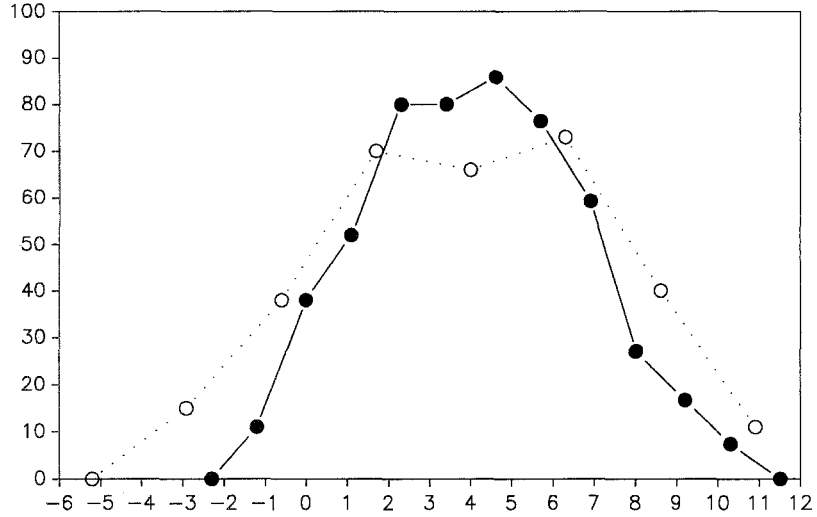

FIG. 1. Solid line: percent of males making oriented upwind flights and contacting gray septa impregnated with 1 on ordinate and logarithm of dose in micrograms on abscissa. Dotted line: equivalent data for 1 previously reported by Preiss and Priesner (1988) from cotton wick dispensers (see text for the manner of aligning the two curves).

of about 80% at 10 μg. From 10 μg to 300 μg the response was approximately constant at about 80%, and from 300 to 100,000 μg the response steadily decreased to zero. From 0.1 to 300 μg the percentage of males contacting the dispenser was virtually identical to the percentage flying upwind; often the two numbers were identical. From 300 to 30,000 μg there was an increasing discrepancy between these numbers; upwind flight was constant at about 80% up to 30,000 μg and decreased to 50% at 100,000 μg. The responses to the septa compared favorably with the response to five caged females (63% response).

Preiss and Priesner (1988) previously reported flight tunnel studies of male codling moth responses to doses of 1. The shape of their response curve was similar to that reported here. Because they used a cotton wick for their controlled release dispenser, their results cannot be compared to ours on the basis of dosage. However, since they also obtained a curve with a plateau, the two plateaus can be aligned to provide a comparison. Their curve aligned with ours is shown in Figure 1. The two curves are remarkably similar, especially in light of the many differences in techniques employed in the two studies. For example, besides the difference in controlled-release dispensers, Preiss and Priesner used a shorter flight tunnel operated at a higher air speed, and they conditioned their males under constant light whereas we used a light–dark cycle. Furthermore, they determined five replicates of 10 males per replicate and changed doses by

a factor of 10, whereas we determined three replicates of nine males per replicate and changed doses by a factor of about 3. The standard deviations of their values were mainly about 8–9%; the standard deviations in this report are mainly about 15–17%.

In the report of Preiss and Priesner (1988), the plateau region extended from a dose of 1 to 100 ng (a factor of 100), and in this report the range is 10–300 μg (a factor of 30). Even though our plateau region was shorter, the alignment of the curves shows our data are consistent with a plateau region of a factor of 100. Their dose of 1 ng aligned with a dose of between 3 and 10 μg in the gray septa. The 3-μg dose corresponded to an evaporation rate of 1 of 0.12 ng for the 10-min test period used in both studies; for the 10-μg dose, the rate would be 0.4 ng/10 min. This range of evaporation rates appears to be reasonable for the 1-ng dose on their cotton wick.

There are some differences between the observations of Preiss and Priesner (1988) and those reported here that deserve discussion. Their data indicated a lower threshold for response than ours. The apparent lower threshold could be caused by the differences in controlled release dispensers. In septa, the pheromone is in solution and the evaporation rate is proportional to dose at all doses, whereas when cotton is the dispenser the pheromone is on the surface, and it is possible that the surface area-to-volume ratio of the pheromone is larger at low doses. This would result in higher evaporation rates per unit of dose as dose was decreased to the lowest values. Another difference is that they reported that the number of males contacting the dispenser was always the same as the number undertaking upwind flight even when the response was decreasing at the higher doses. This is in contrast to our finding of virtually no loss of the percent undertaking upwind flight at the highest doses. For example, the following doses (μg) and upwind flight percentages and the percentages flying upwind and contacting the septum were recorded: 10, 77, 77; 30, 86, 83; 100, 91, 88; 300, 94, 79; 1000, 82, 51; 3000, 80, 33; 10,000, 83, 15; 30,000, 81, 7; 100,000, 50, 0. The differences are probably accounted for by the differences in length of our flight chamber (210 cm) and theirs (80 cm). Although we did not determine the distance males flew before arrestment of flight, an arrestment distance of 80 cm or more from the dispenser would account for the difference in these observations.

Male Response to Three- and Five-Component Mixtures. The responses to the three component lure (1 + dodecanol + tetradecanol) and 1, shown in Figure 2, were essentially identical. The curves overlap in several places and the differences are always well within the standard deviations of the values. As with 1, from 0.1–300 μg, the percentage of males contacting the dispenser was virtually identical to the percentage flying upwind, and the percent flying upwind was constant at about 80% from 10 to 30,000 μg, decreasing to 36% at 100,000 μg.

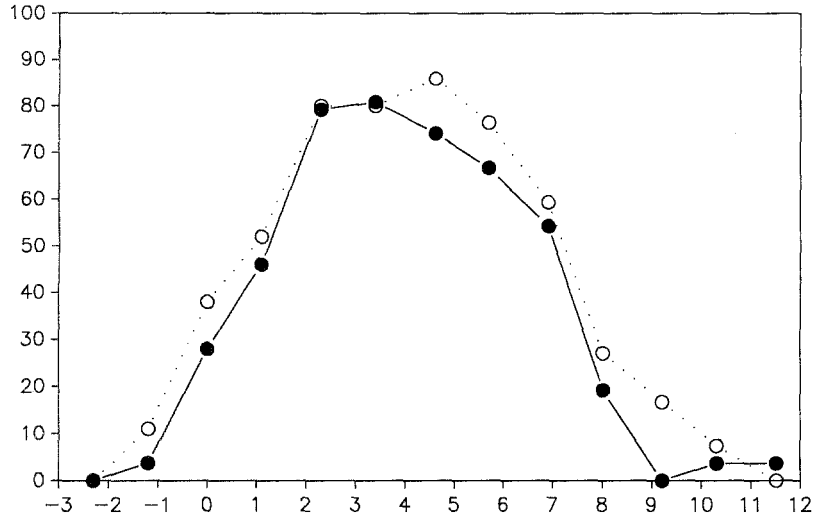

FIG. 2. Solid line: percent of males making oriented upwind flights and contacting septa impregnated with the three-component mixture on ordinate and logarithm of dose of 1 (in the three-component mixture) on abscissa. Dotted line: data for 1 alone (same as in Figure 1) for comparison.

Our data do not support the several published accounts (Einhorn et al., 1984; Arn et al., 1985; Bartell et al., 1988; Rothschild et al., 1988) ascribing behavioral activity to dodecanol and tetradecanol. There also are significant differences among these reports. For example, Arn et al. (1985) reported that when dodecanol was combined with 1, response was increased at both high and low doses compared to 1 alone, whereas Einhorn et al. (1984) reported that dodecanol increased response of 1 only at low doses. Bartell and coworkers (1988) reported that neither dodecanol nor tetradecanol increased response when combined with 1 individually, but that when combined together with 1, they increased response 1000-fold in their wing-flutter bioassay. It is significant that the best response obtained by Arn et al. (70% response over a 100-fold range of doses) occurred when dodecanol was present and was basically the same as that reported by Preiss and Priesner (1988) and by us in the present study for 1 alone, thereby indicating some problem in the determination of the response to 1 alone in their study. The wing-flutter bioassay of Bartell et al. (1988) does not measure the same behavioral responses as the flight tunnel, and therefore their data are not necessarily in contradiction to ours. The responses measured in the flight tunnel (oriented upwind flight toward the pheromone source and contacting the source) are the behaviors involved in disruption of communication between the sexes and therefore indicate that, contrary to the assertions of Roths-

child et al. (1988), the three-component composition is not superior to 1 alone for this purpose.

The five-component composition (three-component blend + decanol + (*E*)-9-dodecen-1-ol) is compared to 1 in Figure 3. Again as with the 1 component, the percentage flying upwind was virtually identical to that contacting the septum up to a dose of 300 μg. The percentage flying upwind was constant at about 80+% from 10 up to 10,000 μg and then decreased to 42% at 30,000 μg and 22% at 100,000 μg. The range of maximum response (a factor of 100) extended from 10 to 1000 μg and was slightly larger than observed for the 1 component. The response to the five-component blend is not different from the 1 component. The slightly enhanced values at some dosages are well within the standard deviations of those values. (*E*)-9-Dodecen-1-ol was considered a good candidate for an active component because active pheromone components of Lepidoptera are most often unsaturated, but here it added no detectable activity. This compound is a biosynthetic precursor to 1 (Lofstedt and Bengtsson, 1988).

Male Response to Composition 4. The response to composition 4 (equilibrium mixture of isomers of 8,10-dodecadien-1-ol), shown in Figure 4, is greatly reduced compared to that of 1. The response (upwind flight and contacting the septum) was initiated at almost as low a dosage level as 1, but increased to a maximum of only 26% at a dose of 30 μg and then decreased to 0% at 3000

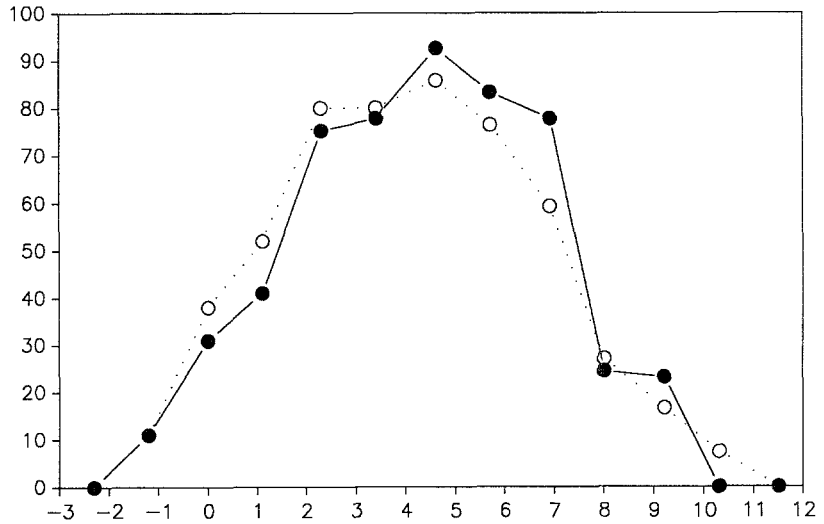

FIG. 3. Solid line: percent of males making oriented upwind flights and contacting septa impregnated with the five-component mixture on ordinate and logarithm of dose of 1 (in the five-component mixture) on abscissa. Dotted line: data for 1 alone (same as in Figure 1) for comparison.

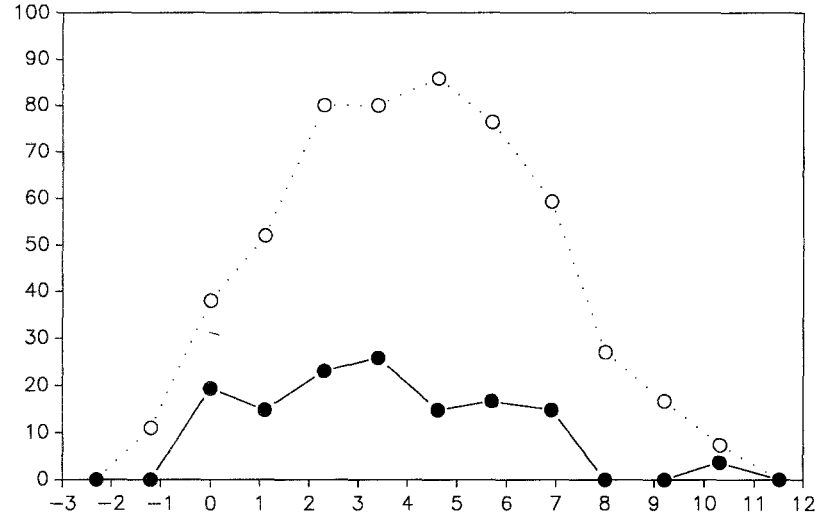

FIG. 4. Solid line: percent of males making oriented upwind flights and contacting septa impregnated with the equilibrium mixture of isomers of 8,10-dodecadien-1-ol on ordinate and logarithm of dose of 1 (in the isomeric mixture) on abscissa. Dotted line: data for 1 alone (same as Figure 1) for comparison.

μg. From 0.3 to 30 μg, the percentage flying upwind and the percentage contacting the septum were almost the same, but the percentage flying upwind increased to 56% at 1000 μg and then decreased to 0% at 100,000 μg. With the other compositions, the percentage flying upwind never increased above the maximum response for those flying upwind and contacting the septa.

The inhibiting effect of the isomers of 1 appeared greatest at higher concentrations. Therefore, high-dosage lures, which are being considered for use for monitoring in mating-disrupted orchards (Charmillot, 1990), should be formulated in dispensers that minimize isomerization.

The inhibitory effect of the nonpheromone isomers has been known since Roelofs et al. (1972) reported decreased trap catch when these isomers were present, but this is the first report of their activity in flight tunnel tests. From these results one would expect the efficacy of mating disruption of the isomerized mixture to be different from 1 above, but it is not possible from this information to predict whether it would be lower or higher.

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PHOTOISOMERIZATION OF ANTIAGGREGATION PHEROMONE VERBENONE: BIOLOGICAL AND PRACTICAL IMPLICATIONS WITH RESPECT TO THE MOUNTAIN PINE BEETLE, *Dendroctonus ponderosae* HOPKINS (COLEOPTERA: SCOLYTIDAE)

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Abstract—Release of the antiaggregation pheromone, verbenone, at 3.8 mg/day from a concentrated source within a multiple-funnel trap completely inhibited response by the mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, to attractive semiochemical lures. When aerial applications were simulated and verbenone was released at the same rate from beads lying in a 2 × 2-m area on the forest floor 15–35 cm below a trap, the response of the MPB was inhibited by only 50%. This reduced inhibition may be explained in part by the photoisomerism of verbenone. When exposed to full sunlight on two occasions, the times required for 50% of verbenone vapors to be converted to chrysanthenone were 75 and 100 min, respectively. Trap and tree-baiting experiments indicated no biological activity of chrysanthenone. Rapid photoisomerization could reduce the concentration of verbenone below biologically active levels and would allow the MPB to colonize trees close to already occupied hosts, contributing to the characteristic clumped distribution of MPB attack. The rate of verbenone photoisomerization may vary according to geographic location, stand elevation and density, and should be considered before verbenone is applied to control the MPB and other bark beetles.

Key Words—Mountain pine beetle, *Dendroctonus ponderosae*, Coleoptera, Scolytidae, antiaggregation pheromone, verbenone, chrysanthenone, photoisomerism.

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INTRODUCTION

The mountain pine beetle (MPB), *Dendroctonus ponderosae* Hopkins, is a destructive pest of pines, particularly lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, in western North America. Its damage is characterized by small patches of dead or dying trees (Safranyik, 1978; Geiszler and Gara, 1978) and has been recognized since the early 1900s when attempts were made to control the beetle by felling and burning beetle-infested trees (Klein, 1978). Successful attack and death of an individual host is a complex sequence of events (Borden et al., 1987) that depends upon the resistance of an individual tree and the number of attacking beetles (Raffa and Berryman, 1983). After selecting a host, the female releases the pheromone, *trans*-verbenol, which attracts males and some other females. As the males arrive, they release the pheromone, *exo*-brevicomin, attracting mostly females to the host (Borden et al., 1987). The attraction to both pheromones is increased by the host tree kairomone, myrcene (Conn et al., 1983). Thus, attack is concentrated on a host tree. Termination of attack is mediated by the reduction in levels of attractive compounds and the production of the antiaggregation pheromone, verbenone (Renwick and Vité, 1970; Raffa and Berryman, 1983; Borden et al., 1987).

Verbenone, 4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one, has been detected in the hindguts of feeding MPBs (Pitman et al., 1969) and in volatiles emanating from male–female pairs (Rudinsky et al., 1974). Its production has also been shown to arise from the metabolism of α-pinene and *trans*-verbenol by symbiotic microorganisms occurring within the beetles' guts (Hunt and Borden, 1989) and their galleries (Hunt and Borden, 1990) in conjunction with autoxidation of the same compounds (Hunt et al., 1989). Ryker and Yandell (1983) found that verbenone had antiaggregative properties for *D. ponderosae* and reported that (−)-verbenone was the most active enantiomer.

Since current control methods involving the removal of susceptible and recently attacked trees are not acceptable in areas such as wildlife corridors and campsites (Borden and Lindgren, 1988), it has been proposed that verbenone could be used as a management tool to prevent the migration of MPBs into these areas and to disperse beetles from susceptible and sensitive stands (Amman et al., 1989, 1991; Lindgren et al., 1989; Gibson et al., 1991; Shea et al., 1992).

Although the results of Amman et al. (1989), Lindgren et al. (1989), and Shea et al. (1992) look very promising from an operational point of view, many trials have produced inconsistent results (Gibson et al., 1991; Shea et al., 1992; B.S. Lindgren, personal communication, Phero Tech Inc., Delta, British Columbia). One reason for this inconsistency may be problems associated with the aerial application of verbenone-impregnated beads (Shea et al., 1992; B.S. Lindgren, personal communication). Another possible reason for inconsistent results

could be the conversion of verbenone to chrysanthenone under ultraviolet light (Erman, 1967) (Figure 1).

METHODS AND MATERIALS

Simulation of Aerial Application with Verbenone-Impregnated Beads. A 10-replicate trapping experiment was conducted in a lodgepole pine forest, approx. 20 km east of Penticton, British Columbia, August 20–24, 1991. In randomized complete blocks, 40, 12-unit multiple-funnel traps (Phero Tech Inc., Delta, British Columbia) were placed 25 m apart. Ten traps were unbaited controls and the others were baited with one of three treatments: (1) MPB funnel lure, releasing myrcene, *exo*-brevicomin, and *trans*-verbenol at 300, 0.01, and 1.7 mg/day, respectively, at a constant temperature of 24°C; (2) MPB funnel lures plus 2 g of verbenone-impregnated polyethylene beads (85% (−) enantiomer, Phero Tech Inc.) within an 8 × 3-cm plastic mesh bag placed within the central column of the trap releasing verbenone at 3.8 mg/day; and (3) MPB funnel lure and 2 g of verbenone beads scattered within a 2 × 2-m area below the centered trap. The bottoms of the traps were placed 15–35 cm off the ground. Captured beetles from each trap were collected, sexed (Lyon, 1958), and counted.

Photoisomerism of Verbenone Exposed to Sunlight. Two experiments were conducted to assess verbenone photoisomerism. In each experiment, a glass capillary (1 mm ID), open at one end and containing 20 μl of verbenone (96% pure, Phero Tech Inc.) was placed in each of 20, 10-ml screw-top Pyrex test tubes. The test tubes were sealed with a Teflon-lined screw-cap and left in the dark at room temperature for 48 hr. Two control tubes were covered with light-tight black tape. After 48 hr, the capillaries were removed, ensuring that only verbenone vapor was present in each tube. The test tubes were placed in a rack at 45° off vertical, and exposed to full sunlight. Exposure in the first experiment began at 1200 hr on September 6, 1990, at Burnaby, British Columbia and in the second experiment, exposure began at 1200 hr on September 23, 1990.

After 0, 10, 20, 30, 40, 50, and 60 min of sunlight exposure on September 6, and after the same time intervals plus 75 and 90 min on September 23, two

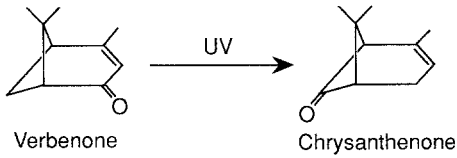

FIG. 1. Conversion of verbenone to chrysanthenone under the influence of ultraviolet radiation.

of the test tubes were removed, placed in a light-tight container, and kept at −15°C. Control tubes were removed at 60 min (September 6) and 60 and 90 min (September 23). The light intensity was measured with a radiometer (Licor, model LI-185, pyranometer sensor) at each of the collection times.

After 24 hr at −15°C, each tube was rinsed with 200 μl of pentane containing 6-undecanone (25 ng/μl) as an internal standard. Two microliters of this rinse were subjected to gas chromatography (GC) employing a Hewlett Packard 5880 gas chromatograph equipped with a DB-1 column (30 m × 0.25 mm ID). The initial oven temperature of 100°C was increased at 5°C/min to 160°C.

Bioactivity of Chrysanthenone. A five-replicate, four-treatment, randomized complete block experiment using 12-unit multiple-funnel traps was conducted in a lodgepole pine forest approx. 35 km east of Penticton, British Columbia from July 25 to August 1, 1990. Within each block, traps were placed 25 m apart and assigned one of four treatments: (1) unbaited control; (2) MPB funnel lure (as above); (3) chrysanthenone (67% pure, Phero Tech Inc., released at approx. 2 mg/day); and (4) MPB funnel lure plus chrysanthenone. A second experiment was conducted August 1–9, 1990. At the end of each experiment captured beetles were collected, sexed (Lyon, 1958), and counted.

On July 26, 1990, a 10-replicate, randomized complete block, tree-baiting experiment was conducted approx. 35 km northeast of Princeton, British Columbia. Lodgepole pine trees with diameter >20 cm at 1.3 m above ground were selected at 33-m intervals and baited with one of two treatments: (1) MPB tree baits, releasing myrcene, *exo*-brevicomin, and *trans*-verbenol at 6.5, 0.2, and 1.7 mg/day at 24°C, respectively; or (2) MPB tree baits plus chrysanthenone (as above). This experiment was evaluated on September 16, 1990, by counting the number of entrance holes in 20 × 40-cm areas at eye level on the east and west sides of each tree.

Statistical Analysis. Because the data for the trapping and tree-baiting experiments were not normally distributed, the nonparametric Friedman test (Friedman, 1937; Conover, 1980) was applied for the statistical analysis employing SAS (Statistical Analysis System) within a Unix environment (Conover, 1980; SAS Institute, 1985). Data on the photoisomerism of verbenone over time were analyzed by linear regression (Conover, 1980).

RESULTS AND DISCUSSION

Simulation of Aerial Application with Verbenone-Impregnated Beads. When verbenone-impregnated beads were contained within a multiple funnel trap, the response of MPBs to the funnel lures was completely inhibited (Figure 2). However, when verbenone was released at the same rate (3.8 mg/day) from beads on the ground, 15–35 cm below the base of the traps, it only partially inhibited the response.

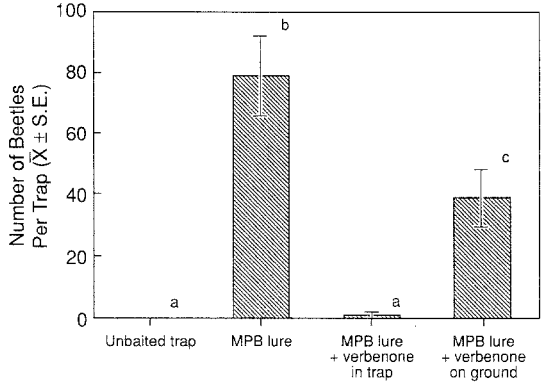

FIG. 2. Response of MPBs to multiple funnel traps baited with attractive semiochemical lures alone and combined with verbenone released from beads within the trap and on the forest floor. $N = 10$. Approx. 20 km east of Penticton, British Columbia, August 20–24, 1991. Bars with same letter are not significantly different, Friedman test, $P < 0.05$. Captured beetles 52% male, 48% female. No difference between sexes in response, chi-square test, $P > 0.05$.

Reduced inhibition may be caused by a number of factors: (1) low volatility may keep verbenone close to the ground, below the flight path of the MPB; (2) diffusion may reduce the dose to suboptimal levels at trap height; (3) the reduction of exposed surface area, caused by the beads lying on the forest floor, may reduce the release rate; (4) a significant amount of verbenone may be adsorbed on soil and surface litter particles; (5) cool temperatures on the forest floor may reduce the release rate or inhibit diffusion; and (6) isomerism of verbenone to chrysanthenone (Figure 1) may contribute to reducing verbenone below optimal levels. Variation in environmental factors, e.g., temperature, relative humidity, precipitation, and wind, could affect the efficacy of verbenone-impregnated beads on the ground. Thus, the effective deterrence of aerially applied, verbenone-impregnated beads, reported by Shea et al. (1992) in one year, could be offset by environmental effects or technological limitations in another year or on another site, as revealed by the inconsistent results when verbenone is applied in this manner (Shea et al., 1992; B.S. Lindgren, personal communication).

Photoisomerism of Verbenone Exposed to Sunlight. Sunlight exposure, on two different days, caused the photoisomerism of verbenone vapor to chrysanthenone, which was the only product of the photolysis detected by GC analysis (Figure 3). On each day, the rate of verbenone conversion was uniform (Figure 4). Isomerism did not occur when light was excluded from the vessels (Figure 3). Missing points in Figure 4 resulted from the loss of volatiles during the experiment, causing a reduction in levels of the compounds below detectable

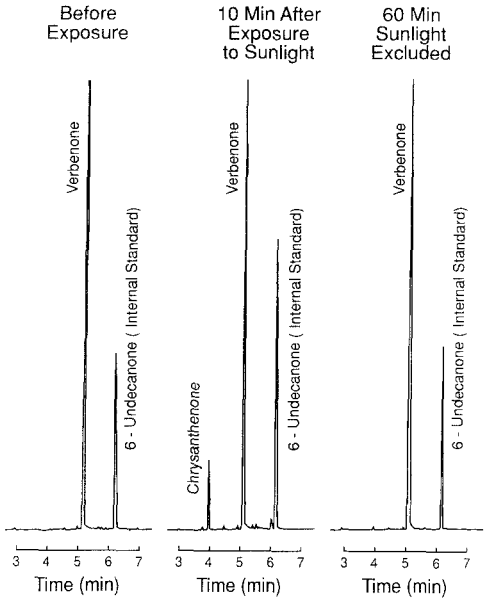

FIG. 3. Representative gas chromatographic traces, showing the conversion of verbenone to chrysanthenone within 10 min of exposure to sunlight.

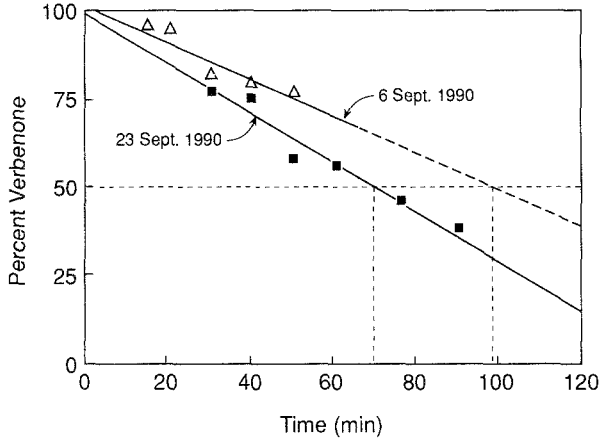

FIG. 4. Time-dependant reduction of verbenone concentration within Pyrex test tubes exposed to sunlight, on two different days in Burnaby, British Columbia, September 6, 1990: $y = 102.1 - 0.53x$, $R^2 = 0.9063$, $P < 0.0034$. September 23, 1990: $y = 99.5 - 0.70x$, $R^2 = 0.9815$, $P < 0.0001$.

thresholds. A subsequent experiment revealed that there was no difference in chrysanthenone production when the verbenone was contained in Pyrex or quartz vessels. The average light intensities on September 6 and 23, 1990, were 196 and 225 W/m^2, respectively. Even though measuring the light intensity at discrete times did not provide a true indication of the average light intensity over the duration of the experiment, increased light intensity on September 23, 1990, apparently caused accelerated isomerism. On both days the weather conditions consisted of bright sunshine with a few scattered clouds.

In addition to chrysanthenone, several minor products were reported when verbenone in a solvent (cyclohexane or acetic acid), at much higher concentrations, was irradiated with a wide-spectrum lamp and a Pyrex filter (Erman, 1967). Other minor products may also have been formed during the exposure of verbenone to sunlight but may have remained below the detectable threshold of the flame ionization detector, due to the small concentrations of verbenone used in these experiments.

Bioactivity of Chrysanthenone. The results of both trapping experiments (Table 1) indicated that chrysanthenone has no behavioral influence on the response of MPB to synthetic attractants. This effect was confirmed by the tree-baiting experiment (Table 2). Although the chrysanthenone tested had some impurities, it is unlikely that any combination of these contaminants could have caused opposing attractant and repellent effects on the MPB that would have resulted in no apparent behavioral activity. One can therefore conclude that

TABLE 1. RESPONSE OF MPB TO MULTIPLE FUNNEL TRAPS BAITED WITH MPB LURES, CHRYSANTHENONE, OR BOTH TOGETHER ON TWO OCCASIONS[a]

Date of experiment	Treatment	Number of MPB/trap (mean ± SE)[b] Males	Females
July 25–Aug. 1, 1990	Unbaited control	1.6 ± 0.7a	0.2 ± 0.2a
	MPB trap bait	158.6 ± 50.6b	83.6 ± 25.0b
	Chrysanthenone	1.0 ± 0.4a	0.4 ± 0.3a
	MPB trap bait + chrysanthenone	181.4 ± 30.8b	94.4 ± 10.6b
Aug. 1–9, 1990	Unbaited control	2.6 ± 0.9a	2.6 ± 0.5a
	MPB trap bait	94.4 ± 20.1b	109.4 ± 23.2b
	Chrysanthenone	3.4 ± 1.4a	4.0 ± 1.5a
	MPB trap bait + chrysanthenone	94.8 ± 27.6b	94.2 ± 27.5b

[a] $N = 5$. Approx. 35 km east of Penticton, BC, July 25–Aug. 1, 1990, and Aug. 1–9, 1990.
[b] Means within a column and experiment followed by the same letter are not significantly different, Friedman test, $P < 0.05$.

TABLE 2. COMPARATIVE ATTACK FREQUENCY AND DENSITY BY MPB ON LODGEPOLE PINES TREATED WITH MPB TREE BAITS ALONE AND WITH CHRYSANTHENONE[a]

Treatment	Attack on baited trees		Number of attacked trees within 10 m of a baited tree (mean ± SE)[b]	
	% mass attacked	Attack density (mean ± SE)[b]	Mass attacked (>31.25/m^2)	Lightly attacked (<31.25/m^2)
MPB tree bait	100	78.3 ± 4.5a	3.3 ± 0.7a	0.8 ± 0.2a
MPB tree bait + chrysanthenone	100	75.2 ± 6.3a	2.5 ± 0.4a	1.2 ± 0.2a

[a] $N = 10$. Approx. 35 km northeast of Princeton, BC, July 25–Sept. 16, 1990.
[b] Means within a column followed by the same letter are not significantly different, Friedman test, $P < 0.05$.

verbenone photoisomerizes to an inert substance that has neither an attractant nor a repellent effect on the MPB.

Implications of Verbenone Photoisomerism. To determine the presence of occupied hosts, the MPB has apparently evolved to use a semiochemical of short longevity. The photosensitivity of verbenone may explain in part why and how the beetle switches hosts (Geiszler and Gara, 1978; Geiszler et al., 1980). MPBs are attracted to trees by kairomones and pheromones, but at close range and high colonization density, they switch to nearby hosts, resulting in small spot infestations (Safranyik et al., 1974; Geiszler and Gara, 1978; Geiszler et al., 1980; Mitchell and Preisler, 1991). MPBs fly in the early afternoon at high light intensities and high temperatures (McCambridge, 1971; Billings and Gara, 1975; Safranyik, 1978) under conditions of little or no air movement when pheromones are at highest concentrations within the forest ecosystem (Aylor et al., 1976; Fares et al., 1980). During afternoon periods of high light intensity, photoisomerism may decrease the concentration of verbenone below a critical response threshold, while the stable attractive semiochemicals maintain a greater active distance. Consequently, beetles may orient to a mass-attacked tree, but in close proximity of the tree, at bioactive concentrations of repellent verbenone, they may reorient to and initiate attack on adjacent trees. The rapid disappearance of verbenone emanating from a mass-attacked tree may allow nearby trees to be colonized without beetles being driven out of the area. The resulting characteristic clumped infestation (Geiszler and Gara, 1978; Mitchell and Priesler, 1991), with dominant trees being killed, would open the forest canopy, increase the light intensity and temperature on the forest floor, and would promote seed release, germination, and growth of new trees (Peterman, 1978).

The rapidity of the sunlight-induced photoisomerism of verbenone to chry-

santhenone (Figures 1, 3, and 4), justifies the use of ultraviolet absorbers used in commercial formulations and devices (Phero Tech Inc.), and the placement of verbenone release devices on the north faces of trees (Lindgren et al., 1989). The degree of canopy closure within the forest, and environmental factors such as wind, cloud cover, and temperature, in conjunction with haze, dust, smog, and humidity will affect the quantity and quality of light penetrating the forest (Koller, 1965), altering the rate of chrysanthenone production. The difference in the quantity and quality of sunlight in lodgepole pine and ponderosa pine stands may have discriminately affected verbenone concentration and might have been a contributing factor in the failure of verbenone to protect ponderosa pine forests from MPBs (Gibson et al., 1991).

The intensity of direct solar ultraviolet radiation increases with rising elevation (Koller, 1965). Since photons in the 300- to 380-nm range (UVA and UVB) are responsible for the photoisomerism of verbenone (Kostyk, 1992), the rate of isomerization will likely increase with rising elevation. Similarly, stands in southern locations would likely have a more rapid isomerization of verbenone than those in northern areas because of the increased light intensity and greater amount of ultraviolet light reaching the ground (Koller, 1965). Therefore, elevation, stand density, and geographic location, in conjunction with prevailing weather conditions, should all be considered in determining the optimum dosage of verbenone in semiochemical-based integrated pest management programs for the MPB and other bark beetles.

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ANTIFEEDANT ACTIVITY OF EXTRACTS FROM NEEM, *Azadirachta indica*, TO STRAWBERRY APHID, *Chaetosiphon fragaefolii*

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Abstract—Leaf disk choice test bioassays demonstrated that formulated neem seed oil (NSO) was equally deterrent to first- and third-instar nymphs and adult strawberry aphids, *Chaetosiphon fragaefolii* (Cockerell). Concentrations of NSO resulting in 50% feeding deterrence were approximately 1.1% for this species. The rapid disruption of aphid feeding (< 1 hr) was not related to the presence of the limonoid azadirachtin, and deterrence likely results from the combined activity of several compounds. Activity to *C. fragaefolii* disappeared within 12–24 hr following application to strawberry in the greenhouse. NSO was deterrent to only half of the six aphid species tested. The antifeedant properties of neem do not appear to contribute significantly to the control of aphids and the viruses they transmit.

Key Words—Antifeedant, *Chaetosiphon fragaefolii*, aphids, Homoptera, Aphididae, azadirachtin, *Azadirachta indica*, deterrent, neem.

INTRODUCTION

Antifeedants offer a novel approach to vector and disease management by rendering plants unattractive or unacceptable to pest insects (Saxena and Khan, 1987). Neem has almost legendary insect repellent and antifeedant properties from its long historical use as a crop protectant in many countries of Asia and Africa (Saxena, 1986). A bioassay based on the antifeedant activity of neem to

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desert locust, *Schistocerca gregaria* Forsk., led to the isolation and determination of the most active ingredient, the limonoid azadirachtin (AZA) (Butterworth and Morgan, 1968, 1971). Subsequently, the antifeedant activity of neem seed extracts or AZA has been reported for numerous insect pests (Warthen, 1989), exemplified by the variegated cutworm, *Peridroma saucia* Hübner (Isman et al., 1990); striped, *Acalymma vittatum* (F.); and spotted, *Diabrotica undecimpunctata* Barber, cucumber beetles (Reed et al., 1982); green rice leafhopper, *Nephotettix virescens* (Distant) (Saxena and Khan, 1985); and brown rice planthopper, *Nilaparvata lugens* (Stäl) (Saxena et al., 1981). Concentrations necessary to significantly deter feeding vary markedly between species, however, and feeding of some insect pests does not appear to be influenced by neem extracts or AZA. For example, AZA offered on sucrose-impregnated filter paper to desert locusts completely inhibited feeding at rates as low as 0.01–0.04 ppm (Butterworth and Morgan, 1968, 1971), whereas the migratory grasshopper, *Melanoplus sanguinipes* (F.), readily consumed leaf disks of cabbage treated with AZA at rates as high as 500 ppm (Champagne et al., 1989).

The antifeedant activity of neem could have contributed to the control of aphids reported from previous laboratory and field studies (Siddig, 1987; Lowery et al., 1993) and might help reduce the spread of aphid-transmitted plant diseases. Previous studies of the antifeedant activity of neem extracts or AZA to aphids have produced contradictory results. A neem-based product, RD-Repelin, was highly repellent to the pea aphid, *Acyrthosiphon pisum* Harris, at concentrations of 1–10% (Hunter and Ullman, 1992), but extracts of neem seeds failed to deter settling and probing of the green peach aphid, *Myzus persicae* (Sulzer) (Griffiths et al., 1989). In order to effectively protect crops, deterrency of neem to aphids should be consistently achieved at concentrations appropriate for use in the field, i.e., approximately 1% neem seed oil (NSO) (Lowery et al., 1993).

The purpose of the present investigation was to evaluate the antifeedant activity of neem to the strawberry aphid, *Chaetosiphon fragaefolii* (Cockerell) and compare the response of this aphid to that of other species. Studies involving various instars and aphid species should help clarify earlier contradictory reports. In order to clarify which component(s) of neem is responsible for the antifeedant activity to *C. fragaefolii*, deterrency of NSO, AZA, and the major volatile component of neem, di-*n*-propyl disulfide (DNPD), were assessed in leaf disk choice bioassays. Furthermore, to be of practical use, the deterrent activity of neem should persist for several days. Persistence of the deterrent activity of neem to *C. fragaefolii* was therefore evaluated under laboratory and greenhouse conditions.

METHODS AND MATERIALS

Plant Material. Strawberry, *Fragaria* X *ananassa* Duch., cv Totem; head lettuce, *Lactuca sativa* L., cv Ithaca; sweet pepper, *Capsicum annuum* L., cv California Wonder; broadbean, *Vicia faba* L., cv Windsor long pod; and mus-

tard cabbage, *Brassica chinensis* L., cv Pakchoi, were grown in plastic pots (10 cm diameter) containing a mixture of sandy loam soil and peatmoss (4:1). Pots were placed in a greenhouse with supplemental lighting supplied by sodium vapor lamps (≈ 1500 ft-c), fertilized biweekly with a water-soluble nutrient mix (20:20:20 N:P:K), and irrigated as required. Leaf disks were removed from young, fully expanded leaves of 6-to 8-week-old plants, except for strawberry, which was maintained in permanent plantings.

Leaf Disk Choice Bioassays. Test conditions for the bioassays were adapted from a method for rearing individual apterous *M. persicae* on leaf disks of potato (Lowery and Sears, 1986). Aphids were placed two per disk on four leaf disks (20 mm diameter) cut from leaves with a cork borer and placed in a Petri dish (9 × 50 mm) having 10 small holes in the tight-fitting lids. Dishes were placed upside down on several layers of moist Kimwipe lining the bottom of clear plastic containers, which were held in a growth chamber (17 ± 2°C) under constant, indirect fluorescent light.

For the choice bioassays, two leaf disks treated with the test material and two disks treated with emulsifier only (1.25 ml/liter Triton X-100, BDH Chemicals, Toronto, Ontario) as a control were allowed to dry and then arranged alternately in each dish with their edges barely touching. Because aphids are very sensitive to the condition of their host plant (van Emden, 1972), the four disks in each dish were excised from the same leaf or leaflet. At concentrations of 0%, all four disks were dipped in emulsifier only, and one pair of opposite disks was randomly assigned to the control treatment. Deterrency of test materials was determined by the proportion of aphids on the treated disks relative to the total number of aphids on treated and untreated disks in each dish.

Deterrent Activity of NSO. To evaluate how rapidly neem affected settling of *C. fragaefolii*, deterrency of a 40% emulsifiable concentrate (EC) of NSO to adults was assessed for bioassays lasting 1, 3, 6, 24, and 48 hr. Deterrency of NSO was tested at five concentrations (0.0, 0.375, 0.75, 1.5, and 3.0%), and control disks were dipped in emulsifier (Mazon BSF19, Mazer Chemicals, Inc., Gurnee, Illinois) at a concentration equivalent to that in the corresponding NSO treatment. Six replicates (dishes) with eight aphids per dish were used for each time interval and concentration.

Deterrency of neem to first- and third-instar and adult *C. fragaefolii* on strawberry was determined in the manner outlined above, except for the following changes. NSO containing approximately 4000 ppm AZA was tested at rates ranging from 0 to 2% (0.0, 0.25, 0.5, 1.0, 2.0%) with the position of the aphids assessed after 24 hr. The entire experiment was replicated twice, resulting in 12 dishes for each concentration of NSO. Bioassays were also conducted with adult *Fimbriaphis fimbriata* Richards and an unidentified *Chaetosiphon* species [most likely *C. thomasi* (Hille Ris Lambers)] on strawberry, *A. pisum* on broad-

bean, *M. persicae* on pepper, and the lettuce aphid, *Nasonovia ribisnigri* (Mosley), on lettuce.

Determination of Active Principle. The constituent of NSO responsible for the deterrent activity was investigated in 24-hr bioassays with adult *C. fragaefolii* feeding on leaf disks treated with NSO containing variable concentrations of AZA (0.0, 0.25, 0.5, 1.0, and 2.0%), pure AZA (0.0, 62.5, 125.0, 250.0, and 500.0 ppm), or DNPD (Pfaltz and Bauer, Inc., Waterbury, Connecticut) (0, 10, 100, 1000, and 10,000 ppm). According to Balandrin et al. (1988), DNPD is the major volatile component of NSO (76% by weight of headspace volatiles). Six replicates were used for each concentration of AZA and DNPD and 12 for each concentration of every oil.

Persistence of Activity. Persistence of the deterrent effect of NSO ($\approx$4,000 ppm AZA) to adult *C. fragaefolii* was determined for bioassays with treated leaf disks maintained in the laboratory and for disks removed from leaflets of strawberry treated in the greenhouse. For the first experiment, leaf disks were treated with NSO (0.0, 0.25, 0.5, 1.0, and 2.0%) and held in Petri dishes under test conditions. Bioassays (24 hr) were then conducted daily for four days using the treated leaf material. On each day, each rate was replicated 12 times.

For the second experiment, one leaflet of an intact strawberry leaf was dipped in NSO (1.0 or 2.0%), while one of the remaining two leaflets was dipped in emulsifier only as a control. Pairs of treated and control leaf disks from an individual leaf were then used for bioassays (24 hr) beginning 0 to 48 hr after the leaf material had dried. Treatments were replicated 10 times in a single trial.

Determination of AZA Concentration. The AZA content of the NSOs (provided by Safer Ltd., Victoria, British Columbia) was determined using reverse-phase gradient high-performance liquid chromatography (HPLC) (Isman et al., 1990). The HPLC system consisted of a Waters model 840 chromatograph (Millipore Canada Ltd., Waters Chromatography Div., Mississauga, Ontario) with a model 490 multiwave UV detector. A comparative standard of pure AZA (>95%) was supplied by J.T. Arnason (University of Ottawa, Ottawa, Ontario).

Statistical Analysis. Proportions of aphids on treated disks were transformed by arcsin $\sqrt{x}$ to normalize the variances (Neter et al., 1985). Transformed values were subjected to analysis of variance (ANOVA) (Wilkinson, 1990) and linear regression analysis. Inverse prediction (Neter et al., 1985) was used to determine the effective concentration required to deter 50% of the aphids (EC_{50}) (i.e., when 25% of the aphids remained on the treated disks). The coefficient of determination (R^2) was partitioned, with the values shown being equivalent to those from regression based on the transformed treatment means. Treatment concentrations were transformed, $\ln_e(x + 1)$, as required, to improve linearity; concentrations in experiments with rates exceeding 100 ppm were scaled to improve precision. For the final bioassay to evaluate the persistence

of NSO applied to intact plants in the greenhouse, following ANOVA, Fisher's least significant difference test was used to determine differences between treatment means (Wilkinson, 1990).

RESULTS AND DISCUSSION

Deterrent Activity of NSO. Deterrency of NSO (40% EC) to adult *C. fragaefolii* did not differ for bioassays lasting from 1 to 48 hr, indicating that the response of aphids occurred rapidly, within the first hour (Table 1). EC_{50} values decreased slightly as the duration of the tests increased from 1 to 24 hr, but the slopes of the regression equations did not differ significantly ($P > 0.05$) for any of the bioassays. Multiple regression analysis involving both oil concentration and time also demonstrated that duration of the bioassay (time) was not significant ($P = 0.968$). These results indicate that bioassays lasting 24 hr were sufficiently long to accurately measure the deterrent activity of neem.

In a previous study, AZA applied to wheat seedlings at concentrations of 250 or 500 ppm reduced probing and increased locomotory activity of English grain aphid, *Sitobion avenae* (F.), and bird cherry-oat aphid, *Rhopalosiphum padi* (L.), during the first 25 min of feeding (West and Mordue, 1992). Antifeedant activity of neem seed extract to desert locust occurred after the insects had examined test filter papers impregnated with neem solutions (Butterworth and Morgan, 1971). Although neem deterred feeding in a very rapid manner, it was not repellent, as the insects crawled upon and tasted the treated papers. On the other hand, fewer adult sweet-potato whitefly, *Bemisia tabaci* (Gennadius), landed on cotton treated with neem seed extract (Coudriet et al., 1985), demonstrating that neem was repellent to this insect.

TABLE 1. EFFECTIVE CONCENTRATIONS OF NEEM SEED OIL (NSO) RESULTING IN 50% DETERRENCY (EC_{50}) TO SECOND-INSTAR STRAWBERRY APHID, *Chaetosiphon fragaefolii*, FOR BIOASSAYS LASTING 1–48 h

Time (hr)	EC_{50} (%NSO)	Slope ± SE[a]	R^2	P (reg.)
1	3.3	−0.187 ± 0.024	0.951	0.027
3	3.1	−0.173 ± 0.066	0.694	0.028
6	2.8	−0.183 ± 0.062	0.743	0.015
24	2.0	−0.280 ± 0.063	0.869	0.004
48	4.8	−0.158 ± 0.036	0.864	0.049

[a] Slopes of regression equations for proportions of aphids on NSO-treated leaf disks vs. $\ln_e$ [NSO] are not significantly different ($P > 0.05$), based on 95% confidence intervals.

Results of the current bioassays demonstrate that NSO deterred *C. fragaefolii* in less than 1 hr, but they do not distinguish between repellent and antifeedant activity. Careful observation of *M. persicae*, *N. ribisnigri*, and *C. fragaefolii* on NSO-treated leaf disks, with the aid of a 10× magnifying lens, indicated that NSO was not repellent to these aphids. During the 20 min of observation, all aphids attempted to probe the treated leaf material at least once (data not shown).

Responsiveness of aphids to volatile substances is generally weak and variable, and most studies have concluded that aphids discriminate between suitable hosts following ingestion of plant fluids (Papaj and Rausher, 1983). Aphids invariably attempt to probe the surface of any substrate they land on (Gibson and Plumb, 1977), and it is during these short test probes that a small amount of fluid is ingested and "tasted" with the precibarial chemosensillae (Backus, 1988).

First- and third-instar and adult *C. fragaefolii* were equally deterred by NSO (Table 2). EC_{50} values were almost identical, around 1.1% NSO, and slopes of the regression equations were not significantly different ($P > 0.05$). However, aphid species were not equally deterred by NSO over the range of concentrations tested (Table 2). In addition to *C. fragaefolii*, NSO deterred *A. pisum* (EC_{50} = 1.7% NSO), and *Chaetosiphon* sp. (EC_{50} = 2.1% NSO), but it was ineffective against *F. fimbriata*, *M. persicae*, and *N. ribisnigri* (regression $P > 0.05$).

Previous studies of the deterrent activity of neem to aphids have produced

TABLE 2. CONCENTRATIONS OF NEEM SEED OIL (NSO) RESULTING IN 50% DETERRENCY (EC_{50}) TO SIX SPECIES OF APHIDS IN 24-h LEAF DISK CHOICE BIOASSAYS

Instar, species,[a] host plant	EC_{50} (%NSO)	Slope ± SE[b]	R^2	P (reg.)
First, *C. fragaefolii*, strawberry	1.1	−0.234 ± 0.041	0.916	<0.001
Third, *C. fragaefolii*, strawberry	1.2	−0.186 ± 0.023	0.957	<0.001
Adult, *C. fragaefolii*, strawberry	1.2	−0.206 ± 0.027	0.950	<0.001
Adult, *A. pisum*, broad bean	1.7	−0.153 ± 0.037	0.848	0.003
Adult, *Chaetosiphon* sp., strawberry	2.1	−0.126 ± 0.015	0.961	0.026
Adult, *F. fimbriata*, strawberry	NS[c]		0.006	0.801
Adult, *M. persicae*, pepper	NS		0.560	0.128
Adult, *N. ribisnigri*, lettuce	NS		0.099	0.888

[a] Strawberry aphid, *C. fragaefolii*; pea aphid, *A. pisum*; unidentified *Chaetosiphon* species; green peach aphid, *M. persicae*; lettuce aphid, *N. ribisnigri*.

[b] Slopes of the regression equations for proportions of aphids on NSO-treated leaf disks vs. [NSO] are not significantly different ($P > 0.05$), based on 95% confidence intervals.

[c] Regression nonsignificant ($P > 0.05$).

contradictory results. RD-Repelin at concentrations of 1–10% repelled *A. pisum* (Hunter and Ullman, 1992), and AZA at concentrations of 500 ppm deterred settling and probing of *R. padi* and *S. avenae* (West and Mordue, 1992). Choice bioassays demonstrated that NSO containing unknown amounts of AZA applied to broad bean leaves at a concentration of 2.5% deterred 42.4% of adult *A. pisum* compared to controls (Wilkins et al., 1990). Contrary to these findings, Griffiths et al. (1989) showed that extracts of neem seeds failed to deter settling or probing of *M. persicae*. Electronic monitoring of alate *M. persicae* feeding on iceburg lettuce treated with the neem-based insecticide Margosan-O revealed a slight reduction in the total amount of time aphids probed treated plants, but there was no difference in the amount of time salivating, walking, or ingesting phloem compared to controls (Braker et al., 1991). Based on the current findings, these opposing results might be explained by differences in behavioral response between the aphid species. In our trials *A. pisum* was deterred by NSO, whereas *M. persicae* was not.

A high degree of variation in the behavioral responses of insects to neem extracts and AZA has been documented previously. Concentrations of AZA necessary to reduce feeding on leaf disks by 70% ranged from 1 ppm for third-instar fall armyworm, *Spodoptera frugiperda* Smith, on lima bean to 250 ppm for second-instar Colorado potato beetle, *Leptinotarsa decemlineata* L., on potato (Wood, 1990). Similarly, a 0.01% hexane extract of neem seeds was deterrent to California red scale, *Aonidiella aurantii* (Maskell), while concentrations of 0.1% and 1.0% were required to deter yellow scale, *Aonidiella citrina* (Coquillet), and citrus mealybug, *Planococcus citri* (Risso), respectively (Jacobson et al., 1978). Significantly fewer female brown rice planthoppers and white-backed planthoppers, *Sogatella furcifera* (Horvath), arrived on rice plants treated with ultra-low volumes of NSO at concentrations of 5–50%, but NSO was not repellent to female green rice leafhoppers (Heyde et al., 1984). Feeding by all three species of homopterans decreased with increasing concentrations of NSO, however, suggesting that the antifeedant and repellent activities of neem are independent.

Determination of Active Principle. Our results show that deterrency of NSO to adult *C. fragaefolii* was not related to the concentrations of AZA. EC_{50} values for oils with AZA contents ranging from <50 to 6877 ppm were very similar, and slopes of the regression lines did not differ significantly ($P > 0.05$) (Table 3), indicating that some other component(s) of NSO contributed to the observed activity.

AZA applied to leaf disks of cabbage was not deterrent to *M. persicae* at rates up to 100 ppm ($R^2 = 0.296$, regression $P = 0.198$), but it was deterrent to *C. fragaefolii* on strawberry ($R^2 = 0.986$, regression $P = 0.001$) with an estimated EC_{50} of 119.5 ppm. At a concentration of 100 ppm, 27% ($\pm 10.5\%$) of *C. fragaefolii* had settled on AZA-treated leaf disks after 24 hr, compared to

TABLE 3. CONCENTRATIONS OF NEEM SEED OILS CONTAINING VARIABLE AMOUNTS OF AZADIRACHTIN (AZA) RESULTING IN 50% DETERRENCY (EC_{50}) TO ADULT STRAWBERRY APHID, *Chaetosiphon fragaefolii*

AZA, (ppm)	EC_{50} (%NSO)	Est. EC_{50} (AZA ppm)	Slope ± SE[a]	R^2	P
<50	0.96	(- -)	−0.413 ± 0.122	0.791	<0.001
1084	0.89	(9.6)	−0.341 ± 0.015	0.994	<0.001
2500	0.80	(20.0)	−0.406 ± 0.025	0.989	<0.001
4000	0.98	(39.2)	−0.370 ± 0.073	0.895	<0.001
4700	1.04	(48.9)	−0.353 ± 0.035	0.972	<0.001
6877	0.86	(59.1)	−0.459 ± 0.067	0.939	<0.001

[a]Slopes of regression equations for proportions of aphids on NSO-treated leaf disks vs. $\ln_e$ [NSO] are not significantly different ($P > 0.05$, based on 95% confidence intervals).

49% (±9.9%) at 0 ppm AZA (data not shown). Although AZA may be responsible for some of the deterrency of NSO to aphids, other components of neem likely make a greater contribution to the deterrent activity.

DNPD applied to leaf disks of strawberry at concentrations up to 10,000 ppm did not deter *C. fragaefolii*, and no dose-dependent effect was evident ($R^2 = 0.075$, $P = 0.108$) (data not shown). According to Balandrin et al. (1988), the presence of volatile organosulfur compounds might partially explain the insect repellent effect of neem leaves and seeds, but purified DNPD, which comprises approximately 76% of the volatile components of neem, does not account for the deterrency of NSO to *C. fragaefolii*.

Choice bioassays with various neem seed extract fractions demonstrated that several components were responsible for the antifeedant activity to the California red scale, yellow scale, citrus mealybug, and woolly whitefly, *Aleurothrixus floccosus* (Maskell) (Jacobson et al., 1978). Schwinger et al. (1984) showed that the neem components salannin and 3-deacetylsalannin were as effective as AZA for the prevention of feeding by Mexican bean beetles, *Epilachna varivestis* Mulsant, with 100% inhibition of feeding occurring at concentrations around 0.01% for all three compounds. In the same study, azadiron, azadiradion, 14-epoxyazadiradion, gedunin, nimbinen, 6-deacetylnimbinien, and melianone were effective deterrents at concentrations 10–100 times higher. Saxena and Rembold (1984) determined that the volatile component of neem seeds repelled adult female cotton bollworms, *Heliothis armigera* (Hübner), prior to contact, whereas neem seed oil was not repellent but did inhibit oviposition on contact. AZA neither repelled female moths nor deterred oviposition.

In light of the large number and types of triterpenoids isolated from neem (e.g., Jones et al., 1989), deterrency of NSO likely results from the combined

activities of several compounds. It is likely that the numerous volatile and nonvolatile components of neem work in concert, producing several behavioral responses that differ in magnitude between insect species.

Persistence of Activity. Deterrency of NSO to *C. fragaefolii* persisted for at least four days when treated leaf disks of strawberry were held in a growth chamber under low light conditions (Table 4). Although the regression line was not significant for bioassays conducted three days after treatment ($P = 0.292$), slopes of the remaining regression lines did not differ significantly for tests conducted on days 0–4 ($P > 0.05$). Estimated EC_{50} values ranged from 1.2% NSO (day 0) to 1.9% (day 1). Under greenhouse conditions, deterrency of NSO 1% and NSO 2% disappeared after 12 and 24 hr, respectively (Table 5). At 24

TABLE 4. DETERRENCY OF NEEM SEED OIL (NSO) TO ADULT STRAWBERRY APHID, *Chaetosiphon fragaefolii*, FOR CHOICE BIOASSAYS BEGINNING 0–4 DAYS AFTER TREATMENT OF LEAF DISKS

Day	EC_{50} (%NSO)	Slope ± SE[a]	R^2	P
0	1.2	−0.206 ± 0.027	0.950	0.001
1	1.9	−0.123 ± 0.033	0.821	0.003
2	1.7	−0.134 ± 0.010	0.983	0.001
3	NS[b]		0.084	0.292
4	1.6	−0.112 ± 0.094	0.320	0.020

[a] Slopes of regression equations for proportions of aphids on NSO-treated leaf disks vs. [NSO] are not significantly different ($P > 0.05$, based on 95% confidence intervals).
[b] Regression nonsignificant, $P > 0.05$.

TABLE 5. DETERRENCE OF NEEM SEED OIL (NSO) TO ADULT STRAWBERRY APHID, *Chaetosiphon fragaefolii*, FOR LEAF DISK CHOICE BIOASSAYS BEGINNING 1–48 hr AFTER TREATMENT OF STRAWBERRY IN GREENHOUSE[a]

	Proportions of aphids on NSO-treated disks (SD)					
Treatment	0 hr	3 hr	6 hr	12 hr	24 hr	48 hr
NSO 0.0%	0.47a	0.40a	0.42a	0.45a	0.47a	0.49a
	(0.09)	(0.19)	(0.07)	(0.10)	(0.11)	(0.11)
NSO 1.0%	0.30b	0.12b	0.18b	0.34ab	0.33a	0.47a
	(0.16)	(0.11)	(0.16)	(0.12)	(0.17)	(0.15)
NSO 2.0%	0.06c	0.21b	0.11b	0.27b	0.34a	0.45a
	(0.04)	(0.13)	(0.06)	(0.18)	(0.21)	(0.09)

[a] For each time interval, means followed by the same letter are not significantly different ($P > 0.05$, Fisher's least significant difference test).

hr after treatment, fewer adult *C. fragaefolii* had settled on disks treated with NSO 1% or 2% compared with disks treated with emulsifier only, but the differences were not significant ($P > 0.05$).

The elevated light intensity and warmer, drier conditions in the greenhouse likely accounts for the rapid loss of deterrency. The rapid decline in activity suggests that neem would have limited value for the prevention of aphid-transmitted plant viruses. Deterrency would likely decline even more rapidly under field conditions.

As with many natural plant products, neem materials readily degrade in the environment (Barnby et al., 1989; Walter and Knauss, 1990). For example, feeding of fall armyworm on corn plants sprayed with 20–100 ppm AZA was significantly reduced for up to seven days when plants were held in the laboratory. After 72 hr in the field, however, AZA residues were no longer deterrent even at application rates as high as 600 ppm (Wood, 1990).

CONCLUSION

Neem extracts have been shown to repel and/or deter several homopteran pests, including leafhoppers and planthoppers on rice (Heyde et al., 1984; Saxena and Khan, 1985), sweet-potato whitefly on cotton (Coudriet et al., 1985), Asiatic citrus psyllid, *Diaphorina citri*, on citrus (Chiu, 1984), and *A. pisum* on broadbean (Hunter and Ullman, 1992).

Systemic application of AZA reduced feeding of *M. persicae* on *Nicotiana clevelandii* Gray, but only at rates of 500–1000 ppm (Woodford et al., 1991). Similarly, West and Mordue (1992) showed that AZA applied to barley at concentrations exceeding 250 ppm reduced probing by *R. padi* and *S. avenae* for up to four days. However, Margosan-O contains approximately 3000 ppm AZA (Larson, 1989), and rates of 8–33% would be required to effectively deter feeding of these species. Margosan-O is recommended at a rate of approximately 1%, or 30 ppm AZA (Larson, 1989; Lindquist et al., 1990).

According to Wood (1990), the commercial significance of the antifeedant activity of neem may be limited. Sensitivity of insects to AZA as an antifeedant appears to vary considerably between species; high concentrations are required to deter feeding by many species; and activity may be short-lived under field conditions. AZA does not appear to be a general inhibitor of insect feeding (Butterworth and Morgan, 1971). Results from our trials with several species of aphids support these statements. At concentrations useful for the control of aphids in the field ($\approx 1.0\%$ NSO) (Lowery et al., 1993), NSO deterred only half the species tested and activity was rapidly lost following applications to plants in the greenhouse.

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INTERACTIONS OF *trans*-CINNAMIC ACID, ITS RELATED PHENOLIC ALLELOCHEMICALS, AND ABSCISIC ACID IN SEEDLING GROWTH AND SEED GERMINATION OF LETTUCE

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Abstract—Phenolic compounds have been identified as the most common allelochemicals produced by higher plants. Inhibitions of cinnamic acid, its related phenolic derivatives, and abscisic acid (ABA) on seedling growth and seed germination of lettuce were studied. *trans*-Cinnamic acid, and *o*-, *m*-, and *p*-coumaric acids inhibited the growth of etiolated seedlings of lettuce at concentrations higher than 10^{-4} M and seed germination above 10^{-3} M. Coumarin inhibited seedling growth and seed germination at 10^{-5} M or above. Chlorogenic acid inhibited seedling growth above 10^{-4} M, but did not inhibit seed germination at 10^{-5}–5×10^{-3} M. Low concentrations (below 10^{-3} M) of caffeic and ferulic acids promoted the elongation of hypocotyls, but higher concentrations (over 10^{-3} M) inhibited seedling growth and seed germination. These phenolic compounds and abscisic acid had additive inhibitory effects both on seedling growth and seed germination. The inhibition on lettuce was reversed by caffeic and ferulic acids at concentrations lower than 10^{-3} M except for the inhibition of germination by coumarin. These results suggest that in nature *trans*-cinnamic acid, *o*-, *m*-, *p*-coumaric acids, coumarin, and chlorogenic acid inhibit plant growth regardless of their concentration. How-

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ever, caffeic and ferulic acids can either promote or inhibit plant growth according to their concentration.

Key Words—Allelopathy, allelochemicals, phenolic compounds, abscisic acid, *trans*-cinnamic acid, coumaric acids, chlorogenic acid, coumarin, caffeic acid, ferulic acid, lettuce, *Lactuca cariola*.

INTRODUCTION

Phenolic compounds have been demonstrated to be widespread growth substances in plants (Milborrow, 1984). Recently, these substances were shown to have important roles in the chemical interactions between plants (Chou and Waller, 1989; Harborne, 1988; Li et al., 1992a,b; Putnam and Tang 1986; Rice, 1984; Waller, 1987). The physiological and biochemical effects of these phenolics on plants and their mechanism of action have been studied by many researchers. Some early work showed that phenolic compounds might be involved in the metabolism of auxin. They may act in vivo as inhibitors or activators of the indole-3-acetic acid (IAA) oxidizing system. Polyphenolic substances such as caffeic acid and ferulic acid synergized the action of IAA in the *Avena* curvature test, in the elongation of coleoptile sections of oat and rice, and in the elongation of hypocotyl or internode of oat, sunflower, and pea, while monophenolic compounds such as *p*-coumaric acid or *p*-hydroxybenzoic acid antagonized IAA in the same bioassays. It was suggested that monohydroxy phenolic acids promoted IAA inactivation and inhibited plant growth and that di- or polyhydroxy phenolic acids inhibited IAA inactivation and promoted plant growth (Sondheimer and Griffin, 1960; Thimann et al., 1962; Tomaszewski and Thimann, 1966; Vendrig and Buffel, 1961; Zenk and Muller, 1963). However, most of these results on the interaction of phenolics and IAA do not stand up to rigorous critical examination. The functions and mechanisms of action of the phenolics are not quite clear (Milborrow, 1984).

Recently, there has been an increase of reports on the roles and modes of action of phenolic compounds in plants (Chou and Waller, 1989, Harborne, 1988; Putnam and Tang, 1986; Rice, 1984; Waller, 1989). Some phenolics were reported to inhibit seed germination, root elongation, leaf expansion, photosynthesis, nutrient absorption, and the accumulation of dry matter in shoots and roots in plants (Blum and Rebbeck, 1989; Kuiters, 1989; Lyu et al., 1990; Lyu and Blum, 1990; Rasmussen and Einhellig, 1977; Reynolds, 1978; Rice, 1984; Wacker et al., 1990; Williams and Hoagland, 1982). In most cases of allelopathy, many of these compounds exist simultaneously in the growth media. Effects of these substances on plant growth and development are due to the interactions of all these compounds. The relationships and the mechanisms of action of these substances are very important in understanding their allelopathic

effects in natural ecosystems. However, little information is available about the relationships of these phenolic compounds among themselves and with abscisic acid (ABA) in the growth of intact plants and in seed germination.

trans-Cinnamic, *o*-, *m*-, and *p*-coumaric, chlorogenic, caffeic, and ferulic acids, and coumarin are structurally related allelochemicals. In this paper, the relative activities of these eight phenolics, their interactions, and their relationships with ABA in seedling growth and seed germination of lettuce were studied. Their roles in natural ecosystems and possible mechanisms of action are discussed.

METHODS AND MATERIALS

Chemicals. Chemicals used were newly purchased and stored at 4°C. *trans*-Cinnamic (No. C-6004), caffeic (No. C-0625), ferulic (No. F-3500), chlorogenic (C-3878), and abscisic acid (No. A-7383, synthetic mixture isomers) were from Sigma Chemical Co. *o*-Coumaric acid (No. 07771-51) and coumarin (No. 07450-30) were from Kanto Chemical Co. *m*-Coumaric acid (Art. 800234) and *p*-coumaric acid (Art. 800237) were Merck products.

Growth Tests of Etiolated Seedlings. Seeds of lettuce (*Lactuca cariola* L. var. *sativa* Bisch) were surface sterilized with 1% sodium hypochlorite for 5 min and washed with distilled water. Germination was carried out on filter paper moistened with distilled water in a plastic tray in the dark at 25°C for 36 hr. Uniform etiolated seedlings with ca. 3 mm hypocotyl and 5 mm root were selected for seedling growth tests. The growth tests were performed in 33-mm-diameter Petri dishes with 1 ml of test solution. For each test, six etiolated seedlings were planted in the dish. The seedlings were cultured for 48 hr in the dark at 25°C. The lengths of roots and hypocotyls were then measured. Each test was repeated at least three times.

Seed Germination Tests. Seed germination tests were done on Toyo No. 1 filter paper in 33-mm-diameter Petri dishes with 1 ml of test solution or control containing 100 ppm Tween 80. All compounds were dissolved in 100 ppm Tween 80. Fifty seeds were added to each dish and germinated in the dark at 25°C. Germinated seeds were counted after a 24-hr incubation. Each test was repeated at least three times.

The data were calculated as percentage of germination or percentage over the control (hypocotyl and root elongation). Significance between treatments was determined by Student's *t* test. Throughout this paper, $P < 0.05$ is considered significant.

RESULTS

Effects of Each Phenolic Compound on Seedling Growth and Seed Germination of Lettuce. Figure 1 shows the effects of *trans*-cinnamic acid, *o*-, *m*-, *p*-coumaric, caffeic, ferulic, and chlorogenic acids, and coumarin on the growth

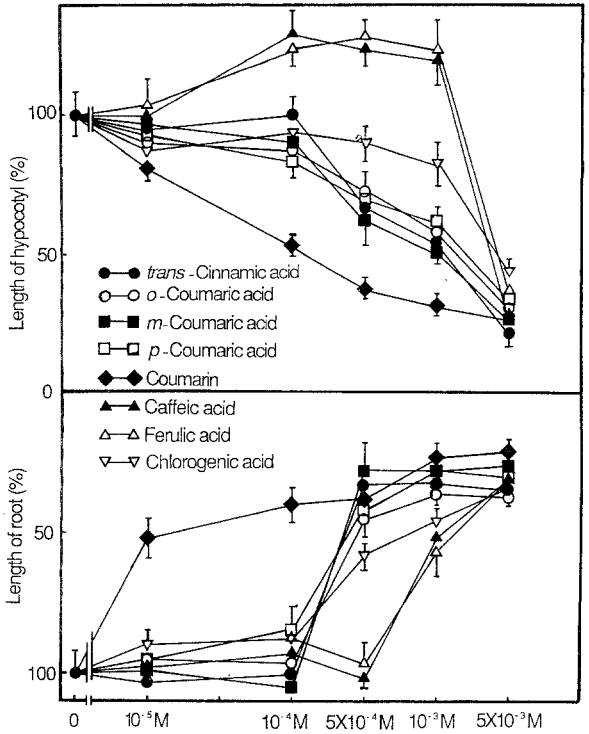

FIG. 1. Effects of phenolic compounds on the growth of etiolated seedlings of lettuce at different concentrations.

of etiolated seedlings of lettuce at various concentrations from 10^{-5} M to 5×10^{-3} M. *trans*-Cinnamic and *o*-, *m*-, *p*-coumaric acids did not show any significant effects ($P > 0.05$, t test) on the elongation of roots and hypocotyls at 10^{-4} M or lower. They inhibited both hypocotyl and root elongation at concentrations higher than 10^{-4} M, and inhibition was stronger on roots than on hypocotyls. At 5×10^{-4} M or more, they completely inhibited root elongation. They had significant inhibitory activity on the elongation of the hypocotyl at 5×10^{-4} M and completely inhibited its elongation at 5×10^{-3} M. The effects of chlorogenic acid on both root and hypocotyl elongation were weaker than with the four phenolics described above. Coumarin caused much stronger growth inhibition than the other compounds tested. It significantly inhibited the elongation of roots and hypocotyls at 10^{-5} M. It completely inhibited seedling growth at 5×10^{-4} M.

Caffeic and ferulic acids at different concentrations had different effects on the growth of lettuce. They significantly promoted the elongation of hypocotyl

in the 10^{-4} M–10^{-3} M concentration range. The maximum promoting activity of these two compounds was about 30%. Neither of them showed any significant promotion ($P > 0.05$, t test) of root elongation at the concentrations tested. However, they strongly inhibited root elongation when the concentrations were higher than 5×10^{-4} M and inhibited the elongation of both root and hypocotyl at concentrations higher than 10^{-3} M (Figure 1).

Except for chlorogenic acid, which did not have any significant inhibition ($P > 0.05$, t test) on lettuce seed germination at 10^{-5}–5×10^{-3} M concentrations, all other compounds tested inhibited germination at varying concentrations. Among these, coumarin was much more active than the others. It significantly inhibited seed germination at 10^{-5} M and completely inhibited seed germination at 5×10^{-4} M or higher. *trans*-Cinnamic, *o*-, *m*-, *p*-coumaric, caffeic, and ferulic acids had almost the same dose–response relationship. They showed little inhibition of seed germination at 10^{-3} M or lower but had strong inhibition activity when the concentrations were higher (Figure 2).

Interactions of Phenolic Compounds. *trans*-Cinnamic, *o*-, *m*-, *p*-coumaric acids, and coumarin demonstrated additive inhibition, in which the combinations of two samples with certain activities of two different compounds or either of

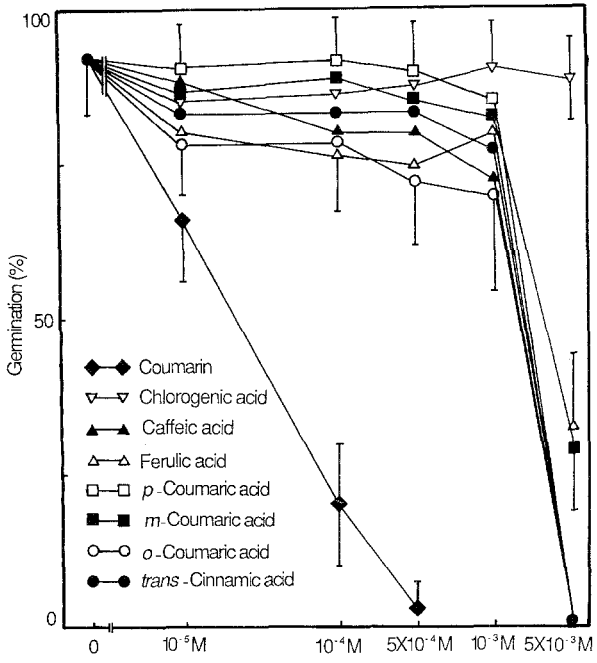

FIG. 2. Effects of phenolic compounds on seed germination of lettuce at different concentrations.

them (two concentrations) produce almost the same inhibition on seedling growth (Table 1) and seed germination (Table 2). Inhibition by combinations of any two compounds on both seedling growth (the elongation of roots and hypocotyls) and seed germination were stronger than with either compound alone. All of them showed additive inhibition with chlorogenic acid on seedling growth (Table 1). Chlorogenic acid alone did not inhibit seed germination (Figure 2); however, it could increase the inhibition of *trans*-cinnamic, *o*-, *m*-, *p*-coumaric acids, and coumarin on seed germination.

Caffeic and ferulic acids at 10^{-4} M completely reversed growth inhibition

TABLE 1. EFFECTS OF INTERACTIONS OF *trans*-CINNAMIC ACID, *o*-, *m*-, AND *p*-COUMARIC ACIDS, CHLOROGENIC ACID, AND COUMARIN ON GROWTH OF ETIOLATED SEEDLINGS OF LETTUCE[a]

Treatment	Length of hypocotyl (%)	Root length (%)
Control	100 ± 8	100 ± 10
trans-Cinnamic acid	63 ± 8	54 ± 6
o-Coumaric acid	67 ± 4	54 ± 6
m-Coumaric acid	65 ± 9	55 ± 8
p-Coumaric acid	63 ± 8	52 ± 8
Chlorogenic acid	88 ± 10	64 ± 6
Coumarin	66 ± 8	54 ± 6
trans-Cinnamic acid		
+ *o*-coumaric acid	52 ± 4	46 ± 6
+ *m*-coumaric acid	54 ± 6	51 ± 8
+ *p*-coumaric acid	52 ± 4	50 ± 8
+ chlorogenic acid	56 ± 6	50 ± 10
+ coumarin	55 ± 4	52 ± 6
o-Coumaric acid		
+ *m*-coumaric acid	54 ± 8	53 ± 9
+ *p*-coumaric acid	50 ± 4	46 ± 6
+ chlorogenic acid	60 ± 8	48 ± 6
+ coumarin	54 ± 8	53 ± 8
m-Coumaric acid		
+ *p*-coumaric acid	52 ± 8	48 ± 10
+ chlorogenic acid	55 ± 10	52 ± 8
+ coumarin	53 ± 6	47 ± 6
p-Coumaric acid		
+ chlorogenic acid	60 ± 8	49 ±6
+ coumarin	61 ± 5	51 ± 8
Chlorogenic acid + coumarin	58 ± 4	46 ± 6

[a]The concentrations used were 5×10^{-5} M for coumarin and 5×10^{-4} M for the others. Each value is the mean of three replicates ± SE.

TABLE 2. EFFECTS OF COMBINATIONS OF *trans*-CINNAMIC ACID, *o*-, *m*-, *p*-COUMARIC ACIDS, COUMARIN, AND CHLOROGENIC ACID ON SEED GERMINATION OF LETTUCE[a]

Treatment	Germination (%)
Control	97 ± 10
trans-Cinnamic acid	28 ± 15
o-Coumaric acid	51 ± 15
m-Coumaric acid	55 ± 10
p-Coumaric acid	52 ± 10
Coumarin	42 ± 6
Chlorogenic acid	98 ± 6
trans-Cinnamaic acid	
+ *o*-Coumaric acid	0
+ *m*-Coumaric acid	3 ± 6
+ *p*-Coumaric acid	3 ± 6
+ Coumarin	0
+ Chlorogenic acid	15 ± 10
o-Coumaric acid	
+ *m*-Coumaric acid	20 ± 10
+ *p*-Coumaric acid	30 ± 6
+ Coumarin	0
+ Chlorogenic acid	42 ± 10
m-Coumaric acid	
+ *p*-Coumaric acid	24 + 10
+ Coumarin	0
+ Chlorogenic acid	35 ± 15
p-Coumaric acid	
+ Coumarin	3 ± 6
+ Chlorogenic acid	43 ± 15
Coumarin + chlorogenic acid	0

[a]The concentrations used were 10^{-4} M for coumarin and 2.5×10^{-3} M for the others. Each value is the mean of three replicates ± SE.

provoked by 5×10^{-4} M of *trans*-cinnamic, *o*-, *m*-, *p*-coumaric acids, and partially reversed inhibition by 5×10^{-5} M of coumarin (Figure 3). A higher concentration (5×10^{-4} M) of caffeic acid and ferulic acid could also reverse inhibition on hypocotyl elongation by 5×10^{-4} M or 10^{-3} M of *trans*-cinnamic and *o*-, *m*-, *p*-coumaric acids, but could not reverse their inhibition on root elongation. Caffeic and ferulic acids at 10^{-3} M could reverse inhibition by 2.5×10^{-3} M of *trans*-cinnamic and *o*-, *m*-, *p*-coumaric acids on seed germination. They did not show any inhibition on seed germination when applied in combination with 2.5×10^{-3} M chlorogenic acid. Caffeic and ferulic acids, however, could not reverse but rather increased the inhibition of coumarin on seed germination (Figure 4).

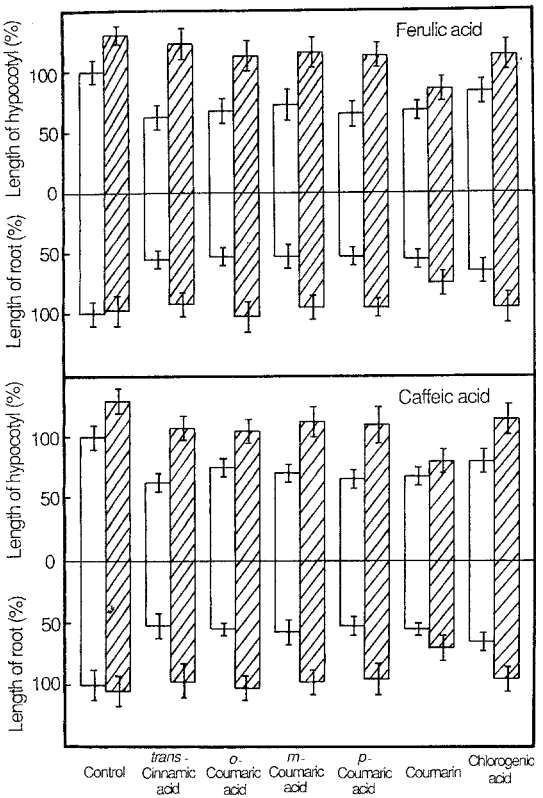

FIG. 3. Interaction of caffeic and ferulic acids with other phenolic compounds on the growth of etiolated seedlings of lettuce. The concentrations used were 10^{-4} M for caffeic acid and ferulic acid, 5×10^{-5} M for coumarin, and 5×10^{-4} M for the others.

Relationships of Phenolic Compounds and ABA. *trans*-Cinnamic, *o*-, *m*-, *p*-coumaric, and chlorogenic acids and coumarin showed additive inhibition with ABA on lettuce seedling growth. All combinations of any one phenolic compound with ABA produced stronger inhibition on seedling growth than for each phenolic compound or ABA alone. However, the combinations of *trans*-cinnamic or *o*-, *m*-, or *p*-coumaric acids with ABA increased their inhibition on hypocotyl elongation when compared to treatments with the same phenolic compounds alone. The combinations of coumarin or chlorogenic acid with ABA increased their inhibition activity on both hypocotyls and roots, when compared to coumarin or chlorogenic acid treatment alone (Figure 5). Caffeic and ferulic acids at 10^{-4} M could reverse the growth inhibition produced by a low concen-

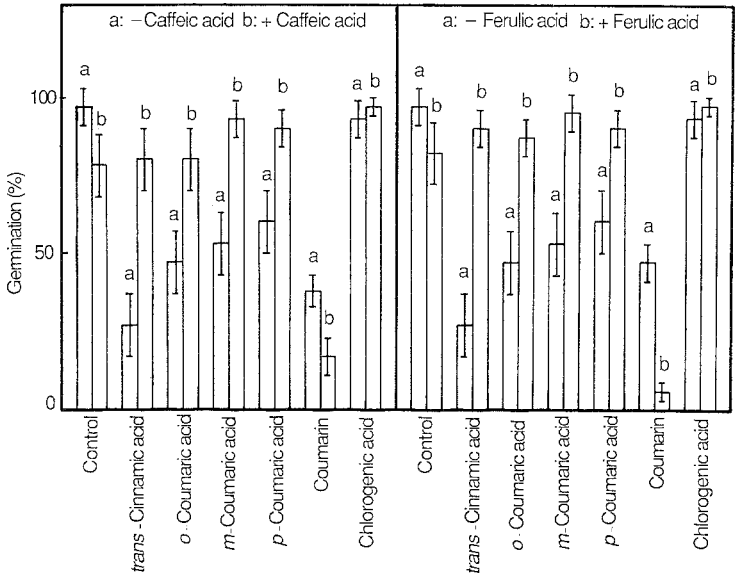

FIG. 4. Interaction of caffeic and ferulic acids with other phenolic compounds on the seed germination of lettuce. The concentrations used were 10^{-3} M for caffeic and ferulic acids, 10^{-4} M for coumarin, and 2.5×10^{-3} M for the others.

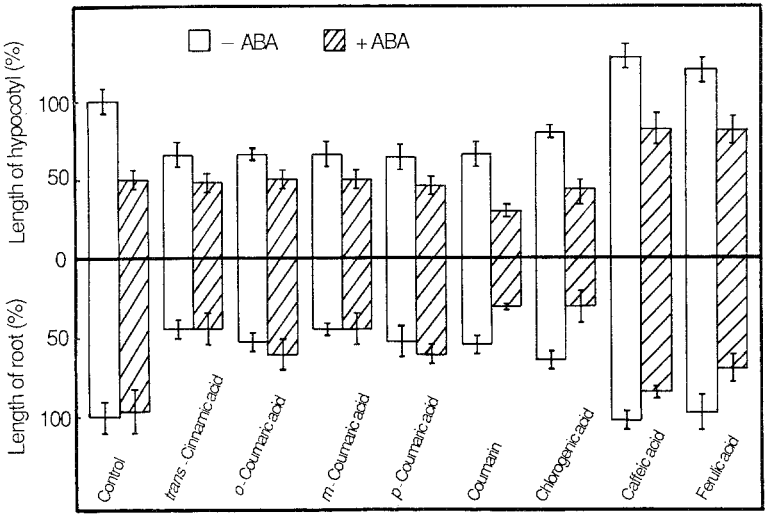

FIG. 5. Interaction of phenolic compounds with abscisic acid on the growth of etiolated seedlings of lettuce. The concentrations used were 10^{-5} M for ABA, 5×10^{-5} M for coumarin, and 5×10^{-4} M for the others.

tration (10^{-5} M) of ABA (Figure 5), but could not reverse the effect of higher concentrations of ABA (data not shown).

trans-Cinnamic and *o*-, *m*-, and *p*-coumaric acids, and coumarin also showed additive inhibition with ABA on lettuce seed germination. Chlorogenic acid increased the inhibition of ABA on seed germination (Table 3). Caffeic and ferulic acids at 10^{-3} M could reverse the inhibition of seed germination produced by a low concentration (10^{-5} M) of ABA (Table 3) but could not reverse the effect of higher concentrations of ABA (data not shown).

DISCUSSION

Comparative studies on the effects and activities of some phenolic compounds showed that *trans*-cinnamic acid and *o*-, *m*-, and *p*-coumaric acids have almost the same effects and activities on seed germination and seedling growth of lettuce. The effective concentrations of these four compounds were over 10^{-4} M or 10^{-3} M in inhibiting seedling growth or seed germination, respectively. These results are consistent with previous work. However, the effects of these

TABLE 3. RELATIONSHIPS OF PHENOLIC COMPOUNDS WITH ABSCISIC ACID (ABA) IN SEED GERMINATION OF LETTUCE[a]

Treatment	Germination (%)
Control	95 ± 6
ABA	34 ± 10
trans-Cinnamic acid	33 ± 6
trans-Cinnamic acid + ABA	0
o-Coumaric acid	48 ± 10
o-Coumaric acid + ABA	0
m-Coumaric acid	52 ± 10
m-Coumaric acid + ABA	0
p-Coumaric acid	58 ± 10
p-Coumaric acid + ABA	0
Coumarin	38 ± 6
Coumarin + ABA	0
Chlorogenic acid	93 ± 6
Chlorogenic acid + ABA	0
Caffeic acid	78 ± 10
Caffeic acid + ABA	88 ± 6
Ferulic acid	90 ± 6
Ferulic acid + ABA	80 ± 10

[a]The concentrations used were 10^{-5} M for ABA, 10^{-4} M for coumarin, 10^{-3} M for caffeic and ferulic acids, and 2.5×10^{-3} M for the others. Data are the means of three replicates ± SE.

phenolics might differ according to the plant species tested. Kuiters (1989) reported that *p*-coumaric acid, etc. had a stimulatory effect at a low concentration (10^{-5} M) and had an inhibitory effect at a high concentration (10^{-3} M or higher) in herbaceous woodland plants. In our experiments, no stimulatory effects of these four phenolic compounds were observed even at low concentrations of 10^{-5} M. Unlike some early work (Hemberg, 1951; Sondheimer and Griffin, 1960; Tomaszewski and Thimann, 1966), chlorogenic acid did not show any growth-promoting effect in our experiments. It only inhibited seedling growth, but not seed germination.

Vendrig and Buffel (1961) proposed that caffeic acid may be a very important growth substance. Previous studies showed that caffeic acid and ferulic acid promoted growth and IAA induced growth in the *Avena* curvature test and in sections of coleoptiles or stems of oat, rice, sunflower, and pea (Hemberg, 1951; Milborrow, 1984; Thimann et al., 1962; Tomaszewski and Thimann, 1966). Our results showed that caffeic and ferulic acids had similar activity on the seedling growth of intact plants. Both of them promoted growth at lower concentrations and inhibited growth at higher concentrations.

Our results on the relationships of these phenolic compounds and abscisic acid in seed germination and seedling growth are consistent with early work, which demonstrated that monohydroxy phenolic compounds inhibit and that di- or polyhydroxy phenolic compounds promote plant growth or auxin-induced growth, although the mechanisms of action are not quite clear (Milborrow, 1984; Sondheimer and Griffin, 1960; Tomaszewski and Thimann, 1966; Zenk and Muller, 1963). Very little information is available about the relationships of phenolic allelochemicals and ABA. Our results showed that a relationship similar to the one existing between phenolics and auxin exists in the case of ABA. That is, monohydroxy phenolics increase, while di- or polyhydroxy phenolics decrease, the action of ABA in both seedling growth and seed germination of lettuce. In the case of lettuce, it is difficult to see whether caffeic acid and ferulic acid could promote seed germination at low concentrations. Whether caffeic acid and ferulic acid only interfered with the action of ABA or they themselves promoted seed germination is not clear.

As Reynolds (1978) reported, coumarin had much higher activity than other phenolics in inhibiting seed germination and seedling growth. Its effective concentrations were lower than 10^{-5} M, similar to those of ABA (data not shown). However, its mode of action may differ from ABA. At low concentration, ABA only inhibited stem elongation, but not root elongation, while coumarin, like other phenolics, inhibited elongation more in roots than in stems. Its relationships with caffeic acid and ferulic acid were also different than with of *trans*-cinnamic acid and *o*-, *m*-, and *p*-coumaric acids. Cornman (1946) reported that coumarin inhibited cell division in onion and lily. Our results also suggested that it may have a different mechanism of action from other phenolic compounds.

In natural ecosystems, allelopathy is affected by environmental factors themselves (Einhellig, 1986), including allelochemicals. Usually, the composition of allelochemicals, derived from various sources (such as root exudates, fallen leaves, and plant residues), is very complex. The allelopathic effects are determined by the interactions of all these allelochemicals, not by any single compound. This study showed that *trans*-cinnamic acid, *o*-, *m*-, *p*-coumaric acids, chlorogenic acid, and coumarin will always have inhibiting effects on seed germination and seedling growth in the case of lettuce as an acceptor plant, regardless of their concentrations. They also exhibit additive inhibition between themselves or with ABA. On the other hand, caffeic acid and ferulic acid may have different effects on plant growth according to their concentrations. At low concentrations, they may promote plant growth or decrease growth inhibition produced by other allelochemicals and ABA. At high concentrations, they may inhibit plant growth or increase growth inhibition by other allelochemicals and ABA. The results on the relationships of these phenolics, among themselves and with ABA, also suggested that they may have regulatory roles on the growth and development of intact plants.

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(*E*4,*E*10)-DODECADIENYL ACETATE: NOVEL SEX PHEROMONE COMPONENT OF TENTIFORM LEAFMINER, *Phyllonorycter mespilella* (HÜBNER) (LEPIDOPTERA: GRACILLARIIDAE)

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Abstract—(*E*4,*E*10)-dodecadienyl acetate (*E*4,*E*10-12:OAc) is a newly discovered sex pheromone component of the tentiform leafminer, *Phyllonorycter mespilella* (Hübner). In apple orchards, traps baited with 1 μg of *E*4,*E*10-12:OAc attracted *P. mespilella* in British Columbia and *P. blancardella* (F.) in Massachusetts and Nova Scotia. The compound was identified in *P. mespilella* by gas chromatographic-electroantennographic analysis (GC-EAD) of pheromone gland extracts, retention index calculations, EAD profiles to *E*3 to *E*10 dodecenyl acetates, and synthesis of candidate pheromone components. Even though *E*4,*E*10-12:OAc was not detected in gland extracts by

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GC-mass spectroscopy, several factors indicate that it is female-produced. Antennal responses to gland extracts coincided with authentic *E*4,*E*10–12:OAc on four GC columns with different retention characteristics. *E*4,*E*10–12:OAc and *E*10–12:OAc, a known female-produced pheromone component, elicited equally strong EAD responses. In field tests, *E*4,*E*10–12:OAc was two to four times more attractive than *E*10–12:OAc. There was no additive or synergistic effect between the two components.

Key Words—Lepidoptera, Gracillariidae, *Phyllonorycter blancardella*, *P. mespilella*, sex pheromone, sex attractant, (*E*10)-dodecenyl acetate, (*E*4,*E*10)-dodecadienyl acetate.

INTRODUCTION

Tentiform leafminers (TLMs), *Phyllonorycter* spp., have become serious pests in commercial apple orchards in North America (Jones, 1991). High populations have been associated with reduced fruit size, premature fruit ripening, and reduced fruit set the following year (Reissig et al., 1982). Insecticides such as pyrethroids and carbamates can be used to control organophosphate-resistant TLMs (Pree et al., 1986), but they are detrimental to integrated management of mites.

Pheromone-based mating disruption is an alternative management strategy for many lepidopteran pests. Mating disruption is used to control several species including codling moth, *Cydia pomonella* L. (Charmillot, 1990); pink bollworm, *Pectinophora gossipiella* (Saunders) (Baker et al., 1990); Oriental fruit moth, *Grapholita molesta* (Busck) (Rice and Kirsch, 1990); and grape berry moth, *Endopiza viteana* Clemens (Dennehy et al., 1990), but it has not yet been evaluated for TLMs.

(*E*10)-Dodecenyl acetate is the only semiochemical known to attract the economically important spotted tentiform leafminer, *Phyllonorycter* (*Lithocolletis*) *blancardella* (F.) (Roelofs et al., 1977) in eastern North America, and *P. mespilella* (Hübner) [taxonomic description by Wagner and Landry (in preparation)] in the Okanagan Valley of British Columbia (G.J.R. Judd, unpublished). *P. mespilella*, formerly considered to be the western tentiform leafminer, *P. elmaella* Doganlar & Matuura, apparently immigrated from Washington State and is now established in the Okanagan Valley (Cossentine and Jensen, 1992). A more complete pheromonal blend could be more effective than a single component in mating disruption programs for *Phyllonorycter* spp. We report the identification and field testing of a new sex pheromone component.

METHODS AND MATERIALS

Insects. Apple leaves infested with overwintering pupae of *P. mespilella* were collected in February–March 1991 in the Okanagan Valley. Pupae were removed from the leaves and kept individually in filter paper-lined Petri dishes at 20°C and a photoperiod of 14:10 hr light–dark.

Pheromone Analysis. One to two hours into the photophase, abdominal tips of 2- to 3-day-old virgin female *P. mespilella* were removed and extracted for approximately 5 min in hexane. Other virgin females were confined in a Pyrex glass aeration chamber, and their effluvia trapped on Porapak Q. Every second day, the females were replaced, accumulating female-released volatiles for seven days. Volatiles were desorbed from the Porapak Q trap with pentane. Gland and Porapak Q extracts were subjected to gas chromatographic–electroantennographic analysis (GC-EAD) (Arn et al., 1975) on four fused silica columns (30 m × 0.25 mm) with different retention characteristics: DB-5, DB-210, and DB-23 (J&W Scientific), and SP-1000 (Supelco, Inc.).

Coupled GC-mass spectroscopy (MS) (HP 5985B) in selected ion monitoring mode (SIM) on a DB-210 column with isobutane for chemical ionization (CI) was conducted to confirm the identification of EAD-active components in female gland extracts. Full-scan CI mass spectra of synthetic candidate compounds were obtained to select diagnostic ions. In sequence, 200 pg of synthetic compounds, hexane, and aliquots of 250 female equivalents (FE) of gland extract were chromatographed in SIM mode, each time scanning for diagnostic ions.

Synthetic monounsaturated (*E*3 to *E*9) dodecenyl acetates were analyzed by GC-EAD to determine their EAD activity relative to (*E*10)-dodecenyl acetate.

Synthesis of Pheromone Components. The dianion obtained from 5-hexyn-1-ol **(1)** and butyllithium in tetrahydrofuran (THF) and hexamethylphosphoramide (HMPA) was coupled with iodomethane to give 5-heptyn-1-ol **(2)** (Figure 1). Reduction of **2** with lithium in liquid ammonia yielded (*E*5)-hepten-1-ol **(3),** which was transferred to 7-bromo-(*E*2)-heptene **(4)** with bromine and triphenylphosphine in acetonitrile (Monson, 1971). Coupling **4** with the dianion obtained from 4-pentyl-1-ol and butyllithium in THF and HMPA gave (*E*10)-dodecen-4-yn-1-ol **(5)** (Henrick, 1977), which was reduced to (*E*4,*E*10)-dodecadiene-

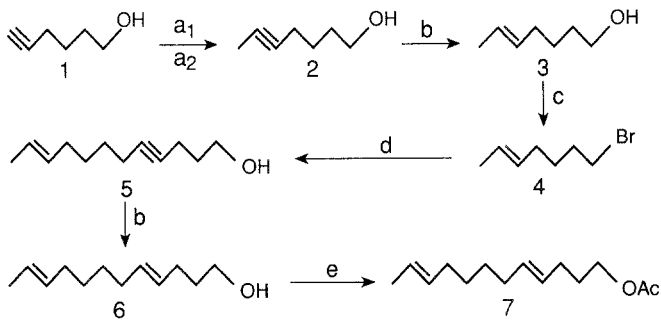

FIG. 1. Scheme for the synthesis of (*E*4,*E*10)-dodecadienyl acetate [a_1: BuLi, THF, HMPA; a_2: CH_3I; b: Li, NH_3 (l); c: PPh_3, Br_2, CH_3CN; d: 4-Pentyn-1-ol, BuLi, THF, HMPA, e: $(AcO)_2O$, Py].

1-ol **(6)** with lithium in liquid ammonia. Acetylation of **6** with acetic anhydride in pyridine gave (*E*4,*E*10)-dodecadien-1-yl acetate **(7).** (*E*10)-Dodecenyl acetate (*E*10–12:OAc) was synthesized as described by Henrick (1977).

Field-tested compounds were more than 99% geometrically and chemically pure. None of the chemical contaminants elicited antennal responses in GC-EAD recordings.

Field Experiments. Experiments were conducted in apple orchards in the Okanagan Valley, British Columbia, in the Annapolis Valley, Nova Scotia and near Amherst, Massachussetts. Wing traps (Phero Tech Inc. Delta, British Columbia, Canada V4G 1E9) were set up in randomized complete blocks with traps and blocks at 15- to 45-m intervals. Traps were suspended 1.5 m above ground, and baited with red rubber septa (Aldrich Chem. Co., Milwaukee, Wisconsin 53233, catalog No. Z12435-4) impregnated with candidate pheromone components in HPLC-grade hexane. Septa were not extracted prior to use and no antioxidants were added to the candidate pheromone components. After 24 hr, traps were removed and captured male *Phyllonorycter* counted. At all three trapping sites, specimens captured in pheromone-baited traps were randomly selected for taxonomic identification (Pottinger and LeRoux, 1971; Landry and Wagner, in preparation).

The first experiment tested synthetic *E*10–12:OAc and *E*4,*E*10–12:OAc alone at 1 μg each and in binary combination at ratios of 1:1, 1:0.1 and 0.1:1 μg/μg, respectively. The remaining experiments (2–4) were conducted only in the Okanagan Valley, British Columbia. The second experiment tested binary combinations of *E*4,*E*10–12:OAc (1 μg) and *E*10–12:OAc at respective ratios of 1:1, 1:0.1, 1:0.01, 1:0.001, and 1:0.0001. The third experiment tested binary combinations of *E*4,*E*10–12:OAc (1 μg) and *E*10–12:OAc at respective ratios of 1:1, 1:10, 1:100, and 1:1000. A final dose–response experiment tested *E*4,*E*10–12:OAc and *E*10–12:OAc alone and at a 1:100 ratio at the following doses: 0.01, 0.1, 1, 10, and 100 μg.

RESULTS

Pheromone Analysis. GC-EAD analysis of Porapak Q and pheromone gland extracts of female *P. mespilella* revealed two significant antennal responses (Figure 2). The first EAD-active compound invariably occurred below the detection threshold of the flame ionization detector, whereas the latter was present at 1–2 ng/FE of gland extract. Identical mass spectroscopic and retention characteristics on four GC columns of female-produced versus synthetic compound identified the major EAD-active component as *E*10–12:OAc. The earlier-eluting EAD-active compound according to its retention index (Figure 2, Table 1) was hypothesized to be *E*4,*E*10–12:OAc. Antennal responses to monounsatur-

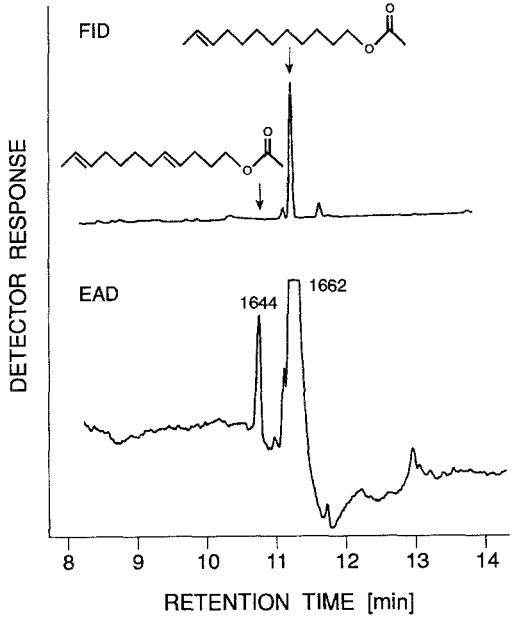

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD) responses to 1 FE of pheromone gland extract of *P. mespilella* from the Okanagan Valley, British Columbia, chromatographed on a DB-210 column (70°C for 1 min, 20°C/min to 140°C, 2°C/min to 220°C).

ated dodecenyl acetates (*E*3 to *E*9), with double-bond position 4 eliciting the strongest response (Figure 3), supported this hypothesis.

GC-MS-SIM of aliquots of 250 FE did not detect *E*4,*E*10–12:OAc. However, retention times of authentic *E*4,*E*10–12:OAc coincided with antennal responses to gland extracts on four GC columns with different retention characteristics. Moreover, 50 pg each of *E*4,*E*10–12:OAc and *E*10–12:OAc elicited equally strong antennal responses.

Field Trapping. Both *E*10–12:OAc and *E*4,*E*10–12:OAc attracted *P. blancardella* in Nova Scotia and Massachusetts, and *P. mespilella* in British Columbia. *E*4,*E*10–12:OAc was two to four times more attractive than *E*10–12:OAc at all three trapping sites (Figure 4). There were no additive or synergistic effects of combining the monoene and diene acetates at any of the ratios tested. In the dose–response test, numbers of captured *P. mespilella* significantly increased with increasing quantities of each of *E*10–12:OAc and *E*4,*E*10–12:OAc or combinations thereof (Figure 5).

TABLE 1. RETENTION INDEX CALCULATIONS AS MEANS TO HYPOTHESIZE DOUBLE-BOND POSITIONS OF STRAIGHT-CHAIN, NONCONJUGATED DIENE ACETATE (*E*4,*E*10-DODECADIENYL ACETATE)[a]

Compound	Column type (30 m × 0.25 mm ID)	Index calculated[b]	Index hypothesized
Dodecenyl acetate	DB-210	1655	
		Δ 17	
(*E*4)—Dodecenyl acetate	DB-210	1638	
(*E*10)—Dodecenyl acetate	DB-210	1662	
(*E*4,*E*10)—Dodecadienyl acetate	DB-210	1644	1662 − 17 = 1645
Dodecenyl acetate	DB-5	1475	
		Δ 12	
(*E*4)—Dodecenyl acetate	DB-5	1463	
(*E*10)—Dodecenyl acetate	DB-5	1479	
(*E*4,*E*10)—Dodecadienyl acetate	DB-5	1467	1479 − 12 = 1467

[a] Calculations with other monounsaturated dodecenyl acetates resulted in retention indices not coinciding with the target index, 1644, of the early EAD response in Figure 2.
[b] Authentic standards were used for calculations of retention indices.

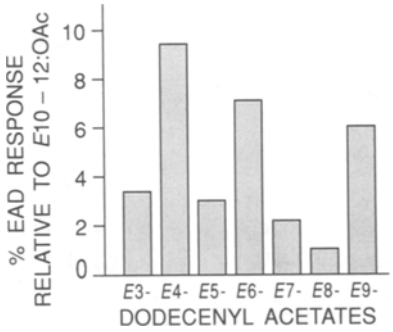

FIG. 3. Mean EAD response profile ($N = 5$) by male *P. mespilella* from the Okanagan Valley, British Columbia, to monounsaturated (*E*3 to *E*9) dodecenyl acetates.

DISCUSSION

Several sex attractants and/or pheromone components have been reported for *Phyllonorycter* spp.. *E*10–12:OAc attracted *P. blancardella* (Roelofs et al., 1977) and *P. mespilellus* (Hrdy et al., 1989). (*Z*10)-Tridecenyl acetate attracted *P. watanabii* (Kumata) (Ando et al., 1977). (*Z*8)-Tetradecenyl acetate and (*Z*10)-tetradecenyl acetate (*Z*10–14:OAc) were attractive to *P. pygmaea* (Kumata) and *P. orientalis* (Kumata), respectively (Ando et al., 1977). *P. kleemanella*

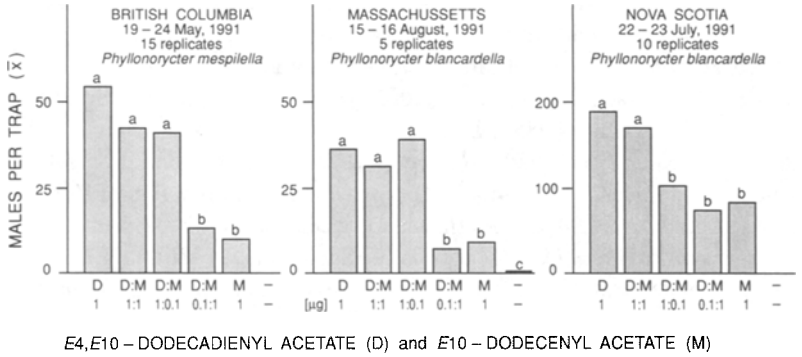

FIG. 4. Attraction of male *Phyllonorycter* spp. to wing traps either unbaited (—) or baited with either (*E*4,*E*10)-dodecadienyl acetate (D), (*E*10)-dodecenyl acetate (M), or binary combinations thereof. For each location, bars superscripted by the same letter are not significantly different ($P < 0.05$). Data from both Massachusetts and British Columbia were transformed by log ($x + 1$) followed by ANOVA and Student-Newman-Keuls' multiple range test. Data from Nova Scotia were transformed by square root ($x + 1$) followed by ANOVA and Tukey's least significant difference test.

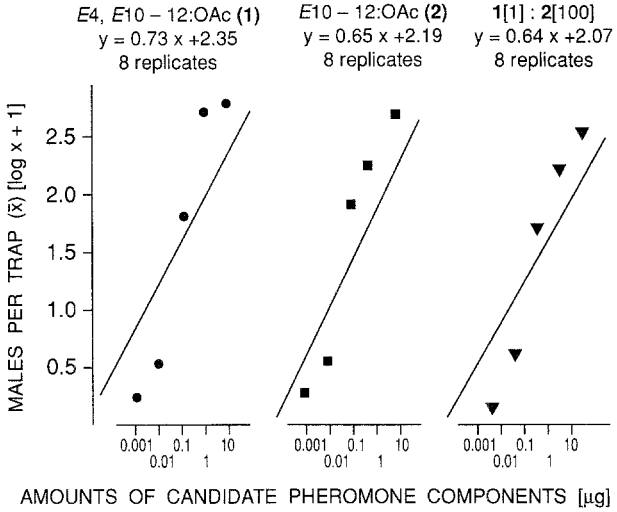

FIG. 5. Dose-dependent attraction of *P. mespilella* to wing traps baited with *E*4,*E*10–12:OAc, *E*10–12:OAc, and a 1:100 binary combination thereof, July 7–14, 1991, Okanagan Valley, British Columbia. Slopes and intercepts of regression lines are not significantly different, ANOVA, $P > 0.05$.

(F.) responded to a mixture of *Z*10- and *E*10–14:OAc (Booij and Voerman, 1984). Traps baited with (*E*8,*E*10)-tetradecadienyl acetate (*E*8,*E*10–14:OAc) captured *P. pulchrum* Kumata in Japan (Ando et al., 1987). *E*8,*E*10–14:OAc and (*Z*8,*Z*10)-tetradecadienal attracted *Phyllonorycter* spp. in North America (Reed and Chrisholm, 1985). *L. corifolliella* (Hübner) was captured in traps baited with (*E*4,*Z*7)-tridecadienyl acetate (Voerman and Herrebont, 1978) or (*E*4)-dodecenyl acetate (Voerman, 1991). A binary combination of (*Z*10)-tetradecenyl acetate and (*E*4,*Z*10)-tetradecenyl acetate has been reported as a sex pheromone of *P. ringoniella* (Matsumura) (Sugie et al., 1986). (*E*4,*E*10)-Dodecadienyl acetate is thus a new sex pheromone component of *P. mespilella* and is also a novel sex pheromone component in the Lepidoptera.

Although *E*4,*E*10–12:OAc was not detected in pheromone gland extracts by GC-MS-SIM, four factors provide evidence that it is a female-produced sex pheromone component. Antennal responses to gland extracts coincided with authentic *E*4,*E*10–12:OAc on four columns with different retention characteristics. Equal amounts of *E*10–12:OAc and *E*4,*E*10–12:OAc elicited equally strong antennal responses by male *P. mespilella*, whereas *Z*4,*E*10–12:OAc was hardly EAD active. *E*4,*E*10–12:OAc was detected by GC-EAD in the effluvia of calling females, indicating that the diene acetate is a female-released pheromone component rather than a pheromone precursor or degradation product present in gland extracts. In field experiments, *E*4,*E*10–12:OAc was more attractive than the previously known *E*10–12:OAc. Even though structurally related compounds may mimic behavioral activity of a sex pheromone (Sarmiento et al., 1972; Millar et al., 1987), nonpheromone structures are not known to exceed attraction to the female-produced pheromone.

Lack of synergism between *E*10–12:OAc and *E*4,*E*10–12:OAc at any of the ratios tested (Figure 4) is unusual. It contrasts with the finding that *P. ringioniella* uses a two-component pheromone blend comprised of (*Z*10)-tetradecenyl acetate and (*E*4,*Z*10)-tetradecenyl acetate (Sugie et al., 1986). However, neither of the two components of *P. ringioniella* was field tested individually, and attraction to the two-component blend could have been caused by either compound alone. Furthermore, field-trapping experiments evaluate only attraction to a pheromone lure but do not determine additional and/or different functional roles of candidate pheromone components. Even though *E*4,*E*10–12:OAc does not appear to enhance attraction to *E*10–12:OAc, the presence of both components may be required during courtship or mating behavior.

The similar rates of capture of moths in traps baited with various pheromone components in the dose–response test (Figure 5) is not surprising. This experiment was conducted at extremely high moth density, during the second and third generation of *P. mespilella* in the Okanagan Valley. Similar rates of trap catches at outbreak population levels have also been experienced with the eastern

spruce budworm, *Choristoneura fumiferana* (Clemens) (C.J. Sanders, Forest Management Institute, Sault Ste. Marie, Ontario; personal communication), the western spruce budworm, *C. occidentalis* (Freeman), (R.F. Shepherd, Pacific Forestry Centre, Victoria, British Columbia; personal communication), the blackheaded fireworm, *Rhopobota naevana* (Hübner) (K.N. Slessor, unpublished), and the western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst) (Krannitz, 1992). Future research on the potential of *E*4,*E*10–12 : OAc for pheromone-based mating disruption of *Phyllonorycter* spp. will therefore be conducted where and when moth populations are low.

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JUST HOW INSOLUBLE ARE MONOTERPENES?

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Abstract—Prior generalizations about the ecological roles of monoterpenes may be misleading if based on the presumed insolubility of monoterpenes in water. We determined the aqueous solubility of 31 biologically active monoterpenes by gas chromatography. While hydrocarbons were of low solubility (<35 ppm), oxygenated monoterpenes exhibited solubilities one or two orders of magnitude higher, with ranges of 155–6990 ppm for ketones and of 183–1360 ppm for alcohols. Many monoterpenes are phytotoxic in concentrations under 100 ppm, well below the saturated aqueous concentrations of oxygenated monoterpenes. Therefore, even dilute, unsaturated solutions of monoterpenes, occurring naturally in plant tissues and soil solutions, may act as potent biological inhibitors.

Key Words—Allelopathy, monoterpenes, *Calamintha ashei*, *Conradina canescens*, ursolic acid, borneol, camphor, juglone, solubility.

INTRODUCTION

Monoterpenes operate as chemical defenses against herbivores (Eisner, 1964) and disease (W.H. Muller, 1965), fragrances attractive to pollinators (Harborne, 1988), and phytotoxins inhibitory to other plants (Muller et al., 1964; Muller and Chou, 1972; Gant and Clebsch, 1975). Chemically, the molecular skeletons of monoterpenes possess 10 carbon atoms derived from two C_5 isoprene units. They exist as hydrocarbons or as oxygenated moieties with aldehyde, alcohol, ketone, ester, and ether functionalities. Furthermore, they may be acyclic,

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monocyclic, bicyclic or tricyclic in structure (Dev, 1982). Owing to the low molecular weight and nonpolar character of these compounds, the group as a whole has been classified as volatile and assumed to have negligible water solubility compared to other classes of organic compounds. For example, Harborne (1984) claims "terpenoids are generally lipid-soluble" whereas "phenolic substances tend to be water-soluble." Despite two older (Rhode, 1922; Seidell, 1940–41) and one modern (Smyrl and LeMaguer, 1980) citation to the contrary, standard chemical references concur on the aqueous insolubility of monoterpenes: the Merck Index (Budavari, 1989) lists borneol, carvone, and pulegone as "almost insoluble" or "practically insoluble" in water, and the CRC Handbook of Chemistry and Physics (Weast, 1976, 1989) registers camphor, geraniol, and pulegone as insoluble, and borneol, carvone, and menthone as slightly soluble in 1976, but none of these compounds are listed as water soluble in 1989.

In studies of allelopathy in the pine forests of the southeastern coastal plain of the United States, we found evidence for the allelopathic effects of two shrubs, *Calamintha ashei* and *Conradina canescens* (Labiatae), which contain monoterpenes as active constituents (Richardson and Williamson, 1988; Tanrisever et al., 1987, 1988; Macias et al., 1989; Williamson et al., 1989). However, the only likely mechanism of allelochemical release was aqueous leaching of foliage and litter—an apparent incongruity with the reputed insolubility of monoterpenes. Because of the alleged insolubility of these compounds, we have proposed that natural detergents such as the triterpene ursolic acid, which is also present in these two mints, may speed solubilization and increase the solubilities of terpenoid compounds. Our previous experimental studies unambiguously established the formation of micelles in water leachates of these plants (Fischer et al., 1988; Tanrisever et al., 1988), although increases in solubilization rates and solubilities were not determined.

Therefore, as part of our ongoing investigations of allelopathic mechanisms in the Florida scrub, we determined the water solubilities of a variety of monoterpenes, eight hydrocarbons and 23 with oxygenated moieties, and compared them to known phenolic phytotoxins (Davis, 1928; Blum and Dalton, 1985; Tanrisever et al., 1987; Harborne, 1988) that are widely presumed to be water-soluble: juglone, ferulic acid, and hydrocinnamic acid.

METHODS AND MATERIALS

Preparation of Standards. The three *Calamintha ashei* natural products (calaminthone, desacetylcalaminthone, and epievodone) were previously isolated and purified in our laboratories. Other compounds were obtained from

commercial sources (Aldrich Chemical Co., Milwaukee, Wisconsin; Eastman Kodak Co., Rochester, New York; Fluka Chemical Corp., Ronkonkoma, New York; Sigma Chemical Co., St. Louis, Missouri; and SCM Glidden Organics, Jacksonville, Florida). Purities of all monoterpenes were determined by gas chromatography. A 10,000 ppm stock solution of each compound was prepared in ethanol and serially diluted to 1000, 100, and 10 ppm. Standard solutions were stored under refrigeration in crimp-top vials with Teflon seals.

Preparation and Analysis of Saturated Aqueous Solutions of Monoterpenes and Phenolics. Saturated aqueous solutions were prepared by adding an excess of each compound to 1.5 ml of water in crimp-top vials with Teflon seals and sonicating for 30 min in a water bath (25–30°C). Vials were inverted and stored for three days at ambient temperature to permit phase separation of the excess organic compound from the aqueous solution, with the exception of juglone, which was analyzed immediately before oxidation occurred. Solutions prepared from solids were filtered prior to analysis.

The effect of ursolic acid on monoterpene solubility was investigated by determining solubilities using two procedures. In the first, solubility was determined in a saturated aqueous solution of ursolic acid using the methods detailed above. Saturated solutions of ursolic acid were prepared by sonication for 30 min (2 mg/20 ml). The ursolic acid solution was allowed to stand at room temperature for 24 hr and filtered through a 0.2-μm nylon membrane. The second procedure used water containing 0.5 mg solid ursolic acid/1.5 ml. The ursolic acid–monoterpene emulsions were filtered (0.2 μm) and stored inverted in a fresh vial for 72 hr.

Monoterpene concentrations were quantified based on peak area, corrected for compound purity, in a Hewlett Packard 5890 gas chromatograph equipped with a split/splitless injector system, a flame ionization detector, and a Hewlett Packard 3393A integrator. Linear detector response over the range of 10–10,000 ng was verified for each compound. Solubilities of the phenolics were determined by UV absorbance with a Perkin-Elmer Lambda 2 spectrophotometer.

Bioassay Procedure. Five milliliters of five concentrations (25, 10, 5, 1, and 0 ppm) of aqueous solutions of borneol, camphor, and juglone were added to 480 ml glass jars lined with one sheet of Whatman No. 1 filter paper and containing 25 seeds of native *Rudbeckia hirta* or commercial lettuce *Lactuca sativa*. Assays were conducted in the dark at room temperature (23–25°C), replicated three times, and terminated after three to five days. Seed were considered to be germinated if the radicle protruded at least 1 mm. Germination in each treatment is presented as a percent of germination in the control (0 ppm). Responses were compared to the controls ($P = 0.05$) using the least squares means of the general linear models procedure of the Statistical Analysis System (SAS) programs (SAS Institute, Inc., 1985).

RESULTS AND DISCUSSION

Solubility among the monoterpenes was extremely variable, ranging from a low of < 10 ppm (parts per million by weight) to a high of 6990 ppm. The eight hydrocarbon monoterpenes had low solubilities, all under 35 ppm (Figures 1 and 2). However, monoterpenes containing oxygen in the form of a ketone, alcohol, ether, or aldehyde had solubilities 10–100 times greater than hydrocarbons with comparable skeletons. Alcohols were somewhat more soluble than comparable ketones in the monocyclic skeletons (Figure 1). In bridged bicyclic monoterpenes, the ketones were more soluble than comparable alcohols, perhaps due to ring strain favoring formation of the geminal diols—for example, camphor (550 ppm) versus borneol (274 ppm) and verbenone (6990) versus myrtenol (1010 ppm) (Figure 2).

The solubility of monoterpenes was not enhanced in saturated ursolic acid solutions or in emulsions of ursolic acid with the monoterpenes (data not shown). Because no increase in solubility was observed with a group of 11 monoterpenes (borneol, camphene, (+)-camphor, cineole, *p*-cymene, *d*-limonene, myrcene, α-pinene, β-pinene, sabinene, and α-terpinene), no other determinations were made. When solid ursolic acid was present, the solubility of monoterpenes was, in fact, reduced. The ursolic acid apparently adsorbed the bulk of the added monoterpene, similar to the action of a solid-phase adsorbent. The two ursolic acid procedures were originally chosen because of uncertainty about how ursolic acid might act to increase monoterpene solubility. Lacking a sensitive analytical method for ursolic acid in aqueous solution, there was doubt about whether the small but unquantifiable amount of ursolic acid present in a saturated solution would be sufficient to have an effect. In the natural situation, the leaves of *Calamintha* and *Conradina* contain large quantities of ursolic acid, hence the treatment with 0.5 mg ursolic acid per vial was included for comparison.

Overall, the prior generalization that monoterpenes are insoluble in water is shown by these results to be invalid. As a class, monoterpenes exhibit a range of solubilities comparable to the common phenolics tested here—juglone (52 ppm), ferulic acid (174 ppm), and hydrocinnamic acid (3490 ppm). Since the true difference between monoterpenes and phenolics is the biochemical pathway of origin, the mevalonic acid and shikimic acid pathways, respectively, the reported generalizations about solubility differences may be false. Given that variation in solubility within the monoterpenes and within the phenolics is greater than differences between the classes, the generalizations about their biochemical activities and ecological functions based on putative differences in aqueous solubilities need to be reexamined. For example, one conclusion of Tukey's (1969) classic foliar leaching studies is that "carbon dioxide, ethylene and terpenes" are released as volatiles, while rain and dew leach "mineral nutrients, carbohydrates, amino and organic acids, and growth regulators." Characterizing the

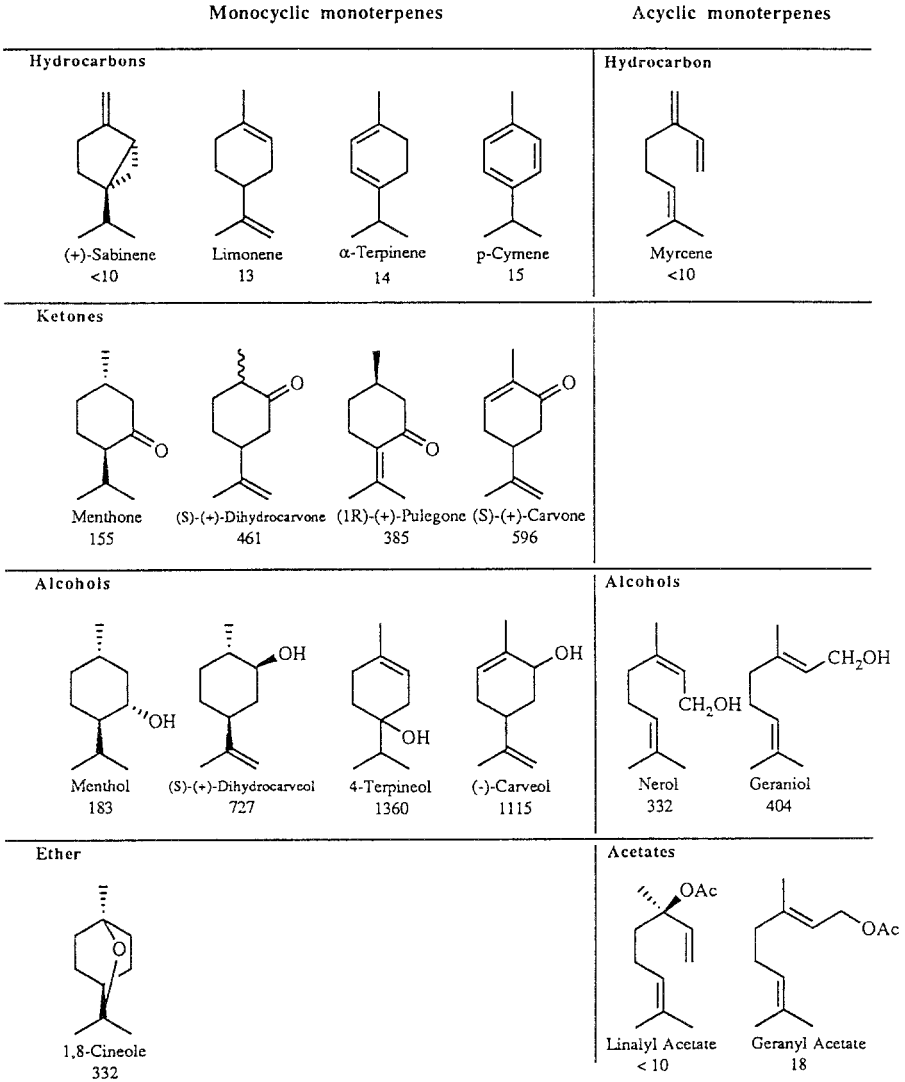

FIG. 1. Monocyclic and acyclic monoterpenes with their aqueous solubilities (ppm).

role of secondary metabolites in litter decomposition, Horner et al. (1988) claimed that "Leaching losses of fairly water soluble components (e.g., most simple phenolics, phenylpropanoids, flavonoids, and tannins) should exceed those of components that are only slightly or negligibly soluble in water (e.g., terpenes and lignin, respectively)." In regard to allelopathy, numerous authors,

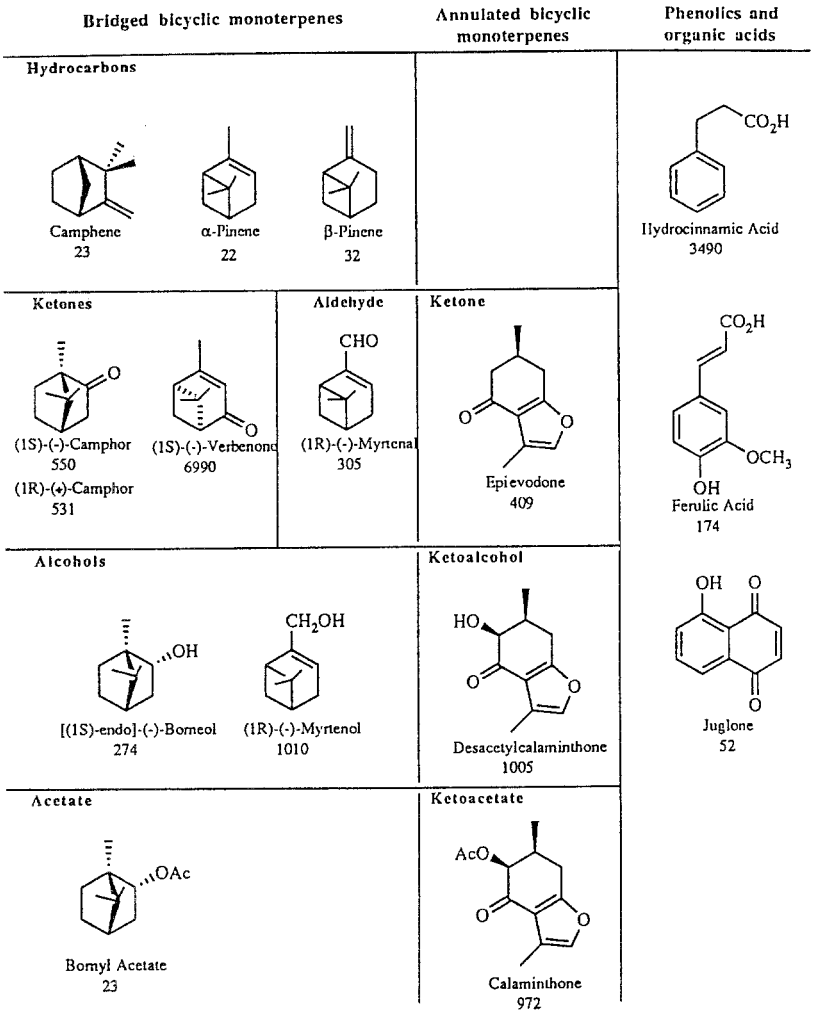

FIG. 2. Bicyclic monoterpenes and phenolic and organic acids with their aqueous solubilities (ppm).

including ourselves, have differentiated the "volatile terpenes" from the "water-soluble" phenolics and aromatic acids (Whittaker, 1971; National Research Council, 1971; Harborne, 1988; Fischer et al., 1989; Williamson et al., 1992)—all apparently based on the pioneering research of C.H. Muller (Muller et al., 1964; C.H. Muller, 1965; McPherson et al., 1971; Muller and Chou, 1972) who found several monoterpenes emitted as volatiles from *Salvia leucophylla*

(Labiatae) and *Artemisia californica* (Asteraceae) and several phenols and organic acids washed from the leaves of *Adenostoma fasciculatum* (Roasaceae) and *Arctostaphylos glandulosa* (Ericaceae).

For monoterpenes, biological activities are as variable as their solubilities, but in many cases compounds are active at apparently low concentrations, i.e., well below their aqueous solubilities (Fischer, 1991). Here, we present the results of bioassays for allelopathy with two monoterpenes, borneol and (+)-camphor, and the phenolic, juglone, the active constituent of *Juglans nigra* (Davis, 1928; Harborne, 1988). Significant ($P < 0.05$) inhibition of germination in both test plants was common at 10 and 25 ppm, less so at 5 ppm, and only rarely at 1 ppm for juglone, borneol, and camphor (Figure 3). At 5 ppm, both borneol and camphor significantly reduced germination of *Rudbeckia*, while juglone had no effect. Thus, in numerous cases, the biological activity of the monoterpenes matched or exceeded that of juglone, the well-known allelopathic agent of black walnut, while the solubility of the two monoterpenes exceeded that of juglone. Like many other monoterpenes (Fischer, 1991), borneol and camphor are active biological inhibitors in concentrations well below their

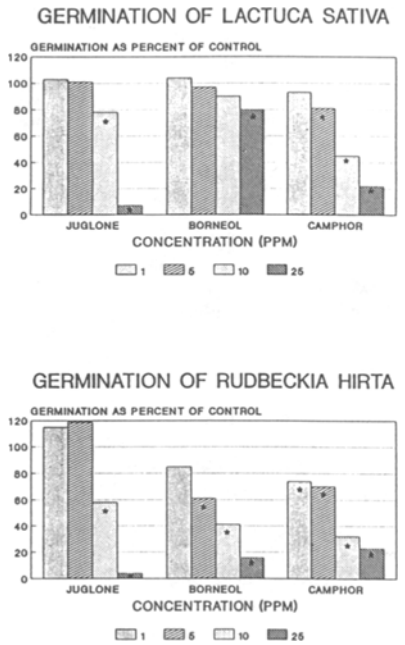

FIG. 3. Germination of *Lactuca sativa* and *Rudbeckia hirta* in 1, 5, 10, and 25 ppm aqueous solutions of juglone, borneol, and camphor, as percentages of the water controls (0 ppm).

aqueous solubilities, 274 and 550 ppm, respectively. Rather than measuring solubility in absolute terms, it is perhaps most appropriate, for biologically active molecules, to consider solubility relative to concentrations needed for biological activity.

Our prior hypothesis of the importance of micelles formed by biological detergents and monoterpenes needs revision on several counts: first, ursolic acid did not increase the water solubilities of monoterpenes; and second, monoterpene solubilities alone are sufficient for biological activity. Effects of ursolic acid on solubilization rate were not investigated in this study. Our preliminary studies (unpublished) using sonication to solubilize the monoterpenes show no effect on solubilization rate. The effect of ursolic acid on the biological activity of monoterpenes was also not investigated in this study. In previous bioassays (Fischer et al., 1988), epievodone (250 ppm) in a saturated ursolic acid solution strongly inhibited *Schizachyrium* germination, while epievodone in aqueous solution at the same concentration was strongly stimulatory. The results of this earlier study suggest that ursolic acid may play a role in facilitating transport of monoterpenes to target seeds or seedlings. Clearly, the ecological significance of the formation of micelles in water leachates of allelopathic shrubs of the Florida scrub community (Fischer et al., 1988; Tanrisever et al., 1988) needs further investigation.

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Review

ROLE OF CHIRALITY IN OLFACTORY-DIRECTED BEHAVIOR: AGGREGATION OF PINE ENGRAVER BEETLES IN THE GENUS *Ips* (COLEOPTERA: SCOLYTIDAE)

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Abstract—There has been a renaissance of interest in the significance of enantiomeric composition in biological systems. Three chiral monoterpene alcohol aggregation pheromone components (ipsenol, ipsdienol, and *cis*-verbenol) commonly isolated from engraver beetles (*Ips* spp.) provide a paradigm for this theme as it relates to olfactory-guided insect behavior. The literature pertaining to this system is reviewed and the effects of the enantiomeric composition of these semiochemicals on the *Ips* spp. community is explored on two trophic levels. Hypotheses generated from the well-studied aggregation pheromone production and response patterns for *I. paraconfusus* Lanier and *I. pini* (Say) are generalized to the North American species in the genus. Despite the progress with *I. paraconfusus* and *I. pini*, substantial deficiencies exist in our understanding of the role of enantiomeric composition in pheromonal/allomonal effects in different subgeneric groups, in the regulation and mechanisms of stereoselective biosynthesis of the monoterpene alcohols, and in the benefits derived by individual insects that produce relatively large proportions of inactive or interruptive enantiomers with attractive enantiomers.

Key Words—Coleoptera, Scolytidae, *Ips*, enantiomeric composition, chirality, aggregation pheromone, pheromone biosynthesis, *Ips pini*, *Ips paraconfusus*, *cis*-verbenol, *cis*-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol, ipsenol,

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2-methyl-6-methylene-7-octen-4-ol, ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol.

SCIENTIFIC SIGNIFICANCE

Ligand–receptor-mediated chemosensation is a fundamental phenomenon that occurs at all levels of biological organization. In the prokaryotes, *Escherichia coli* and *Salmonella typhimurium*, bacterial chemotaxis can be elicited when ligands such as serine, aspartate, or glucose are placed into the medium (Adler, 1976; Koshland, 1981). The mating process of the a- and α-haploid mating cell types of common baker's yeast, *Saccharomyces cerevisiae*, a unicellular eukaryote, involves oligopeptide pheromones. These externally secreted ligands specifically arrest the cells of opposite mating types in the G_1 cell cycle phase so that fusion can occur (Herskowitz and Oshima, 1981; Nasmyth, 1982). Chemosensation likely pervades all internal and external functions of multicellular organisms. Lymphokine ligands secreted by helper T cells of the mammalian immune system coordinate many of the cell-mediated immune responses and, of course, foreign antigens act as eliciting ligands for the humoral antibody response. Neurotransmission, endocrine responses, and developmental and growth responses are all regulated by ligand–receptor complexes (Alberts et al., 1983). Haldane (1955) recognized an evolutionary continuum of chemical communication extending from protozoa to metazoa and realized the significance of chemical signals to communication between (and even within) cells of a metazoan. He noted that "a mass of mutually adherent cells is not an organism unless they can influence one another" and that cells in a metazoan communicate chemically by both transitory (e.g., neurotransmitters) and more permanent (e.g., hormones) signals. Externally, multicellular animals employ chemosensation for olfaction and taste, while conceivably even phenomena such as induced chemical defenses and allelochemical effects, noted in multicellular plants (Whittaker and Feeney, 1971; Whittaker, 1972), as well as plant–microbial molecular signal interactions, are mediated by ligand–receptor complexes (all three topics reviewed in Harborne, 1989).

As dictated by the tertiary structure of proteinaceous receptors, the three-dimensional structure of molecular ligands has an important influence on chemosensation. Subtle variation in three-dimensional ligand structure achieved through constitutional (structural) isomerism and stereoisomerism can elicit remarkably different sensory responses. In a comprehensive review of insect pheromones, Brand et al. (1979) summarized this phenomenon as it relates to stereoisomers [including geometric and optical isomers, (Eliel, 1962)] under the heading "stereobiology." In a milieu of chiral protein receptors constructed of chiral amino acids, perhaps the most subtle difference in elicitors exists between simple enantiomeric ligands that derive their handedness from a single asym-

metric carbon atom. The effect of different enantiomeric ligands is amplified through the formation of diastereomeric complexes with the same chiral receptor. The significance of chirality to the elementary particles of physical matter as well as to the basic components of life systems (Hegstrom and Kondepudi, 1990; Gaffield, 1990; Bonner, 1991) is well illustrated by the enzyme chymotrypsin, which contains 251 separate chiral carbon atoms. This compound could exist as a possible 2^{251} optical isomers, yet is found as only one isomer in biological organisms (Brown, 1987).

In human taste, the chirality of chemical stimuli can determine flavor perception. The umami or savory taste is based on L-α-amino acids such as monosodium glutamate and monosodium aspartate (Yamaguchi, 1979), and sweetness or bitterness of amino acids or peptides is often a function of their chirality (Belitz et al., 1979). In perhaps the earliest recognition of receptor sensitivity to enantiomers, Pasteur (in Piutti, 1886) suggested *nervous tissue dissymmetry* as the source of the sweet taste for (+)-asparagine and of the insipid taste for (−)-asparagine, resolved and tested by Piutti (1886). Nowhere is the enantiomeric sensitivity of human taste better demonstrated than in the sugars in which chirality is reflected in the common nomenclature (e.g., *dex*trose, *lev*ulose, *dex*tran, *lev*an).

In human olfaction, the enantiomeric composition of the stimuli can significantly influence the perception of the odor. In 1971, three groups demonstrated that humans discriminate between the odors produced by the enantiomers of carvone (Friedman and Miller, 1971; Leiterig et al., 1971; Russell and Hills, 1971). The (+)-enantiomer emits an odor reminiscent of caraway, while the (−)-enantiomer represents spearmint. Similar discrimination occurs with limonene [(+)-orange; (−)-lemon; Hegstrom and Kondepudi, 1990]. While odor perception can elicit behavioral responses and mammalian pheromones with chiral carbon atoms have been described [e.g., 5α-androst-16-en-3α-ol and its ketone in the boar, *Sus scrofa* (reviewed in Harborne, 1988)], behavioral studies to reveal possible enantiomeric effects have not been reported. Albone et al. (1986) address the problems associated with semiochemical behavioral assays involving animals of higher cognitive ability.

The relationship between chirality and behavior-modifying chemicals perceived through olfaction has been most thoroughly investigated in insects (reviewed in Silverstein, 1979, 1985, 1988; Mori, 1984, 1989). Yet, despite a recent abundance of studies of the impact of pheromone chirality on insect behavior, the significance of pheromone chirality to behavioral activity was not immediately recognized in insect pheromone biology. The first four insect pheromone structures to be reported had isomeric configurations. The sex pheromone of the silkworm moth, *Bombyx mori* L. (*trans*-10, *cis*-12-hexadecadien-1-ol; Butenandt et al., 1962), the incorrectly identified sex pheromone of the gypsy moth, *Lymantria dispar* (L.) (*d*-10-acetoxy-*cis*-7-hexadecen-1-ol; Jacobson et

al., 1960), and the honeybee, *Apis mellifera* L., queen mandibular gland pheromone, (9-hydroxy-*trans*-2-decenoic acid; Butler et al., 1964) were all reported as specific geometric isomers (diastereomers). The incorrectly identified sex pheromone of the American cockroach, *Periplaneta americana* L. (2,2-dimethyl-3-isopropylidenecyclopropyl propionate; Jacobson et al., 1963) did not exhibit geometric isomerism. However, both the latter and the *L. dispar* sex pheromone contained chiral carbon atoms (C-1 and C-10, respectively), a fact that was noted in the original reports by attempts to include the optical rotations among the chemical data reported to characterize the natural products. The *P. americana* pheromone showed no optical rotation upon measurement (Jacobson et al., 1963), while an optical rotation of $[\alpha]_D^{23} = +7.9°$ ($c = 1.0$, $CHCl_3$) was reported for the pheromone of *L. dispar* (Jacobson et al., 1960). The latter authors implied that in this system chirality was not important to behavioral activity by offering evidence that both the naturally occurring *d* form and the synthetic *d/l* form elicited the same level of activity from male *L. dispar* in the field. Neither the *P. americana* nor the *L. dispar* pheromone structure was correctly isolated and identified until the 1970s. Ironically, however, the authentic sex pheromones of *P. americana* [(1*Z*,5*E*)-1,10(14)-diepoxy-4(15),5-germacradien-9-one (periplanone-B) (Persoons et al., 1979) and periplanone-A (Persoons et al., 1982)] and of *L. dispar* [*cis*-7,8-epoxy-2-methyloctadecane (disparlure) (Bierl et al., 1970)] are also chiral structures. The absolute configuration of disparlure isolated directly from *L. dispar* has never been reported, but is assumed through studies of the activity of synthesized material [(7*R*,8*S*)-(+); Iwaki et al., 1974; Yamada et al., 1976; Vité et al., 1976; Cardé et al., 1977]. Similarly, the absolute configuration of periplanone-B isolated from *P. americana* has not been determined, but is also assumed through studies of the activity of the synthesized material [(1*R*,2*R*,5*E*,7*S*,10*R*), Persoons et al., 1979; Adams et al., 1979; Persoons et al., 1982]. The structure of periplanone-A remains controversial (Okada et al., 1990, 1991). Recent studies of sex pheromones of chafer beetles (Coleoptera: Scarabaeidae) by Leal and colleagues, employing newly developed chiral GC columns, elegantly demonstrate the determination of the enantiomeric composition of naturally derived pheromone compounds (Leal, 1991; Leal et al., 1992).

During the isolation and identification of the first coleopteran pheromone from the California five-spined ips, *Ips paraconfusus* Lanier, formerly *I. confusus* (LeConte), the chirality of each component in the ternary blend of monoterpene alcohols (4*S*)-(−)-ipsenol (2-methyl-6-methylene-7-octen-4-ol) **(2)**, (4*S*)-(+)-ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) **(3)**, and (1*S*,2*S*)-(+)-*cis*-verbenol (*cis*-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol) **(5)** (Figure 1) was acknowledged by reporting the optical rotations (Silverstein et al.,

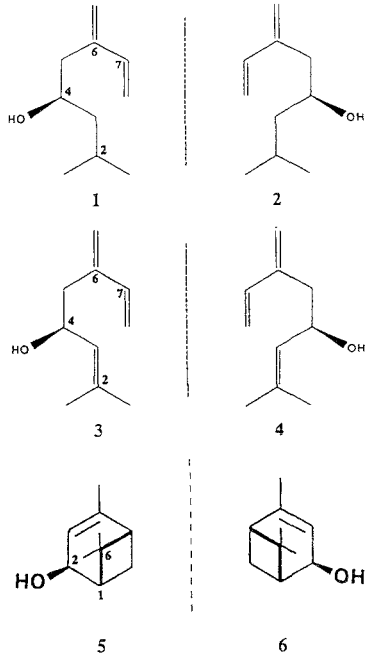

FIG. 1. The enantiomers of monoterpene alcohol aggregation pheromone components isolated from *Ips paraconfusus* Lanier: ipsenol [(4*R*)-(+)-2-methyl-6-methylene-7-octen-4-ol **(1)** and (4*S*)-(−)-2-methyl-6-methylene-7-octen-4-ol **(2)**]; ipsdienol [(4*S*)-(+)-2-methyl-6-methylene-2,7-octadien-4-ol **(3)** and (4*R*)-(−)-2-methyl-6-methylene-2,7-octadien-4-ol **(4)**]; and *cis*-verbenol [(1*S*,2*S*)-(+)-*cis*-4,6,6-trimethyl bicyclo[3.1.1]hept-3-en-2-ol **(5)** and (1*R*,2*R*)-(−)-*cis*-4,6,6-trimethyl bicyclo [3.1.1]hept-3-en-2-ol **(6)**]. The optical rotation of **5** is dextrorotatory (+) in acetone or methanol, but levorotatory (−) in chloroform. The optical rotation of **6** is levorotatory (−) in acetone or methanol, but dextrorotatory (+) in chloroform (Mori et al., 1976).

1966a,b).[2] Riley et al. (1974) reported an early example of the influence of chirality on alarm behavior in the leaf-cutting ant *Atta texana* (Buckley). Pioneering studies by Borden et al. (1976), Wood et al. (1976), and Tumlinson et al. (1977) each demonstrated the same behavioral outcome of insect aggregation, but in each case the response was elicited by an "active" enantiomer

[2]The optical rotation of (1*S*,2*S*)-*cis*-verbenol is dextrorotatory (+) in acetone or methanol, but levorotatory (−) in chloroform (Mori et al., 1976). See also legend of Figure 1. Throughout this paper, the author associates the (+) (i.e., acetone or methanol) rotation with the (1*S*,2*S*) configuration of *cis*-verbenol because the rotation of the original isolate was reported from acetone ($[\alpha]_D^{21} = +4°$, $c = 0.1$, CH_3COCH_3).

and an antipode with different relative properties. With the ambrosia beetle, *Gnathotrichus sulcatus* (LeConte), Borden et al. (1976) provided an example where the male emits both enantiomers and together they yield the maximal aggregation (synergism). With the western pine beetle, *Dendroctonus brevicomis* LeConte, Stewart et al. (1977) found that the female and male appear to release predominantly one enantiomer of each of two compounds. Wood et al. (1976) concluded that, other than diluting the naturally occurring enantiomer, the presence of the opposite enantiomer had no effect on aggregation. Finally, in the case of the Japanese beetle, *Popillia japonica* Newman, Tumlinson et al. (1977) demonstrated that the female appears to release predominantly one enantiomer, but the presence of the antipode dramatically reduces aggregation of responding males (interruption[3]). In the latter case it is not clear whether the insect produces any of the interruptive opposite enantiomer.

To summarize the possible behavioral effects of chiral semiochemicals on insects, Silverstein (1979) constructed nine production–response categories that include all possible production–response outcomes in a simple, continuous system of one enantiomer and its antipode. The model was designed around the communication between an individual producing insect and an individual responding insect without regard to possible underlying genetic variation in pheromone production and response in populations (Löfstedt, 1990; Miller, 1990; Teale, 1990). Also implicit in this model is the assumption that if an insect produces both enantiomers in a given ratio, another member of its species will respond optimally to that enantiomeric blend. The foresight of Silverstein in considering these issues in insects is evident in the current concerns in the pharmaceutical industry regarding the pharmacological activity of optically active drugs (Borman, 1990) and in the emergence of the nascent chirotechnology industry (Stinson, 1992; Nugent et al., 1993). Gaffield (1990) describes the status quo in biological chemistry as a "general awakening" to the significance of absolute stereochemistry in the subdisciplines of pharmacology, agrochemistry, and natural products chemistry. This increased awareness has been reflected in a debate between September 1990 and the present over stereochemical terminology in more than a dozen letters to the editor of *Chemical and Engineering*

[3] *Interruption* of mating or aggregation is the statistically significant reduction in response at traps baited with attractants or attractant-producing substrates due to the effect of interruptant compounds or natural substrates (Wood, 1977). Other authors (Birch, 1978; Borden, 1985; Byers, 1989) use the term *inhibition* to describe the same phenomenon. In the parlance of chemical ecology, the source of the chemical interruptant can be intraspecific (pheromonal) or interspecific (allomonal) (Nordlund and Lewis, 1976; Nordlund, 1981). Borden (1985) terms pheromonal interruptants as *antiaggregation pheromones* (epideictic or spacing pheromones) and allomonal interruptants *as territorial allomones*. Wood (1977) argues that the term *interruptant* is appropriate from a behavioral standpoint because it indicates that the behavioral and physiological mechanisms underpinning the reduced response are not fully understood; the terms disruption, inhibition, confusion, and antiattraction indicate a deeper level of mechanistic understanding. The molecular equivalents of attractants and inhibitors are agonists and antagonists.

News. The debate has apparently culminated with the informal acceptance of the new terms enantiopure, enantioenriched, and nonracemic into the lexicon of the stereochemist (Halevi, 1992).

A phenomenon similar to the Japanese beetle response was demonstrated by Birch et al. (1980) with western populations of the pine engraver beetle, *Ips pini* (Say), and the enantiomers of its aggregation pheromone, ipsdienol. Ethanol solutions of (4*R*)-(−)-ipsdienol **(4)** were attractive in the lab and field, while solutions of (4*S*)-(+)-ipsdienol were interruptive. In this system, however, progress in techniques of analytical and preparative enantiomeric resolution of ipsdienol (Slessor et al., 1985; Kubo et al., 1986) have made biological experimentation at the limits of the enantiomeric spectrum possible. Mustaparta et al. (1980, 1985) have augmented the behavioral work on *I. pini* with electrophysiological studies on the antennal club. In several populations of *I. pini* they have identified cells that appear to be specific for each enantiomer of ipsdienol. Both cell types respond to semi-enantiopure solutions of each enantiomer of ipsdienol, but each type exhibits a higher response at a lower stimulant dose to the preferred enantiomer. Through studies with semi-enantiopure ipsdienol on antennal preparations from western *I. pini*, Mustaparta et al. (1980) also demonstrated that the antagonistic action of (+)-ipsdienol does not occur at the receptor level. Rather, it is thought to occur during the integration process in the central nervous system.

The enantiomeric composition of insect semiochemicals may also impact higher levels of ecological organization. Birch and colleagues (Birch and Wood, 1975; Birch and Light, 1977; Light and Birch, 1979; Birch et al., 1977, 1980) extended behavioral studies of aggregation to the level of community ecology to show that (−)-ipsdienol produced by *I. pini* interrupts the aggregation of the sympatric and cohabiting species, *I. paraconfusus*, while (+)-ipsdienol and (−)-ipsenol produced by *I. paraconfusus* interrupt the aggregation of *I. pini*. Importantly, the respective enantiomers of these compounds are components of the aggregation pheromone for the producing species. In essence the compounds are simultaneously functioning as a stereospecific chemical interruptant or chemical "no trespassing" sign (allomone) as well as a conspecific attractant (pheromone) in the competitive colonization of pine phloem tissue by these two cohabiting species (Borden, 1985).

In addition to *I. pini* and *I. paraconfusus*, the genus *Ips* DeGeer contains 25 other species in North America (S.L. Wood, 1982; Lanier, 1987; Lanier et al., 1991). The genus was originally divided into 11 subgeneric groups (Hopping, 1963; Lanier and Cameron, 1969). Currently, nine subgeneric groups are recognized (S.L. Wood, 1982). Many *Ips* spp. have sympatric distributions and can colonize the same host species (S.L. Wood, 1982), increasing the probability of competition and attempted interspecific matings. Merrill (1991) placed the

role of pheromone specificity into perspective among pre- and postmating barriers to hybridization between heterospecifics.

Wood (1970) and Lanier and Burkholder (1974) hypothesized that the specificity of aggregation pheromones maintains breeding isolation between sympatric species from different subgeneric groups [e.g., *I. pini* (*pini* group) and *I. paraconfusus* (*grandicollis* group)]. Later, they studied the behavioral interactions among many of the species in these groups in the laboratory and found minimal cross-attraction between species in different groups (Lanier and Wood, 1975). As is the case with *I. pini* and *I. paraconfusus*, the chemical basis for the pheromonal/allomonal effects between species from different subgeneric groups may be determined by the relative enantiomeric compositions of ipsenol and/or ipsdienol produced by the species.

The role of pheromone specificity (and thus the relative enantiomeric compositions of ipsenol and ipsdienol) in maintaining breeding isolation between consubgeneric species cooccurring in narrow zones of sympatry (Lanier and Burkholder, 1974) is less clear. Wood (1970) suggested that (especially) sympatric consubgenerics must prefer their own pheromones (thereby reducing the probability of inviable matings), but Lanier and Wood (1975) demonstrated considerable within-group cross-attraction. Lanier and Burkholder (1974) reasoned that because these species are cross-attractive, breeding isolation breaks down in zones of sympatry and the species are forced into largely allopatric distributions as a result of wasteful matings in the blend zone. In contrast, based on laboratory studies of proximal mating behavior between *I. paraconfusus* and *I. confusus* (LeConte) (both *grandicollis* group), Merrill (1991) suggested that limited heterospecific pairings, although inviable, may still be adaptive for these polygynous *Ips* spp. For example, an egg gallery constructed by a heterospecific female may reserve more space in the phloem for offspring from the conspecific females in the harem. This food tissue might otherwise be usurped during gallery construction by parents of a competing brood or consumed by the larvae of an additional conspecific female of the same brood.

In the context of a two-year study of bidirectional cross-attraction between *I. paraconfusus* and *I. confusus* in two hosts in a zone of sympatry in southern California, Cane et al. (1990) presented evidence that female *I. confusus* preferred their conspecific attractant produced in their own host. However, neither species discriminated well and cross-attraction to the heterospecifics pooled over both hosts exceeded 50% for female *I. paraconfusus* in 1983 and female *I. confusus* in 1985. Cane et al. (1990) proposed that prolonged contact of closely related species results in the evolution of sexual asymmetry in pheromone discrimination followed, ultimately, by the development of mutual interruption mechanisms like those known for more distantly related species such as *I. pini* and *I. paraconfusus* (Birch and colleagues cited above). Thus, similarities in

pheromone production, such as presence, absence, and enantiomeric composition of ipsenol and ipsdienol, may reflect the recent ancestry of consubgenerics.

Partitioning of habitat by olfactory signals can also occur among competing species in different genera. Byers and Wood (1980, 1981a) and Byers (1982) investigated interruption between *I. paraconfusus* and *D. brevicomis*, which cohabit *Pinus ponderosa*$_{\text{Laws.}}$ and *Pinus coulteri*$_{\text{D. Don}}$. They have found that among other compounds, (+)-ipsdienol, produced by males of both species, plays a role in interspecific interruption. Thus, "interspecific pheromone interactions may explain in part the spatial and temporal relationships among bark beetles on the same host tree" (Wood and Bedard, 1977).

Predatory coleoptera such as *Enoclerus lecontei* (Wolcott) (Cleridae) and *Temnochila chlorodia* (Mannerheim) (Trogositidae) will respond to natural bark beetle aggregations and to synthetic pheromones (Wood, 1970). In this capacity, ipsenol and ipsdienol function as kairomones for the predators leading them to a bonanza of prey. Chirality may also play a role in kairomonal activity. Raffa and Klepzig (1989) have recently demonstrated that two predators of eastern populations of *I. pini*, *Thanasimus dubius* (F.) (Cleridae) and *Cylistix cylindrica* (Paykull) (Histeridae) can discriminate between enantiomeric blends of ipsdienol in field studies. However, in studies in the same region, Herms et al. (1991) reported a considerably weaker stereospecific response for *T. dubius*.

Based on the model of Silverstein (1979), one might expect that the optimal response to a chiral aggregation pheromone would be elicited by the blend of enantiomers that reflects the mean enantiomeric composition produced by the population. This is the case for populations of *I. pini* from eastern North America (Lanier et al., 1980; Teale, 1990). They have enantiomeric production–response characteristics that are similar to *G. sulcatus*, i.e., both enantiomers are produced and necessary for maximal response. However, in cases of enantiomeric interruption of aggregation, as illustrated by western *I. pini* (Birch et al., 1980) and *P. japonica* (Tumlinson et al., 1977), it has generally been assumed that the insect does not produce the interruptive isomer. Surprisingly, ipsdienol has been extracted from abdomens of individual male *I. pini* to reveal wide variation in the enantiomeric composition from individuals in western populations (Miller et al., 1989).

The absolute preference of both sexes of western *I. pini* for (−)-ipsdienol (Birch et al., 1980), coupled with the discovery that there are males in the population that contain up to 80% (+)-ipsdienol in their tissues (Miller et al., 1989), suggests that the enantiomeric composition of male-produced ipsdienol is not tightly controlled. Factors that limit tight regulation could include dependence on biosynthesis by endosymbiotic microorganisms (Byers and Wood, 1981b; Conn et al., 1984; Hunt and Borden, 1989 for *I. paraconfusus*), genetic variation, or selection pressures such as predation or interspecific competition. Alternatively, the presence of significant proportions of (+)-ipsdienol in some

individual males could be a methodological artifact. While bark beetle pheromones are known to be associated with hindgut tissue (Byers, 1989) and the original isolates were obtained from extracts of the frass (a mixture of digested and undigested phloem and xylem tissue fragments), compounds collected from the air are the most realistic representation of the stimuli that evoke olfactory behavior (see Silverstein, 1985, p. 124 for discussion of this issue). Compounds extracted from insect tissue close to the metabolic pool may in fact be precursors or by-products that have little bearing on what is released into the air to guide behavior. Thus, chemical or stereochemical analyses based on tissue extraction may not accurately predict behavioral signals. Collection of volatile compounds from individual insects has been frequently employed in studies of Lepidoptera (e.g., Haynes et al., 1984; Schal et al., 1987; Barrer et al., 1987; Du et al., 1987; Haynes and Baker, 1988; Witzgall and Frérot, 1989; Ono et al., 1990; Shani, 1990; Haynes and Hunt; 1990a,b; Hunt et al., 1990; Kou and Chow, 1991), but applied less frequently in the Coleoptera (Ma et al., 1980; Gries et al., 1988, 1990; Birgersson et al., 1988; Birgersson and Bergstrom, 1989; Teale, 1990).

The mechanisms that determine the enantioselective biosynthesis of *Ips* spp. aggregation pheromone components in tissue and, ultimately, their enantiomeric composition in the volatile headspace are poorly understood. Ipsenol and ipsdienol have been shown to accumulate in hindgut (Hughes, 1974), mid- and hindgut (Byers et al., 1979; Byers, 1983), and abdominal (Hendry et al., 1980; Hunt and Borden, 1989) tissues of male *I. paraconfusus* in response to treatment with myrcene. *cis*-Verbenol has been shown to accumulate in hindgut (Renwick et al., 1976), mid- and hindgut (Byers, 1983), and abdominal (Hunt and Borden, 1989) tissues of male *I. paraconfusus* in response to treatments with α-pinene. In addition, *cis*-verbenol was found in hindgut (Renwick et al., 1976) and abdominal (Hunt and Borden, 1989) tissue of α-pinene-treated female *I. paraconfusus*. Production of *cis*-verbenol was demonstrated by Byers (1983) in gut extracts from mature and immature (teneral) adult male and female *I. paraconfusus* exposed to α-pinene vapors. Females and teneral males produced neither ipsenol nor ipsdienol following exposure to myrcene vapors (Byers et al., 1979; Byers, 1983). Vanderwel (1991) confirmed the results of the labeling studies of Hendry et al. (1980) by detecting the presence of deuterated ipsdienol and ipsenol in whole body extracts of *I. paraconfusus* after the mature male insects had been aerated with deuterated myrcene. Similarly, male *I. pini* converted D-myrcene to D-ipsdienol (Vanderwel, 1991). In *I. paraconfusus* the precursor–product relationships are thought to be myrcene to ipsdienol to ipsenol (Fish et al., 1979, 1984; Hendry et al., 1980; Vanderwel, 1991). The ketone derivative of ipsdienol, ipsdienone, appears to be involved in the biosynthesis (Fish et al., 1979, 1984; Vanderwel, 1991), but a role for the ketone derivative

of ipsenol, ipsenone, remains questionable (Fish et al., 1984; Byers and Birgersson, 1990; Vanderwel, 1991).

While precursors besides α-pinene and myrcene may be involved in the biosynthesis of *cis*-verbenol, ipsenol, and ipsdienol by male *I. paraconfusus* (Hendry et al., 1980; Byers and Birgersson, 1990), these two components of pine oleoresin appear to be involved in different possible mechanisms for enantioselective biosynthesis. In the case of α-pinene, there are two enantiomers, and the absolute configuration of this precursor appears to dictate the isomeric composition of the verbenol product. Because Renwick et al. (1976) found that male *I. paraconfusus* treated with (1*S*)-(−)-α-pinene produced (1*S*,2*S*)-(+)-*cis*-verbenol ($[\alpha]_D^{23} = +5.6°$, $c = 0.07$, CH_3OH), while those treated with (1*R*)-(+)-α-pinene produced (1*R*,2*S*)-(+)-*trans*-verbenol ($[\alpha]_D^{23} = +125°$, $c = 0.34$, CH_3OH), they proposed that the host range of *I. paraconfusus* may be restricted to those host species or individuals that contain the proper enantiomeric composition of pheromone precursors. By pooling results from the European congeners, *I. typographus* (L.) and *I. amitinus* Eichh., Klimetzek and Francke (1980) found a correlation between the chirality of α-pinene (determined from the optical rotation) and the *cis*/*trans* ratio of verbenols present in extracts of crushed beetles. In this study they utilized six different synthetic blends of (+)- and (−)-α-pinene as well as eight different oleoresin samples from host and nonhost conifers. α-Pinene was isolated from the latter samples and its rotation was measured to estimate the enantiomeric composition for the experiment. In a later study using male *I. typographus*, Lindström et al. (1989) also found a correlation between the chirality of α-pinene in host phloem tissue and the *cis*/*trans* ratio of verbenols found in their hindguts. However, in this experiment the male *I. typographus* were recovered after they had colonized living specimens of *Picea abies* (L.) Karsten, and the tree specimens provided the source of phloem for the α-pinene analysis. In all of these cases the enantiomeric composition of α-pinene appears to influence the isomeric composition of the verbenols, but not the enantiomeric composition of *cis*-verbenol per se.

Myrcene, unlike α-pinene, is achiral. It is therefore difficult to envision how it can influence the enantiomeric composition of its biosynthetic products, ipsdienol and ipsenol. However, there is clear evidence of enantioselectivity in the biosynthesis of ipsdienol and ipsenol by male *I. paraconfusus*. The predominant enantiomers produced by *I. paraconfusus* are (4*S*)-(+)-ipsdienol and (4*S*)-(−)-ipsenol (Silverstein et al., 1966a; Fish et al., 1984). Vanderwel (1991) determined that D-myrcene is oxidized to predominantly D-(4*S*)-(+)-ipsdienol [94% (+)], while earlier, using unlabeled substrates, Fish et al. (1979) had provided evidence that only (4*R*)-(−)-ipsdienol [95% (−)] is converted to ipsenol (enantiomeric composition undetermined) by *I. paraconfusus*. Thus, the overall conversion of myrcene to (−)-ipsenol and the biosynthetic step from

(−)-ipsdienol to (−)-ipsenol have not been proven unequivocally through coupled labeling and stereochemical analyses.

A possible mechanism for the relatively enantioselective production of ipsdienol by *I. paraconfusus* was suggested by Fish et al. (1984). In a study involving unlabeled precursors and products, these authors isolated a nearly racemic blend of the enantiomers of ipsdienol [64% (−)] and predominantly (−)-ipsenol [93% (−)] from male *I. paraconfusus* abdominal tissue after the beetles had been exposed to the ketone analog of ipsdienol, ipsdienone. They proposed an in vivo asymmetric oxidation–reduction equilibrium whereby only (−)-ipsdienol could be oxidized to ipsdienone, while both enantiomers could be generated by reduction of the latter. Thus, (+)-ipsdienol would accumulate in the tissues, while the titer of the (−)-enantiomer would be depleted by the asymmetric equilibrium and conversion to (−)-ipsenol. However, although Vanderwel (1991) confirmed that an oxidation–reduction equilibrium between ipsdienol and ipsdienone does exist in vivo in *I. paraconfusus*, this equilibrium is apparently not responsible for the predominance of (+)-ipsdienol in *I. paraconfusus*. Rather, the enantiomeric composition of ipsdienol produced by *I. paraconfusus* may be determined solely by selective conversion of (−)-ipsdienol to ipsdienone followed by the stereospecific synthesis of (−)-ipsenol through an ipsenone intermediate (Vanderwel, 1991).

In contrast to the proposed mechanism for enantioselective biosynthesis of ipsdienol in *I. paraconfusus*, labeling studies of males from a western population of *I. pini* indicated that the enantiomeric composition of ipsdienol ultimately emitted by this insect may be regulated by enzymatic conversion of (+)-ipsdienol to (−)-ipsdienol (Vanderwel, 1991). Directional conversion of the enantiomers of ipsdienol and ipsenol to their antipodes may be a general feature in *Ips* spp. pheromone biosynthesis, but the enzymes involved in synthesis and interconversion are entirely unknown.

In insects, as in other organisms, monooxygenases [also called mixed function oxidases (MFOs) or polysubstrate monooxygenases (PSMOs)] are enzyme systems normally associated with the detoxification of xenobiotics (Ahmad and Forgash, 1973; White et al., 1979, 1980; Agosin, 1985; Hodgson, 1985; Ahmad, 1986; Watanabe and Groves, 1992). Several authors (White et al., 1979, 1980; Vanderwel and Oehlschlager, 1987; Hunt and Smirle, 1988) have suggested that PSMOs have been modified from detoxification and dedicated to pheromone biosynthesis in bark beetles. In *Ips* spp. modification of the PSMOs would have to result in pheromone production that is stereospecific, mature adult male-specific, and inducible by juvenile hormone III or its analogs. Stage-specific and age-specific differences in xenobiotic metabolism by PSMOs have been noted for some insects (Agosin, 1985). Generally, larvae contain less activity than adults, but larvae do contain detectable PSMO activity. If pheromone biosynthesis is truly a highly modified detoxification mechanism in *Ips* spp., then

evolutionary traces of it might be present in immatures or females, both of which feed and reside in the same substrate as pheromone-producing males.

It is conceivable that a second enzyme system may function with the PSMOs to allow *Ips* spp. to convert one enantiomer of ipsdienol or ipsenol to its antipode or to enzymatically catalyze racemization. Isomerases are the class of enzymes that catalyze geometric or structural changes within one molecule (International Union of Biochemistry, 1984). This class contains a subclass called racemases and epimerases (Adams, 1972; Glaser, 1972; International Union of Biochemistry, 1984) and these enzymes may play a role in the biosynthesis of *Ips* spp. pheromone components. Like all enzymes, both types of isomerases can generate both product and substrate; i.e., both members of an enantiomeric or diastereomeric pair. Nonetheless, in vivo, one member can be favored by subcellular biochemical equilibria. Thus, through the association of a racemase function with suitable biochemical equilibria, a particular species of *Ips* could exert biosynthetic control over the enantiomeric composition of each pheromone component in the blend, leading to species-specific blends. Although amino acid racemases have been investigated most intensely, particularly with regard to mechanism (Choi et al., 1992; Yamauchi et al., 1992), racemases and epimerases that act on noncarbohydrate, asymmetric carbons with secondary hydroxyl moieties have also been characterized (Dahm et al., 1968; Wainwright et al., 1990; Powers et al., 1991). Further studies of the stereospecific biosynthesis of pheromone components of *Ips* spp. should indicate the extent to which enzymes in the isomerase class are involved.

Aggregation pheromones in bark beetles are thought to have evolved through exploitation of sex pheromones as a host-finding strategy by opportunistic members of the same sex as the colonizing beetle (Alcock, 1982; Schlyter and Birgersson, 1989; Raffa et al., 1993). An additional, much-debated argument for the evolution of aggregation in bark beetles is that in addition to attracting mates for reproduction, an individual needs conspecifics of both sexes to help overcome the resistance of the host under colonization (see Alcock, 1982; Schlyter and Birgersson, 1989; Raffa et al., 1993, for discussions of this issue from various perspectives). Since *Ips* spp. often colonize physiologically weakened or fallen trees or tree parts, the host resistance argument is not as convincing as it is with the tree-killing *Dendroctonus* spp. (Wood, 1972; Wood and Bedard, 1977).

The behavioral effects of the (+)-isomer of ipsdienol in western populations of *I. pini* (Birch et al., 1980) raise questions about its value to the individual in an aggregation. Given equivalent rates of ipsdienol production, a rare individual male western *I. pini* that produces relatively more (+)-ipsdienol [= "(+)" male] than its neighbor [frequently a "(−)" male] may be less likely to attract females for reproduction or male and female conspecifics for assistance in overcoming host resistance. However, if an individual (+) male succeeds in attract-

ing a harem of females to a susceptible host, he may reserve more space under the bark for his offspring by generating more of the interruptant and reducing the probability of luring males to initiate new galleries near to his. Finally, the (+) males may be viewed simply as opportunistic "satellites" or "sneakers" that exploit an aggregation to increase the probability of encountering females (Schlyter and Birgersson, 1989). Whatever the selection pressures are for this variability, the enantiomeric composition of ipsdienol within populations of *I. pini* is likely a heritable phenotypic character (Teale, 1990) that is strongly tied to the survival of the insect. This raises intriguing questions about the evolution and genetics of this system.

ECONOMIC SIGNIFICANCE

Bark beetles in the family Scolytidae are the most destructive pests of sawtimber and pulpwood growing stock in the Northern Hemisphere. Members of the genera *Ips*, *Dendroctonus*, and *Scolytus* are responsible for losses of billions of cubic feet of coniferous standing timber per year (Furniss and Carolin, 1977; Drooz, 1985; Waters, 1985). One characteristic behavior that all species in these three genera have in common is that they mass attack or aggregate as they colonize tree tissue. Aggregation pheromones or exocrine attractants play an indispensible role in the process of colonization. Colonization begins with the exposure of beetles to compounds in the tree tissue that are used as precursors for the chemical signals and ends with the termination of aggregation on the tree (Wood, 1982). The nutritive phloem of a fully colonized tree becomes completely deteriorated through gallery construction and feeding by adult and larval beetles, as well as through invasion by pathogenic and nonpathogenic fungi associated with the beetles.

One promising avenue for the protection of stands of conifers from mortality inflicted by bark beetles is the use of aggregation pheromones as management tools. These compounds can be used to monitor the density of the population or as a mass trapping and/or aggregation interruption tactic (Wood, 1979; Silverstein, 1981; Bakke, 1981; Bedard and Wood, 1981; Borden, 1984; Wood et al., 1985). To fully exploit pheromone-mediated bark beetle aggregation from a management standpoint, a thorough understanding of the origin, expression, and activity of these signals in the target species is important. Also significant is the role that related organisms play in the colonization process, as the semiochemicals that join or partition the organisms may have utility in pest management of the target species. This physiological and ecological understanding could lead to the development of receptor antagonists that resemble the pheromone structurally, but through their action as competitive ligands, interrupt the aggregation of the target species. Alternatively, molecules might be behaviorally

antagonistic but function through more complex neural routes through a different ligand–receptor complex. Whatever their origin or molecular mode of action, such interruptants could be used to protect important individual trees or could be used on a large scale to interrupt aggregation in forested stands.

Aggregation pheromones are being used operationally in a variety of tactics in British Columbia for management of populations of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Borden, 1990), and they merit serious attention for management of all pestiferous bark beetle species. However, they should never be considered as a panacea for the complex problem of bark beetle control. Indeed, resistance to pheromones, first suggested for bark beetles by Lanier et al. (1972), has become an important theoretical consideration for pheromone biologists (Haynes et al., 1984; Haynes and Baker, 1988). Because of the dual nature of many aggregation pheromones as kairomones, deleterious effects on natural enemies resulting from frequent applications of aggregation pheromones are predictable.

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Note

EMETIC VOIDANCE OF STOMACH LINING INDUCED BY MASSIVE BEETLE INGESTION IN A BELUGA WHALE[1]

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In July of 1988, Sea World of Texas, in San Antonio, received a young female beluga whale, *Delphinapterus leucas*. The animal has shown a persistent tendency to ingest foreign material that happens to land in her pool. In the spring of 1989 and 1990, the whale manifested episodes of acute inappetence. Endoscopy revealed oak leaves and insects in her forestomach (a nonsecretory chamber, derived from the esophagus; the first of three stomach compartments) (Green, 1972). She was treated symptomatically and recovery was uneventful. In late 1990, she was moved to a different pool to prevent access to leaves. However, inappetence reoccurred in May 1991. Endoscopy revealed the forestomach to be laden with insects. Two days following the examination, the whale vomited most of the lining of her forestomach, voiding the remainder two days later (Figure 1).

Approximately 0.5 liters of insects were expelled with the gastric lining. Three samples of these insects were sent out for identification. All specimens

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turned out to be beetles of two families, Carabidae and Scarabaeidae. The carabids, of which there were species from six genera present (Table 1), were the most numerous. One species, *Harpalus caliginosus*, predominated by far. The scarabs were of two genera only (*Phyllophaga*, *Cyclocephala*). The frequency distribution of the various beetles in two of the samples was as follows: sample 1 (10 *H. caliginosus*, 4 other carabids, 2 scarabs), sample 2 (13 *H. caliginosus*, 2 other carabids, 2 scarabs).

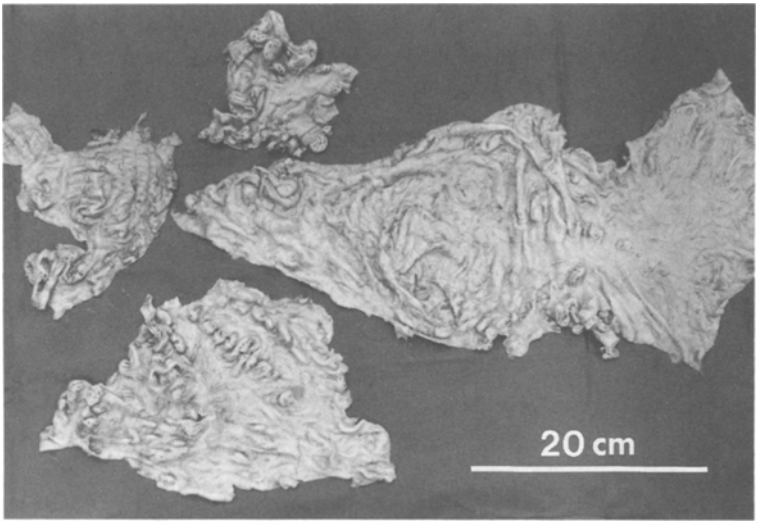

FIG. 1. Inner-surface view of several pieces of forestomach lining vomited by the beluga.

TABLE 1. GENERA OF BEETLE FAMILY CARABIDAE REPRESENTED IN VOMIT OF BELUGA[a]

Genus	Components of defensive secretion
Anisodactylus	formic acid
Amara	methacrylic acid, tiglic acid, *n*-decane, *n*-undecane, *n*-tridecane
Brachinus	1,4-benzoquinones
Calosoma	methacrylic acid, tiglic acid, salicylaldehyde, caproic acid
Harpalus	formic acid
Discoderus	(chemistry unknown)

[a] The chemicals are those identified by various authors (references in Weatherston and Percy, 1978) from species in these genera.

Histology of the gastric lining showed it to consist of stratified squamous epithelium. Flattened cells with indistinct nuclei characterized what was judged to be the luminal surface of the lining; the basal surface, where detachment had presumably occurred, consisted of swollen, sometimes partly detached cells with pycnotic nuclei. There were scattered infrequent bacterial colonies present, but no evidence of inflammation, either histologically or hematologically. The beluga recovered fully over a period of three months. During the spring of 1992, presumably as a consequence of abnormal weather conditions, the usual nocturnal swarms of insects that were attracted to lights near the pool failed to materialize. For the first time since arrival at Sea World of Texas, the beluga remained asymptomatic.

We suggest that the gastric episodes of the beluga were caused by beetle ingestion. Insects are hard-shelled and potentially abrasive and, therefore, capable of inducing mechanical irritation upon ingestion. However, beluga whales appear able to tolerate arthropodan prey, since they are known to include Crustacea in their diet (the forestomach, in fact, is thought to function as a gizzard) (Brodie, 1989; Green, 1972). The most likely possibility is that chemical irritation was involved, induced by the potent defensive secretions characteristically produced by Carabidae. Scarabs, as a rule, lack defensive glands analogous to those which in carabids take the form of a pair of capacious sacs opening on the abdominal tip (Weatherston and Percy, 1978). Judging from what is known of the defensive chemicals of carabid species congeneric with those ingested by the beluga, it is clear that the beluga could have been exposed internally to a rather formidable array of substances (Table 1). Compounds such as formic acid, methacrylic acid, tiglic acid, and benzoquinones are general toxicants capable of inducing severe irritation. Carabids commonly produce these chemicals in quantities of several percent of body mass. While we have no precise count of the number of carabids ingested by the beluga prior to emesis, we judge from the bulk vomited that the quantity was substantial. Gram amounts of the toxicants, particularly formic acid from *H. caliginosus*, a large beetle 2.5 cm in length, must thus have been liberated in the beluga's forestomach. Beetles that were still struggling live in the water when taken by the beluga can be expected to have ejected their secretion as they were being swallowed. Other specimens, taken dead, might have given up their secretion gradually, by leakage from the gland openings.

The oceans, to our knowledge, offer no diversity of small prey that, upon disturbance, discharge concentrated low-molecular-weight general toxicants comparable to those ejected by carabids. Whales may therefore have no protection against enteric exposure to such chemicals. The gastric tissue vomited by the beluga might thus have been chemically damaged and for that reason discarded. However, we cannot rule out the possibility that natural undesirable prey are also voided by emesis in belugas, together perhaps with sloughings of

the lining of the forestomach. Alternatively, it is possible that belugas have an intrinsic aversion to chemically protected prey that occur in their natural environment and that they ordinarily ignore such prey.

A note of caution is in order, especially for institutions housing cetaceans in open pools. Among beetles that occasionally swarm to light and could potentially accumulate in such pools are Dytiscidae (diving beetles), which secrete a diversity of steroids from defensive glands in the thorax (Scrimshaw and Kerfoot, 1987). The compounds include pregnanes such as cortexone, estrone, estradiol, and testosterone, known to have hormonal and anesthetic properties (their primary defensive function is as feeding deterrents to fish) (Gerhart et al., 1991). Single beetles can store astounding quantities of these steroids (for example, an individual *Dytiscus* may contain 0.4 mg cortexone, a quantity equivalent to that obtainable from over 1000 bovine adrenal glands) (Schildknecht et al., 1966). Ingestion of such beetles could obviously be of unforeseen consequence to a cetacean and should be taken into consideration.

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CHIRAL SPECIFICITY IN RESPONSES BY THE BARK BEETLE *Dendroctonus valens* TO HOST KAIROMONES

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Abstract—The attraction of the red turpentine beetle, *Dendroctonus valens*, to the resin volatiles of its host, *Pinus ponderosa*, is elicited by three chiral monoterpenes. In field assays response was greatest to (*S*)-(−)-β-pinene; 92% (*S*)-(−)-α-pinene found in *P ponderosa* resin was not attractive. However, 75% (*R*)-(+)-α-pinene, which occurs in *Pinus lambertiana*, a sympatric host of *D. valens*, was attractive. (*S*)-(−)-α-Pinene interrupted response to (*R*)-(+)-α-pinene. (*S*)-(+)-3-Carene from both hosts was attractive at the (*R*)-(+)-α-pinene level. Three sympatric coniferous nonhosts each have the same attractive monoterpenes but produce less resin. These studies demonstrate the importance of chirality of host compounds in the host finding behavior of this bark beetle.

Key Words—*Dendroctonus valens*, coleoptera, Scolytidae, host selection, chiral, enantiomer, kairomone, *Pinus ponderosa*, α-pinene, β-pinene, monoterpene, olfaction.

INTRODUCTION

Several genera of bark beetles (e.g., *Dendroctonus*, *Ips*, *Scolytus*) aggregate on their hosts, which results in the death of the tree. Through this aggregation behavior these insects are able to reproduce and secure food for their progeny

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(D.L. Wood, 1982). Although considerable progress has been made in understanding pheromone-mediated aggregation of bark beetles (Borden, 1985; D.L. Wood, 1982; Lewis, 1984), little is known about how these insects find their hosts prior to the production of attractant pheromones. Chénier and Philogène (1989) list five scolytids (*Ips grandicollis* Eichoff, *Tomicus minor* Hartig, *Tomicus piniperda* L., *Hylastes ater* Paykull, and *Dryocetes autographus* Ratzeburg) that are attracted to pure conifer monoterpenes. The odor of oleoresin or pure turpentine, a variable mixture of mostly monoterpenes, is attractive to several scolytid species, including *Dendroctonus terebrans* Olivier (Fatzinger, 1985; Payne et al., 1987; Phillips et al., 1988) and *Dendroctonus valens* Leconte (Vité and Gara, 1962). Turpentine or pure monoterpenes increase attraction of seven species of *Dendroctonus* (*D. adjunctus* Blanford, *D. brevicomis* Leconte, *D. ponderosae* Hopkins, *D. rufipennis* Kirby, *D. pseudotsugae* Hopkins, *D. frontalis* Zimmerman, *D. terebrans*) to their respective pheromones (Borden, 1985; Phillips et al., 1990). Many conifer monoterpenes are optically active but chiral specificity to kairomones has not been previously tested in any scolytid species and is not generally known among insect herbivores (E. Bernays, personal communication). Our recent investigation shows that *D. valens* is attracted to two sympatric host species, *Pinus ponderosa* Lawson and *P. lambertiana* Dougl., by three chiral monoterpene hydrocarbons.

Female *D. valens* select host trees in the genus *Pinus* in which to excavate egg galleries. Males and females are attracted to resin exuding from wounded and/or diseased hosts (Vité and Gara, 1962; Owen, 1985; Moeck et al., 1981; Goheen et al., 1985). No pheromone has been identified for *D. valens*, in contrast to several of its congeners (D.L. Wood, 1982; Borden, 1985). *D. valens* rarely mass attacks *P. ponderosa* or *P. lambertiana* and is not an aggressive killer of these species (Smith, 1971). It is often found colonizing the base of trees that are being mass attacked by other scolytids (e.g., *D. brevicomis* and *D. ponderosae* in California) although Owen et al. (in preparation) did not find attraction of *D. valens* to the pheromone of *D. brevicomis*. The objective of our study was to identify which volatile compounds in the resin of ponderosa pine were attractive to *D. valens*, and to explain the mechanism of *D. valens* host selection.

METHODS AND MATERIALS

We analyzed the attraction of *D. valens* to the resin of *P. ponderosa* using resin distillates and individual compounds in field tests using methods described in Birch et al. (1980). Initially, attractive compounds were isolated through steam distillation to produce an attractive volatile fraction. Resin was collected by wounding trees with a V-notch cut into the xylem, which was allowed to

flow into open 100-ml containers overnight. The resin was distilled with steam for 2 hr and collected using a Liebig condenser. Water was removed from the distillate by adding methylene chloride to the mixture and isolating the nonpolar compounds in a separatory funnel. Methylene chloride was removed from the distillate using a rotary evaporator. Responses to distillate and residue were assayed singly and in combination by placing baits on flight traps (Lindgren, 1983) in a complete randomized block design. Eight-funnel Lindgren traps were placed a minimum of 20 m apart in blocks separated by a minimum 100 m. Treatments were placed randomly each day in each block. One-milliliter quantities of test compounds were released from four 1.5-ml, 9-mm ID, plastic Eppendorf centrifuge tubes (West Coast Scientific, Emeryville California) for each compound on each trap. Beetles were collected and sexed each day.

The monoterpene concentrations of the distillate were determined by gas chromatography (GC) on two capillary columns as described below: a 54-m × 0.5-mm-ID OV-17 and a 43-m × 0.5-mm-ID Carbowax 20 M support-coated (SCOT). After diluting the distillate in *n*-pentane, split injection (20:1) was used, with injectors at 220°C. The carrier gas was helium (25 cm/sec flow rate) and both columns were run at 70°C for 5 min, then programmed at 6°C/min to the upper limit (220°C). Detection was by flame ionization (FID); detectors were at 250°C. The monoterpenes were identified by comparison of retention times with those of the authentic substances analyzed under the same conditions. The composition was calculated from the peak areas, normalizing all detected compounds to 100%. The stereochemistry of chiral monoterpenes was determined by analysis on a 30-m × 0.25-mm-ID Cyclodex B chiral column (J&W Scientific, Folsom, California), carrier gas helium (25 cm/sec), temperature 75°C. Whole oleoresin was analyzed for each of five trees (except for *Libocedrus decurrens* Torr., which was from three trees).

The five principal monoterpenes comprising 91% of the steam distillate were tested in two field assays. All five compounds were tested against blank controls in a six-choice test. A subtractive field assay was conducted with five mixtures of the monoterpenes using the concentrations found in the distillate of the initial resin. Each combination of monoterpenes was mixed in a single vial. In the subtractive assay, each compound was successively replaced by a solvent, decane, and tested against blank controls and a complete mixture of the five compounds. The release rate, determined gravimetrically, was approximately 0.8 ml/hr at 23°C for all test compounds but varied in the field with changes in ambient temperature, air pressure, isolation etc.. Chemicals were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Their chemical purity was: (*R*)-(+)-α-pinene 98%, (*S*)-(−)-α-pinene 98%, (*S*)-(+)-3-carene 95%, myrcene 85%, (*R*)-(+)-limonene 97%, (*S*)-(−)-limonene 97%, and (*S*)-(−)-β-pinene 99%. In tests 2 and 3 the natural concentration of enantiomers present in *P. ponderosa* resin was mixed with the solvent decane. (For example, in test

2 the α-pinene treatment was 14.3% (*S*)-(−)-α-pinene, 0.9% (*R*)-(+)-α-pinene, and 84.8% decane. In test 3 the XB treatment had the complete mix of compounds shown for ponderosa pine in the concentrations given in Table 5 (below) with 35.8% decane substituted for (*S*)-(−)-β-pinene).

Because α-pinene was the only monoterpene in which both enantiomers were present in greater than 0.1% of resin volatiles, the attractiveness of each isomer was tested individually. We tested (*R*)-(+)- and (*S*)-(−)-α-pinene (optical purity > 96%) and varying mixtures of the two enantiomers by substituting decane for one of the enantiomers. In addition to ponderosa pine resin, the results of the α-pinene test prompted our analysis of the resin of the only other host species of *D. valens* in our test area, sugar pine (*P. lambertiana*), and of all three coniferous nonhosts: white fir, *Abies concolor* (Gord. & Glend.) Lindl. ex Hildebr.; incense cedar, *Libocedrus decurrens*, and Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco.

A dose–response test was conducted for (*S*)-(−)-β-pinene using release rates of 0.0, 0.7, 7, and 70 ml/day. (*S*)-(−)-β-Pinene was released from open glass tubes attached to flight traps and treatments were assigned each day in a randomized block with a total of 10 replicates for each treatment.

Results from the six field assays were tested with Friedman's two-way analysis for block designs with correction for multiple comparisons setting the experiment-wise type I error rate at the 0.05 level. If significant differences existed among all treatments, then treatments were compared with a Wilcoxon test with α set at 0.05 (SAS Institute, Cary, North Carolina).

RESULTS

The distillate of *P. ponderosa* resin was about 40 times more attractive to *D. valens* than the residue. The residue was not significantly different from the blank control (Table 1). Treatments caught equal numbers of males and females, and the responses of both sexes were pooled for analysis. Chemical analysis of the distillate revealed that the principal monoterpenes of the resin of *P. ponderosa* were: β-pinene [>98% (*S*)-(−)], 3-carene [>99% (*S*)-(+)] and limonene [>98% (*S*)-(−)]. In contrast to the relatively pure enantiomeric composition of these compounds, α-pinene was present in the *P. ponderosa* resin as a mixture with mean 95% (*S*)-(−) and 5% (*R*)-(+) (range of 10 trees sampled: 92%/8%–97%/3%).

Field tests 2 and 3 revealed a strong attraction of both sexes to (*S*)-(−)-β-pinene (Table 2). The dose test shows an increase in response with increasing dose of (*S*)-(−)-β-pinene. Catch at each dose was significantly different from the next highest dose (Table 3). There was a reduced but still significant attraction to (*S*)-(+)-3-carene (Table 2, tests 2 and 3). The catch at the two less

TABLE 1. CATCH OF *D. valens* IN TRAPS BAITED WITH *P. ponderosa* RESIN DISTILLATE OR RESIDUE (BLODGETT FOREST, EL DORADO COUNTY, CALIFORNIA, MAY 1988, TEST 1)[a]

Treatment	Mean catch	SE
Distillate	18.09a	4.42
Residue	0.45b	0.24
Recombination	18.63a	4.67
Control	0.36b	0.36

[a] Distillate ≠ residue; Friedman's test $\alpha = 0.05$, Wilcoxon test $\alpha = 0.05$, means followed by the same letter are not significantly different; 11 replicates/treatment

TABLE 2. CATCH OF *D. valens* IN TRAPS BAITED WITH TEST MONOTERPENES (BLODGETT FOREST, EL DORADO COUNTY, CALIFORNIA, APRIL 1990, TESTS 2 AND 3)[a]

Test 2			Test 3		
Monoterpene treatment	Mean catch/trap/day[b]	SE	Mixture minus one monoterpene	Mean catch/trap/day[b]	SE
(*S*)-(−)-β-Pinene	61.1a	10.44	XB [no (*S*)-(−)-β-pinene]	0.9a	0.27
(*S*)-(+)-3-Carene	11.2b	3.47	XC [no *S*-(+)-3-carene]	14.9b	2.98
Myrcene	2.5c	0.62	XM (no myrcene)	20.8c	3.79
α-Pinene	0.7d	0.27	XA (no α-pinene)	25.0c	4.01
Limonene	0.8d	0.36	XL (no limonene)	13.5bc	2.58
Blank control	0.4d	0.28	Complete mixture	20.2c	2.76
			Blank control	0.1d	0.08

[a] Chiral monoterpenes were tested using the natural blend of enantiomers found in ponderosa pine resin, 16 replicates/treatment in test 2; 22 replicates/treatment in test 3.
[b] Friedman's test $\alpha = 0.05$, Wilcoxon test $\alpha = 0.05$, means followed by the same letter are not significantly different.

abundant monoterpenes in *P. ponderosa* resin, α-pinene and limonene, were not significantly different from the blank control. A third less abundant monoterpene, myrcene, showed statistically significant but minor attraction in our initial test (Table 2, test 2) but was not significantly attractive in the subtractive assay (Table 2, test 3). In both tests 4 and 5 (Table 4) the (*R*)-(+) enantiomer of α-pinene was found to be significantly attractive to *D. valens*. A mixture of decane and the (*R*)-(+) enantiomer was more attractive than a similar mixture

TABLE 3. DOSE RESPONSE OF *D. valens* TO INCREASING DOSE OF (*S*)-(−)-β-PINENE (BLODGETT FOREST, EL DORADO COUNTY CALIFORNIA, JUNE 1990, TEST 6)[a]

Release rate of (*S*)-(−)-β-pinene	Mean catch/trap/day*	SE
70 ml/day	68.7a	26.53
7.0 ml/day	27.5b	15.32
0.7 ml/day	2.2c	0.73
Control	2.2c	1.56

[a]Friedman's test $\alpha = 0.05$, Wilcoxon test $\alpha = 0.05$, means followed by the same letter are not significantly different.

containing the (*R*)-(+) and (*S*)-(−) enantiomers (Table 4, test 4), thus proving that the (*S*)-(−) enantiomer interrupts attraction.

Resin of sugar pine, like ponderosa pine, had a large proportion of the most attractive monoterpene, (*S*)-(−)-β-pinene. However, the largest proportion of its resin volatiles was composed of α-pinene (48%), of which, two thirds was the attractive (*R*)-(+) enantiomer (Table 5). The three nonhosts also each contain large amounts of attractive monoterpenes: (*S*)-(−)-β-pinene in white fir and (*R*)-(+)-α-pinene in all three species. Very little (*S*)-(+)-3-carene was found in these nonhost conifers.

DISCUSSION

The remarkable stereospecificity of *D. valens*' attraction to (*R*)-(+)-α-pinene suggests that enantioselectivity of host compounds may be a significant component of bark beetle host selection behavior. Renwick et al. (1976) found that exposure of *Ips paraconfusus* Lanier to the two optical isomers of α-pinene led to the production of different verbenols, (+)-*trans*-verbenol from (*R*)-(+)-α-pinene and (+-*cis*-verbenol from (*S*)-(−)-α-pinene. If pheromone production by other species of scolytids is similarly influenced by the chirality of host compounds, then stereospecificity in response of beetles to optically active kairomones may be a key element in species-isolating mechanisms and host race (biotype) formation. White et al. (in preparation) studied the antennal response of *D. valens* to resin fractions and to individual components from the resin of *P. ponderosa* using the electroantennogram (EAG) technique (Schneider, 1957; White and Birch, 1987). They concluded that there was a clear difference between the responses to (*R*)-(+)-α-pinene and (*S*)-(−)-α-pinene.

Our field results parallel those of Birch et al. (1980), who found that the response of *Ips pini* Say to its pheromone, (−)-ipsdienol, is interrupted by the

TABLE 4. CATCH OF *D. valens* IN TRAPS BAITED WITH ENANTIOMERS OF α-PINENE [BLODGETT FOREST, EL DORADO COUNTY CALIFORNIA, JUNE 1990 (TEST 4) AND JUNE 1991 (TEST 5)]

Test 4			Test 5		
Treatment	Mean catch/trap/day[a]	SE	Treatment	Mean catch/trap/day[a]	SE
96% (*R*)-(+)	8.8a	2.99	(*S*)-(−)-β-Pinene	27.8a	5.25
75% (*R*)-(+)/25% solvent	6.8a	1.88	(*S*)-(+)-3-Carene	2.6b	0.88
75% (*R*)-(+)/25% (*S*)-(−)	1.5b	0.92	(*R*)-(+)-α-Pinene	2.2b	0.63
Control	0.2b	0.12	(*S*)-(−)-α-Pinene	0.1c	0.08
			Control	0.6c	0.32

[a] Friedman's test $\alpha = 0.05$, Wilcoxon test $\alpha = 0.05$, means followed by the same letter are not significantly different; 9 replicates/treatment in test 4; 20 replicates/treatment in test 5.

TABLE 5. MONOTERPENES PRESENT AS >1% OF RESIN VOLATILES OF 5 TREES FOR EACH SPECIES COLLECTED AT BLODGETT FOREST, EL DORADO COUNTY, CALIFORNIA

	Percent of total volatiles, mean (SD)				
Monoterpene	*Pinus ponderosa*	*Pinus lambertiana*	*Pseudotsuga menziesii*	*Libocedrus decurrens*[a]	*Abies concolor*
(*S*)-(−)-β-Pinene	35.8(4.3)	20.3(9.7)	12.1(3.6)	0.7(0.3)	39.9(16)
(*S*)-(+)-3-Carene	34.4(4.2)	4.7(2.3)	<0.1(0.1)	0(0)	0.2(0.1)
Myrcene	7.0(2.1)	4.5(2.4)	1.1(0.4)	2.0(1.3)	2.3(1.7)
(*S*)-(−)-Limonene	5.5(4.2)	0.1(0.2)	1.8(1.1)	1.0(0.3)	3.7(4.8)
(*R*)-(+)-Limonene	<0.1(0.1)	0.1(0.1)	0.2(0.2)	0.2(0.3)	0.1(0.1)
(*S*)-(−)-α-Pinene	14.3(2.3)	21.1(7.1)	25.6(3.4)	37.3(7.3)	14.3(3.4)
(*R*)-(+)-α-Pinene	0.9(0.4)	47.9(19.2)	55.2(6.5)	55.7(10.8)	30.3(9.9)
(*S*)-(−)-β-Phellandrene	0.6(0.1)	0.3(0.1)	1.9(0.6)	0.9(1.3)	7.9(3.3)

[a] Only three trees sampled.

antipode of this terpene alcohol. Chiral specificity of *Dendroctonus spp.* to their pheromones is summarized by Phillips et al. (1990). *D. terebrans* and *D. brevicomis* respond to (+)-*exo*-brevicomin and (−)-frontalin. Antipodes of these compounds do not interrupt response. *D. ponderosae* produces frontalin and *exo*-brevicomin and responds to both enantiomers of each compound. The response can be aggregation or antiaggregation, depending on release rate (see synopsis in Phillips et al., 1990, p. 256). Although we have shown that (*S*)-(−)-α-pinene interrupts response only to (*R*)-(+)-α-pinene, there is an indica-

tion that it also interrupts attraction to the complete mixture of volatiles from the host. When α-pinene [94% (*S*)-(−)] was removed from the complete mixture, the mean catch increased, although not significantly (Table 2, test 3). The strong attraction to (*R*)-(+)-α-pinene and the interruption of attraction exhibited by its antipode are particularly interesting when the relative proportions of these enantiomers in the resin of *P. lambertiana* and *P. ponderosa* are considered.

The response of the beetles parallels the distribution of the major resin components of their two host species in this forest type, i.e. (*S*)-(−)-β-pinene in *P. ponderosa* and (*R*)-(+)-α-pinene in *P. lambertiana* (Table 5). The second most abundant monoterpene in the volatiles of each host species was also attractive to *D. valens*, i.e. (*S*)-(+)-3-carene in *P. ponderosa* and (*S*)-(−)-β-pinene in *P. lambertiana*. Neither myrcene nor limonene was abundant in either host, and the beetle response to them was weak or not significantly different from controls (Table 2, tests 2 and 3). No discrimination was evident between the two enantiomers of limonene in EAG studies (White et al., in preparation). While attractive compounds may be present among the 49 present in the untested 9% of the volatiles, none of these was greater than 1% of the total volatiles present in the resin (Cool, unpublished).

The broad host and geographic range of *D. valens* (most North American pines; S.L. Wood, 1982) raises intriguing possibilities of host and regional specialization. However, it remains to be established whether or not local adaptation to host kairomones occurs for *D. valens*. EAG, behavioral, and genetic tests with beetles emerging from known sympatric hosts would be required to prove host race formation.

The possibility that beetles may be attracted by widely distributed compounds such as (*S*)-(−)-β-pinene and interrupted by inhibitory compounds such as (*S*)-(−)-α-pinene in the same mixture would be an example of the complex signal discussed by Dethier (1954). Other authors (Renwick and Radke, 1983; Thorsteinson, 1960) have suggested that host selection in generalist herbivores may rely upon deterrent compounds to avoid nonhosts, while specialists search for key stimuli found only in the correct combination in their host. Quantitative differences in resin production might partially explain the lack of colonization of nonhosts. Incense cedar produces very little wound resin. White fir produces and exudes cortical resins, especially in its mid- and upper bole, but very little resin is typically present in the lower bole and root collar where *D. valens* would be expected to feed (Hobson, unpublished). Douglas fir xylem resin is released with breakage, but it is not as abundant as the resin of the two host pines (Hobson, unpublished). In addition to the quantitative differences in host versus nonhost resin production, it seems likely that nonhost status might be assessed by other cues such as olfactory interruptants and/or visual information or, after landing, by gustatory antifeedants and/or tactile cues (Visser, 1986; Lewis, 1984; Elkinton and Wood, 1980). Further research is needed to determine

whether or not compounds that interrupt attraction are present in nonhost conifers that occur in forests with the hosts of *D. valens*.

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ISOLATION OF NEW BROMINATED SESQUITERPENE FEEDING DETERRENTS FROM TROPICAL GREEN ALGA *Neomeris annulata* (DASYCLADACEAE: CHLOROPHYTA)

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Abstract—We investigated the natural products chemistry and feeding deterrent effects of brominated sesquiterpenes produced by Pacific collections of the calcareous tropical green alga *Neomeris annulata*. Assays conducted with whole algae showed that *N. annulata* was not susceptible to grazing by natural populations of herbivorous fishes on Guam. Crude extracts and column chromatography fractions containing the brominated sesquiterpenes deterred feeding by herbivorous fishes at natural concentrations in field assays on Guam. Two major *N. annulata* sesquiterpenes isolated from Guam collections and three related sesquiterpenes previously reported from Bermuda all deterred fish feeding at the high end of their natural concentration ranges, with the exception of one metabolite from Bermuda collections of the alga that differed structurally from the other compounds. The results support our hypothesis that the compounds produced by *Neomeris* function as chemical defenses against herbivores. The alga produces both structural defenses ($CaCO_3$ in the form of aragonite) and secondary metabolites that defend against herbivory by reef fishes.

Key Words—*Neomeris annulata*, Chlorophyta, brominated sesquiterpene, chemical defense, calcified seaweed, herbivory, herbivorous fishes, coral reefs.

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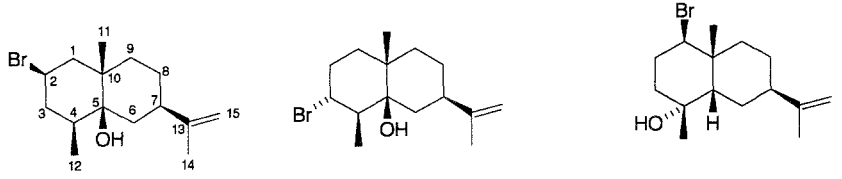
INTRODUCTION

Intense grazing by herbivorous fishes has been shown to directly affect the species composition and abundance of seaweeds in tropical habitats (reviewed by Horn, 1989; Hay, 1991; Choat, 1991). Many tropical seaweeds contain both secondary metabolites and calcium carbonate as defenses against the diverse assemblages of coral reef herbivores (Hay, 1984; Paul and Hay, 1986). Morphological or structural defenses alone do not appear to adequately defend many species of algae in areas of intense grazing. Many reef herbivores, such as parrotfishes, are well adapted for the consumption of calcareous prey, and up to 90% of the gut contents of these fishes may be composed of calcium carbonate (Hiatt and Strasburg, 1960; Randall, 1967; reviewed by Hutchings, 1986). Many tropical green algae are both chemically and structurally defended (Paul and Fenical, 1987; Paul, 1992), and *Halimeda* spp. are among the best studied examples of seaweeds with multiple defenses including aragonite and secondary metabolites (Paul and Fenical, 1986; Hay et al., 1988; Paul and Van Alstyne, 1988a,b, 1992).

Our continuing interest in the chemical adaptations of tropical calcified seaweeds has led us to investigate the natural products chemistry, in situ palatability, and chemical defenses of the calcareous green alga *Neomeris annulata* Dickie. *N. annulata* is a diminutive seaweed widely distributed in shallow inshore waters of many coral reef habitats in both the tropical Indo-Pacific and Caribbean. Previous studies of the natural products chemistry of *N. annulata* collected in Bermuda led to the isolation of three unique brominated sesquiterpenes, the first halogenated sesquiterpenes to be reported from any green algae (Barnekow et al., 1989). The metabolites showed toxicity to brine shrimp and caused necrosis toward johnsongrass in a cut-leaf phytotoxicity assay (Barnekow et al., 1989). Their effects as chemical defenses toward cooccurring herbivores were not examined. Because of the similarity between *Neomeris annulata* and other green algae that are both calcified and chemically defended, we hypothesized that the halogenated sesquiterpenes found in *Neomeris annulata* function as chemical defenses against coral reef herbivores.

In this study, we first investigated the natural products chemistry of Pacific collections of the seaweed *Neomeris annulata* from Kwajalein Atoll and Guam. We found a unique trio of brominated sesquiterpenes (Scheme 1, compounds **1, 2,** and **3**) that were structurally related to the compounds previously reported from Bermuda collections of this alga (Scheme 2, compounds **4, 5,** and **6**) (Barnekow et al., 1989). We describe here the chemical structures of these three new metabolites. We then investigated the defensive role of the *Neomeris* metabolites against herbivorous fishes in field assays conducted on Guam. We examined the in situ palatability of *N. annulata* compared with other preferred and nonpreferred seaweeds. We also tested crude organic extracts, flash column



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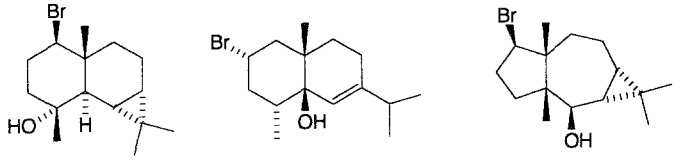
SCHEME 1



4 5 6

SCHEME 2

chromatography fractions, and isolated metabolites from Guam and Bermuda *N. annulata* toward natural populations of herbivorous fishes on Guam.

METHODS AND MATERIALS

Chemical Analyses. Collections of *Neomeris annulata* were made in Agat Bay, Guam (13°25′N, 144°55′E) and at Kwajalein Harbor on Kwajalein Atoll (8°43′N, 167°44′E) in shallow, reef flat habitats (1–2 m depth). Plants were found attached to coral rubble. *Neomeris* collected on Guam was extracted immediately after collection. Samples from Kwajalein Atoll were frozen and extracted two weeks after collection. All samples were extracted three times in 1:1 dichloromethane–methanol, and solvents were removed by rotary evaporator. Crude extracts were fractionated by vacuum flash silica gel chromatography with hexane–ethyl acetate mixtures to obtain fractions for testing in field feeding assays and for isolation of active metabolites. Final purification of the *Neomeris* compounds was achieved by silica gel high performance liquid chromatography (HPLC) with 10% ethyl acetate–90% hexane as the solvent.

IR spectra were recorded with a Nicolet FT-IR spectrophotometer and high-resolution mass spectra (EIMS) were obtained with a VG 7070 EHF mass spectrometer. Proton nuclear magnetic resonance (^{1}H NMR) and ^{13}C NMR spectra were recorded with a Bruker AC 300 spectrometer instrument.

Feeding Preference Assays. Feeding preference assays using whole algae as well as assays with the organic extracts, chromatography fractions, and purified metabolites were conducted on a shallow (3- to 5-m) patch reef in Cocos Lagoon, Guam (described in Paul, 1987). Among the most common fishes seen on the reef were juvenile *Scarus sordidus*, while *Amblyglyphidodon curacao*, *Naso lituratus*, *Zebrasoma flavescens*, and *Siganus argenteus* also commonly occur and were observed to feed during the assays.

Susceptibility of *Neomeris annulata* and three other species of green algae (*Enteromorpha clathrata*, *Halimeda macroloba*, and *H. discoidea*) to grazing by herbivorous fishes was examined by weaving small (3- to 4-cm-long) pieces of thalli, at 5-cm intervals, into a 0.5-m length of three-strand polypropylene line that was fastened to the reef ($N = 21$ replicate lines). When an herbivorous fish encountered a rope, all species of algae should have been equally apparent and available. At the end of the experiment, each species on each rope was recorded as either still present or partially or totally consumed. These methods measure only the relative susceptibility of a species to being eaten by the herbivorous fishes present in the habitat. They do not differentiate among fish species, and they do not yield preference data for any given species or type of fish. Data were analyzed by R × C contingency table analysis supplemented by the simultaneous test procedure for homogeneity of replicates tested for goodness of fit to determine which species were significantly more susceptible than others (Sokal and Rohlf, 1981).

Field feeding preference assays were conducted to determine the effects of the *Neomeris* secondary metabolites on feeding by natural populations of herbivorous fishes. These methods have been previously described (Paul, 1987; Paul and Van Alstyne, 1988a). Briefly, pieces of the palatable green alga *Enteromorpha clathrata* (approximately 250 mg wet mass) were coated with a solution of the extract, fraction, or isolated metabolite to be tested dissolved in diethyl ether. Four pieces of treated *Enteromorpha* were inserted into the feeding rope (0.5-m-long, three-stranded yellow polypropylene rope) and paired with four control pieces (diethyl ether only) inserted into another rope. Replicate pairs ($N = 10$–20 pairs) were placed on the reef for 15–60 min until approximately half of the total algal pieces were consumed by fish. The results were scored as number of pieces completely eaten and were analyzed by the Wilcoxon signed-ranks test for paired comparisons (one-tailed test) (Sokal and Rohlf, 1981).

RESULTS

Chemical Analyses. Crude extract yields from extractions of large collections of the alga ranged from 0.9% to 2.6% dry mass; however, we considered these yields to be low because sand and gravel were often included in the dry

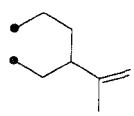
mass measurements. When individual plants from Guam were carefully cleaned and extracted, crude extract yields ranged from a mean of 5.06% dry mass (N = 20, range: 1.6–15.3%) in February 1990 to a mean of 13.9% dry mass (N = 20, range: 6.0–23.3%) in December 1990 (Meyer, 1991). Considerable intra-plant variation in extract concentrations was also observed. Tips of plants had highest extract yields of 12.9% dry mass (N = 4, range: 5.0–21.5%), while bases had much lower concentrations of 0.7% dry mass (N = 4, range: 0.3–0.9%).

Compound **1** was isolated as a colorless oil, $[\alpha]_D$ −10.5° (c 3.8, $CHCl_3$); high resolution EIMS m/z 300.1070 (M^+, 11%, $C_{15}H_{25}BrO$ requires 300.1083), 282(11), 221(15), 220(7); IR ν_{max} $CHCl_3$ 3600, 2935, 2860, 1670, 1450, 1340, 1000, 965, 890 cm^{-1}. Concentrations of compound **1** averaged 0.95% dry mass in the tips (N = 3, range: 0.77–1.16%), while concentrations in the bases averaged only 0.11% dry mass (N = 3, range: 0.09–0.13%) (Meyer, 1991).

Compound **2** was also isolated as a colorless oil, $[\alpha]_D$ +1.43° (c 6.6, $CHCl_3$); high resolution EIMS m/z 300.1083 (M^+, 2%, $C_{15}H_{25}BrO$ requires 300.1083), 282(1), 221(25), 220(11); IR ν_{max} $CHCl_3$ 3605, 2940, 2860, 1680, 1450, 1375, 1095, 980, 890 cm^{-1}. Concentrations of compound **2** averaged 0.58% dry mass in the tips (N = 3, range: 0.27–1.10%), while concentrations in the bases averaged 0.06% dry mass (N = 3, range: 0.04–0.09%) (Meyer, 1991).

Compound **3** was also a colorless oil; high resolution EIMS showed m/z 300.1059 (M^+, 2%, $C_{15}H_{25}BrO$ requires 300.1083), 285(11), 282(2), 203(5), 202(12). Compound **3** was a minor metabolite in Guam and Kwajalein collections of *Neomeris annulata*. Its concentrations were not quantified, and not enough material was isolated to conduct feeding assays with this compound.

Accurate mass measurements (EIMS) established that all three compounds were constitutional isomers with the formula $C_{15}H_{25}BrO$. Three sites of unsaturation were required; the absence of the carbonyl absorptions (IR, ^{13}C NMR) and the presence of only two olefinic carbons (^{13}C NMR) indicated a bicyclic molecule. A tertiary hydroxyl functionality was present (ν_{max} ~3600 cm^{-1}, δ70–77, s) in each compound. The now conventional approach of ^1H–^1H COSY and ^1H–^{13}C HETCOR analyses (HMQC, HMBC) was employed to establish the gross structure of each molecule. All three compounds shared a common substructure (Scheme 3).



SCHEME 3

The other half of each molecule differed in position of the bromine and, in the case of **3**, the hydroxyl group. The position of the bromine was readily established from characteristic ^{1}H and ^{13}C NMR chemical shifts and by the number and magnitude of couplings to the methine bearing bromine. ^{13}C and ^{1}H NMR data for the three sesquiterpenes are shown in Table 1.

The relative stereochemistry of each ring could be determined by analysis of coupling constants and was confirmed by difference nuclear Overhauser effect (nOe) experiments. These nOe studies also established ring juncture stereochemistry and conformation. In the case of **1** and **2**, the bromine, C-4 methyl, and C-7 isopropenyl substituents assumed equatorial dispositions, as would be expected. However, these two compounds differed from the previously identified *Neomeris* sesquiterpenes **4–6** (Barnekow et al., 1989) in the conformation of the ring juncture. In **1** and **2**, the ring juncture methyl group (C-11) was found to reside in an equatorial disposition; the C-5 hydroxyl group, therefore, had to be axial. These assignments followed from nOe relationships between H-11 and H-6a and between H-12 and H-6e in both compounds. In **1**, H-2a also had an nOe relationship with H-9a. In compound **3**, the ring conformation reverted to that of **5**. In this case, nOes from methyl groups attached to C-4 and C-10 to H-2a indicated that these groups were axial, while the coupling constants of H-5 indicated that it was equatorial. The C-1 bromine and C-4 hydroxyl group were also equatorial. Table 2 summarizes the relevant nOe data.

Feeding Preference Assays. Neomeris annulata whole plants were not susceptible to grazing by natural populations of herbivorous fishes in field assays on Guam (Table 3). When the number of pieces that were wholly or partially consumed were compared among algal species, *Neomeris annulata* ranked with species of *Halimeda*, which are of low preference to most generalist herbivores, in its susceptibility to being grazed by fishes.

Whole extracts coated on the palatable seaweed *Enteromorpha* significantly deterred feeding by herbivorous fishes at concentrations of 4% dry mass but not at 2% dry mass (Figure 1). Similarly, the hexane-soluble portion of the extract was not deterrent at 2% dry mass (Figure 1). As mentioned previously, the natural range of crude extract concentrations for individual *Neomeris* plants on Guam was 2–20% dry mass with mean concentrations of approximately 5% (Meyer, 1991). When extracts were fractionated by silica gel chromatography, fraction 1, containing mostly triglycerides, did not deter fish feeding, but fraction 2, containing the brominated sesquiterpenes **1** and **2**, significantly deterred feeding by fishes at concentrations of $\geq 1\%$ dry mass (Figure 2). Fraction 4, which contained terpenes **2** and **3**, deterred fish feeding at a lower concentration of 0.4% dry mass. Similar results were obtained for fractions of extracts of *Neomeris* collected on Guam and in Kwajalein (Figures 2 and 3).

Both of the major metabolites, **1** and **2**, from Pacific collections of *N. annulata* marginally deterred feeding by herbivorous fishes in field assays

TABLE 1. NMR DATA OF *Neomeris* SESQUITERPENES (**1, 2, 3**)[a,b]

Carbon No.	1	2	3[b,c]
1	47.8 (CH_2) 1.68 (ddd, J = 2, 4, 13) 2.24 (t, 13)	35.8 (CH_2) 0.90 (dt, J = 14, 3.5) 1.68, J = 5, 14	68.6 (CH) 3.66 (dd, J = 4, 13)
2	48.5 (CH) 4.03 (tt, J = 4, 4, 12.5, 13)	33.4 (CH_2) 1.83–1.97 (m, J = 4)	30.8 (CH_2) 1.88 (ddd, J = 4, 7, 13) 2.47 (dq, J = 4, 13)
3	42.0 (CH_2) 1.77 (m) 2.06 (q, J = 12.5)	65.5 (CH) 4.02 (dt, J = 5, 11)	42.3 (CH_2) 1.03 (m, J = 4, 13, 14) 1.20 (ddd, J = 3, 4, 14)
4	34.4 (CH) 1.46 (m, J = 3, 6.5)	41.6 (CH) 1.99 (m)	70.7 (C)
5	73.0 (C)	76.5 (C)	52.3 (CH) 0.69 (dd, J = 3.5, 13)
6	36.4 (CH_2) 1.10 (t, J = 13) 1.44 (dd, J = 13, 3)	37.6 (CH_2) 1.10 (t, J = 13) 1.49 (dd, J = 4, 13)	41.9 (CH_2) 0.84 (ddd, J = 4, 13, 13.5) 2.02 (dt, J = 13.5, 3.5)
7	41.5 (CH) 1.58 (m)	41.5 (CH) 1.64 (m)	46.4 (CH) 1.75 (tt, J = 4, 12)
8	26.6 (CH_2) 1.26–1.29 (m)	26.7 (CH_2) 1.27 (dddd, J = 4, 12.5, 12.5, 13) 1.25–1.41 (m)	27.0 (CH_2) 1.40–1.55 (m)
9	33.3 (CH_2) 0.78 (dd, J = 2.5, 9.5) 1.25 (m)	32.7 (CH_2) 0.79 (m, J = 3, 4) 1.56 (dt, J = 5, 13)	30.2 (CH_2) 1.35–1.40 (m)
10	41.5 (C)	37.5 (C)	39.9 (C)
11	22.1 (CH_3) 0.77 (s)	22.3 (CH_3) 0.74 (s)	14.9 (CH_3) 1.28 (s)
12	14.4 (CH_3) 0.58 (d, J = 6.5)	11.8 (CH_3) 1.08 (d, J = 6.5)	29.7 (CH_3) 0.70 (s)
13	149.1 (C)	149.1 (C)	150.0 (C)
14	20.8 (CH_3) 1.58 (s)	20.9 (CH_3) 1.58 (s)	20.7 (CH_3) 1.64 (s)
15	108.9 (CH_2) 4.72 (s) 4.74 (s)	109.0 (CH_2) 4.70 (s) 4.74 (s)	109.1 (CH_2) 4.78 (d, J = 1.5) 4.80 (d, J = 1.5)

[a]Recorded in C_6D_6 at 300 MHz (^1H), 125 MHz (^{13}C); number attached H from Distortionless Enhancement Polarization Transfer (DEPT).
[b]Some coupling constants (^1H) not determined because of spectral overlap; J values in Hertz.
[c]Recorded in C_6D_6 at 500 MHz (^1H), 125 MHz (^{13}C); number attached H from DEPT.

TABLE 2. SELECTED nOe RELATIONSHIPS FOR COMPOUNDS **1–3**[a]

Compound **1**	Compound **2**	Compound **3**
$H_{2a} \rightarrow H_{9a}$	$H_7 \rightarrow H_4$	$H_{11} \rightarrow H_{2a}$
$H_{9a} \rightarrow H_7$	$H_{9a} \rightarrow H_7$	$H_{12} \rightarrow H_{2a}$
$H_{11} \rightarrow H_{6a}$	$H_{11} \rightarrow H_{6a}$	
$H_{12} \rightarrow H_{6e}$	$H_{12} \rightarrow H_{6e}$	

[a]All nOe values 4–10%.

TABLE 3. RESULTS OF FEEDING PREFERENCE ASSAYS WITH WHOLE ALGAE PIECES EATEN CONDUCTED AT A PATCH REEF IN COCOS LAGOON, GUAM[a]

	Pieces eaten	Pieces not eaten
Enteromorpha clathrata	18	3
Neomeris annulata	4	17
Halimeda macroloba	3	18
Halimeda discoidea	0	21

[a]Results were analyzed by contingency table analysis with the G test ($G = 47.384$, $P < 0.001$). Overall results were significantly different among the algal species for the number of pieces of algae eaten and not eaten. *Neomeris annulata*, *Halimeda macroloba*, and *H. discoidea* formed a nonsignificant subset of the data ($G = 6.277$, $P = 0.10$).

when coated on the palatable seaweed *Enteromorpha* at 1% dry mass (Figure 4). The 1% dry mass concentration used in the assays is at the high end of the range of natural concentrations of these metabolites. Compound **3** was a minor metabolite, and insufficient quantities were isolated to test in field assays. Both of the major metabolites from Bermuda collections, **4** and **5**, significantly deterred feeding by herbivorous fishes at concentrations slightly below 1% dry mass (Figure 4). The Bermuda metabolite **6** did not deter fish feeding and instead showed a trend toward being a feeding attractant at a concentration of 0.7% dry mass (Figure 4).

DISCUSSION

The brominated sesquiterpenes produced by *Neomeris annulata* appear to function as chemical defenses against herbivorous reef fishes: natural concentrations of the whole extracts, fractions of extracts containing the terpenes, and some isolated metabolites deterred feeding by natural assemblages of herbivorous fishes in field assays on Guam. These results support our hypothesis that

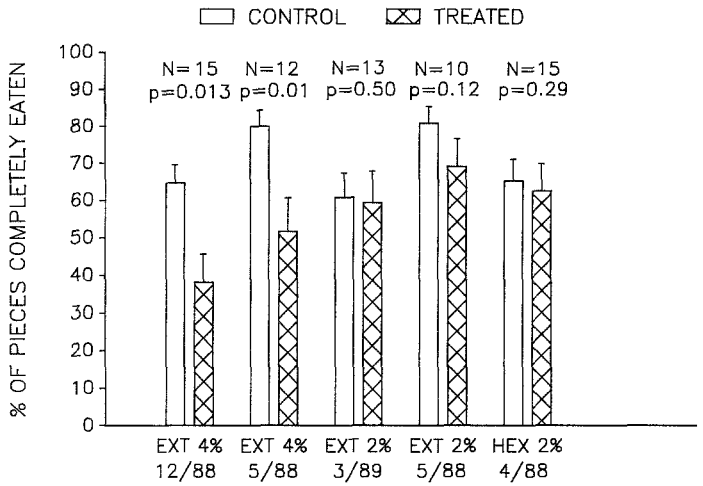


FIG. 1. Results of field feeding assays with crude extracts of *Neomeris annulata* collected from Agat Bay, Guam. Data are presented as percent of *Enteromorpha* pieces completely consumed ($\bar{X}$ + 1 SE). EXT = crude organic extract; HEX = hexane-soluble extract; percent concentrations are percents of *Enteromorpha* dry mass. Dates at the bottom of the histograms are the dates the assays were conducted. Data were analyzed by the Wilcoxon signed-ranks test for paired comparisons (one-tailed). N = number of replicate pairs.

Neomeris is chemically defended against herbivores, in addition to using calcium carbonate as a potential defense against some grazers.

Paul and Hay (1986) showed that many calcified reef seaweeds also produce secondary metabolites and that many of the seaweeds with multiple defenses are grazer resistant. The need for many calcified reef seaweeds to supplement morphological or structural defenses with secondary metabolites is not surprising since some reef herbivores, such as parrotfishes, are well adapted for the consumption of calcified prey (Hutchings, 1986; Choat, 1991). Schupp and Paul (1993) have observed that not only are some parrotfishes not deterred by calcium carbonate in their diets, they may even show a preference for calcified foods. *Neomeris annulata* extracts and a mixture of the major terpenes **1** and **2** did deter feeding by the parrotfish *Scarus sordidus* in aquarium assays on Guam (Paul, work in progress). Thus, although calcification in *Neomeris* may not deter grazing by parrotfishes, the secondary metabolites do. Lubchenco and Gaines (1981) have previously noted that any particular defense is likely to become less effective as a plant is subjected to attack by different types of herbivores. Thus, on tropical reefs where herbivores are both numerous and diverse, multiple defenses may prove to be the rule rather than the exception.

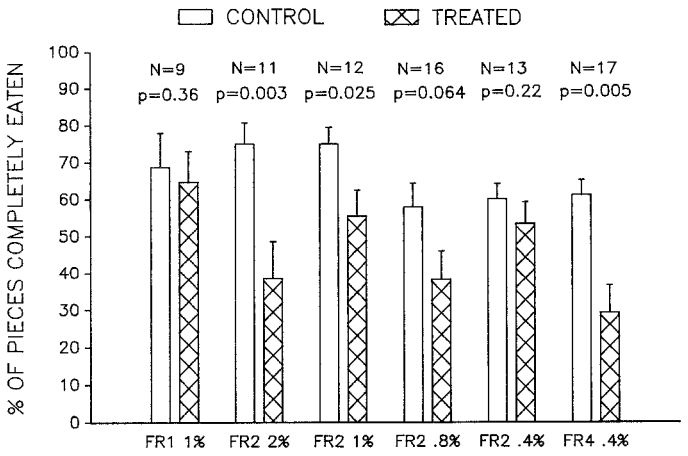


FIG. 2. Results of field feeding assays with column chromatography fractions of extract of *Neomeris annulata* from Agat Bay, Guam. Symbols and data analysis are as in Figure 1. FR = fraction number from column chromatography; concentrations are percents of *Enteromorpha* dry mass. Fraction 2 was tested at several different concentrations because it contained the major metabolites **1** and **2**. Fraction 3 was not available in sufficient quantities to test in the field assays.

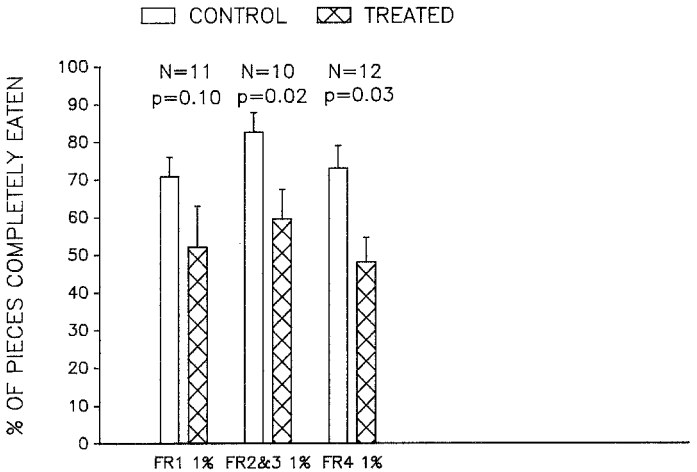


FIG. 3. Results of field feeding assays with column chromatography fractions of extract of *N. annulata* from Kwajalein Atoll. Symbols and data analysis are as in Figure 1. All fractions were tested at 1% of *Enteromorpha* dry mass.

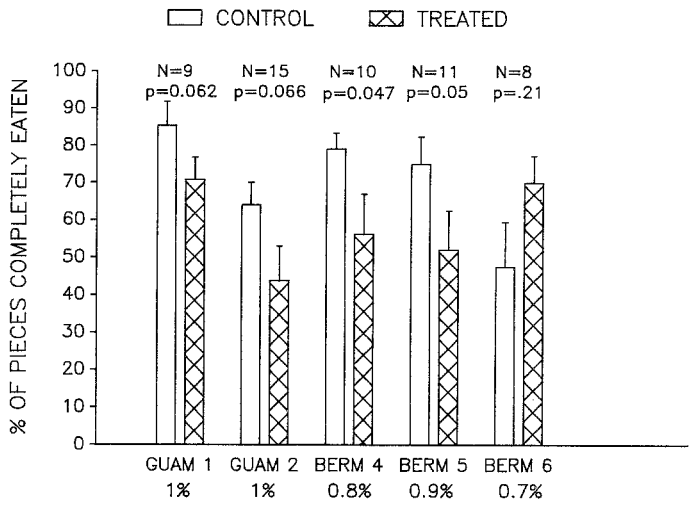


FIG. 4. Results of field feeding assays with isolated metabolites from *Neomeris annulata*. GUAM 1 and 2 are major metabolites **1** and **2** from *Neomeris* on Guam and Kwajalein. BERM 4, 5, and 6 were major metabolites **4, 5,** and **6** from collections made in Bermuda. Symbols and data analysis are as in Figure 1. A two-tailed *P* value is reported for compound **6** because this metabolite appeared to be an attractant.

The same secondary metabolites, compounds **1, 2, 3,** were found in collections from both Guam and Kwajalein. These compounds differed only slightly from the terpenes **4, 5,** and **6** previously described from Bermuda collections of the alga (Barnekow et al., 1989). We do not know whether the source of this variation is genetic, since *Neomeris annulata* is distributed pantropically. However, many marine plants show site-to-site variation in the secondary metabolites they produce; for example, the red alga *Portieria hornemanni* produces many different halogenated monoterpenes (Ichikawa et al., 1974; Burreson et al., 1975a,b; Woolard et al., 1978; Paul et al., 1987; Coll and Wright, 1989; Fuller et al., 1992) depending on where the alga is collected.

When the purified metabolites were tested individually against herbivorous fishes, most of the metabolites deterred feeding at the high end of their natural concentration range, concentrations found often only in the terminal tips of the plants or in young plants (Figure 4). Interestingly, the two major Bermuda metabolites, **4** and **5,** appeared to be slightly more deterrent to fishes on Guam than the two major Pacific metabolites **1** and **2** (Figure 4). Bermuda metabolite **6,** which was structurally the least similar to the other compounds and possessed a different carbon skeleton, was the only compound that did not deter fish grazing. In fact, this metabolite showed a trend toward being an attractant (Fig-

ure 4). No compounds similar to Bermuda terpene **6** were found in the Guam or Kwajalein collections of *Neomeris*.

We do not know if the compounds function together synergistically, but our results do not suggest that they do. Crude extracts, which contain all three compounds in their natural concentration ratios, were only deterrent at concentrations of 4% dry mass (Figure 1). Similarly, mixtures of compounds **1** and **2** that were present in fraction 2 from Guam and Kwajalein were only deterrent at $\geq 1\%$ dry mass (Figures 2 and 3), the same concentrations that were deterrent for the pure metabolites (Figure 4). The only exception to this pattern was for fraction 4 of the Guam collection, which contained a mixture of compounds **2** and **3.** This fraction was deterrent at a concentration of 0.4% dry mass (Figure 2). Fraction 4 was also more deterrent than other fractions in aquarium assays with the herbivorous surgeonfish *Naso lituratus* (Meyer et al., 1993). When purified compounds were tested individually, Neomeris terpene **1** significantly deterred feeding by *N. lituratus*, but terpene **2** did not. These results suggest that compound **3** is a very potent deterrent, or that the mixture of **2** and **3** is much more potent than compound **2** alone. The effects of these metabolites against different herbivores may vary, and further testing of isolated terpenes and mixtures is clearly necessary to determine their effects on different herbivores and whether the metabolites act additively or synergistically.

It has been suggested that chemical variability itself may be an adaptation against herbivores and pathogens, because it would be relatively easy for specialists and rapidly evolving herbivores and pathogens to adapt to any particular defensive compound but more difficult for them to adapt to a complex mixture of secondary metabolites (Dolinger et al., 1973; Whitham, 1983; Whitham et al., 1984). *Neomeris annulata* may be particularly well-adapted against herbivory because it produces a mixture of several different secondary metabolites, the types of compounds vary across its geographic range, and the alga combines chemical and structural defenses to protect against the variety of herbivores found on coral reefs.

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FOLIAR PHENOLICS OF NEBRASKA SANDHILLS PRAIRIE GRAMINOIDS: BETWEEN-YEARS, SEASONAL, AND INTERSPECIFIC VARIATION

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Abstract—Because of their potential as antiherbivore defenses, plant phenolics elicit considerable attention. We made quantitative and qualitative analyses of phenolics, alkaloids, cyanogenic glycosides, and saponins in the dominant graminoids of a Nebraska Sandhills prairie. We examined the foliage of seven species: *Agropyron smithii* Rydb., *Andropogon hallii* Hack., *Andropogon scoparius* Michx., *Bouteloua gracilis* (H.B.K) Lag. ex Griffiths, *Calamovilfa longifolia* (Hook.) Scribn., *Carex heliophila* Mack., and *Stipa comata* Trin & Rupr. Their leaves contain low levels of phenolics that vary significantly among species. A more detailed examination of the three species with the highest levels of phenolics showed among-year, seasonal, and spatial heterogeneity in the levels of total phenolics. In all seven species, the majority of the specific phenolics present have the chromatographic properties of phenylpropanoids and are likely to be present as sugar-linked derivatives such as free glycosides or cell wall-bound phenolics. These species do not contain condensed tannins. The absence of other common kinds of secondary metabolites indicates that these graminoids are unlikely to have significant chemical defenses, at least in terms of substances likely to be active against mammalian herbivores. In this, they exemplify the situation thought typical for prairie graminoids.

Key Words—Phenolics, seasonal variation, graminoids.

INTRODUCTION

Plant phenolics have excited considerable attention because of their potential role as allelochemicals. In this role they may function against herbivores, plant

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pathogens, or even other plants (Harborne, 1988). There is a wealth of literature on this group of plant secondary metabolites, but work has been primarily focused on dicotyledonous species. Relatively little attention has been given to grasses or to other monocots with the same growth form. Indeed, there has often been a perception that such species (graminoids) are relatively devoid of chemical defenses (Jung et al., 1979; Owen and Wiegert, 1981; McNaughton, 1983). However, this view has not gone unchallenged (Redak, 1987), and there remains the necessity to analyze species of particular interest for secondary metabolites.

In addition to work focused on ways in which plant phenolics may mediate interactions between organisms, there has also been an interest in the physiological ecology of resource allocation to plant secondary metabolism versus other processes involved in plant growth and development (Bryant et al., 1983; Chapin et al., 1986; Jonasson, 1986; Waterman and Mole, 1989). Phenolics absorb UV light strongly, and further physiological interest in their production has centered on their potential role as photoprotectants (Rhoades, 1977), particularly in the context of global climate change (Caldwell, 1981).

After a pilot study in which we detected phenolics in seven graminoids sampled from a single Nebraska Sandhills prairie, we chose the three species with the highest levels of phenolics for an extended quantitative analysis. From these analyses, we present the longest run of data for variation of the total phenolics content of graminoids in any natural or managed temperate grassland system: We present seven years worth of data for foliage samples collected at intervals during successive growing seasons. Two years worth of data are also presented for samples taken along a topographic gradient.

In addition, we performed qualitative analyses on the phenolics of all seven species sampled from the site. These show the likely biosynthetic groups of phenolics present. We also report on the absence of several types of nonphenolic secondary metabolites from all seven species.

METHODS AND MATERIALS

Our field site was at Arapaho Prairie in the Sandhills grasslands of western Nebraska (Arthur County sections 31 and 32, T18N, R38W). This 1280-acre reserve is representative of the larger Sandhills area (18,000 sq mi) (Weaver, 1965; Kaul, 1975), considered either as the most western extension of the tallgrass prairie (French, 1979) or as a unique mixture of tall-, mid-, and shortgrass types. Plant productivity is intermediate between tall- and shortgrass types and is predominantly determined by precipitation. However, local heterogeneity in topography, soil texture, and mineral nutrients also influences plant production (Barnes and Harrison, 1982). Since 1987, a continuously recording climate monitoring station has been in operation at Arapaho Prairie. All our samples

were collected close to this weather station site, which is situated in an interdune valley at the prairie.

The collection of samples of three species for long-term analysis of total phenolics was begun in 1985 and continued through 1991. Samples of leaves of two grasses (*Andropogon hallii*, *Andropogon scoparius*) and one sedge (*Carex heliophila*) were collected. Live (green) tissues were cut off at leaf bases and hand sorted to exclude dead material and reproductive tissues from the samples. Each sorted sample contained in excess of 100 leaves and was preserved on site by immediately flash freezing in liquid nitrogen. Subsequently, samples were lyophilized, and stored in dry sealed packages over a desiccant. Collections were made approximately twice a month during each growing season (exact dates were recorded). For each species, five replicate samples were taken several meters apart to eliminate repeated sampling from within clones.

For both 1990 and 1991, two additional groups of five replicate collections were made for each species along a topographic gradient, one midway up the slope and a second on the dune ridge-top ("slope" and "ridge" samples, respectively). Samples were collected at the same times as samples collected from the "valley" (weather station) site. Samples of four other grasses (*Agropyron smithii*, *Bouteloua gracilis*, *Calamovilfa longifolia*, and *Stipa comata*) were also collected by the same methods from the same sites during 1985–1991. These species were included in our qualitative analysis of plant phenolics but were not systematically analyzed for total phenolics.

Prior to chemical analysis, the lyophilized plant samples were ground in a Wiley mill to pass through a 40-mesh screen. Total phenolics were extracted from the ground plant material by their extraction at room temperature using 100-mg samples and 5 ml of 50% methanol, which were then shaken together overnight. Aliquots of the extract were then analyzed by the Prussian blue method for phenolics (Price and Butler, 1977). Results are expressed as percentages of total phenolics in leaf samples, calculated from the equivalent weight of tannic acid expressed on a dry weight of leaf basis. Tannic acid (Sigma Chemical Co.) is the most commonly accepted standard for this assay (Mole et al., 1989) and was used for this reason, even though it is not present in these species. A UV spectrum was recorded over the wavelength range for UV(B) radiation (280–320 nm) for one extract of material collected at each date during the 1987 season.

Qualitative analyses of phenolics were by TLC following the methods adapted from Harborne (1984). By combining 100 mg of ground sample from each collection made in 1991, a large aggregate sample was constructed for each species. One-gram samples of these aggregates were either extracted by: (1) being shaken overnight in water at room temperature or (2) being subjected to acid hydrolysis in 2 M HCl for 30 min at 100°C. These two extracts are subsequently referred to as the unhydrolyzed and hydrolyzed extracts, respec-

tively, and it is important to note that the hydrolysis was performed with cell-wall material present so as to release phenolics bonded (esterified) to it. Each fraction was partitioned against diethyl ether to give an ether-extractable fraction. The remaining aqueous layer was then partitioned against ethyl acetate and then again against amyl alcohol to give ethyl acetate and amyl alcohol fractions, respectively. The remaining aqueous fractions were then lyophilized so that the phenolics contained in them could be concentrated. This was achieved by dissolving the freeze-dried residue in a small quantity of 50% methanol. Organic solvent was removed from the other fractions by evaporation under a stream of nitrogen gas. Small volumes of these concentrated fractions were applied to TLC systems for side-by-side analyses of samples from the seven different species.

TLC solvent systems are numbered for reference below. Those used with silica gel plates (Silica gel/F, Sigma Chemical Co.) were: (1) acetic acid–chloroform (1:9) and (2) ethyl acetate–benzene (9:11); TLC solvent systems used with cellulose plates (Sigma Chemical Co.) were: (3) 100% water, (4) butanol–ethanol–water (4:1:2.2), (5) butanol–acetic acid–water (4:1:5, upper layer used), and (6) "forestal," which is hydrochloric acid–acetic acid–water (3:30:10). Phenolics were detected on the plates by examinations under short- and longwave UV light, with and without fuming the plates with ammonia. Plates were also sprayed with the Prussian blue reagents (Price and Butler, 1977) so as to detect substances contributing to the total phenolics assay. Plates developed for simple phenolics were also sprayed with 10% (w/v) vanillin in hydrochloric acid as an additional detection reagent (Harborne, 1984).

Analyses made for nonphenolic secondary metabolites involved testing fresh material in the field for cyanogenisis early in the 1989 field season (May). This was done by enclosing samples of crushed leaves with Feigl-Anger test paper (Fiegl and Anger, 1966). In the lab, aggregate samples constructed like those for the phenolics analyses (see above) were also tested for the presence of alkaloids. This followed the classic technique of extraction into aqueous acid (1 M HCl) followed by making the extract alkaline extract with ammonia and then partitioning the alkaloid into chloroform. Concentrated samples of this extract were applied to TLC plates (Silica gel G, solvent: methanol–ammonia, 200:3), and both the Dragendorff and the iodoplatinate reagents were used for detection. A more specific search for indole alkaloids was made using the techniques of Mulvena and Slaytor (1982). Finally, a test for saponins was made using the "froth" test method of Darnley-Gibbs (1974).

Statistical analyses were performed using DOS SYSTAT 5.0. Means presented in the text are followed by standard errors in parentheses. The analyses of covariance presented in Tables 1 and 2 each have Julian date as the covariate and total phenolics content as the dependent variable. Kruskal-Wallis ANOVAs used to establish rank orderings of species mean phenolic levels within years

were applied factorally to all pairwise species comparisons, after first establishing an overall species effect for each year.

RESULTS

Temporal and Species-Specific Responses. On the basis of an initial study, we selected three species with relatively high levels of extractable phenolics for an intensive study: *Andropogon hallii*, *Andropogon scoparius*, and *Carex heliophila*. Table 1 presents a statistical analysis of the total phenolics contents of samples of these species collected from the valley site over the period 1985–1992. From this it can be seen that there are both species-specific differences in total phenolics content and among-year differences. Julian date was used as a covariate in the analysis because phenolics levels show some tendency to increase during the growing season (see below).

Over this seven-year period, *A. hallii*, *A. scoparius*, and *C. heliophila* exhibited mean levels of 1.31% (0.020), 1.37% (0.020), and 0.96% (0.021) total phenolics, respectively. *C. heliophila* contained significantly less phenolic material relative to the other two species ($P < 0.001$), which do not differ significantly from each other in total phenolic content. These statistical tests result from pairwise comparisons of means made subsequent to the ANCOVA presented in Table 1 (Bonferoni procedure).

Figure 1 shows the levels of phenolics in these three species for the different years of the study. Both *A. hallii* and *C. heliophila* show little variation, particularly relative to each other, while *A. scoparius* is variable. By inspection of these data, the most frequent pattern seems to be for 1987, 1988, and 1990, where the rank order of the species means for phenolic content is *A. scoparius* > *A. hallii* > *C. heliophila*. This result is statistically significant for each of these years ($P < 0.002$; Kruskal-Wallis ANOVA). While the pattern is similar for 1985 and 1991, the means for the two *Andropogon* species cannot be dis-

TABLE 1. ANCOVA FOR TOTAL PHENOLICS CONTENT OF THREE DOMINANT SANDHILLS GRAMINOIDS

Source	*df*	MS[a]	*P*
Year	6	2.250	0.001
Species	2	8.755	0.001
Species * Year	12	0.941	0.001
Julian date	1	10.083	0.001
Error	659	0.080	

[a] MS = mean squares.

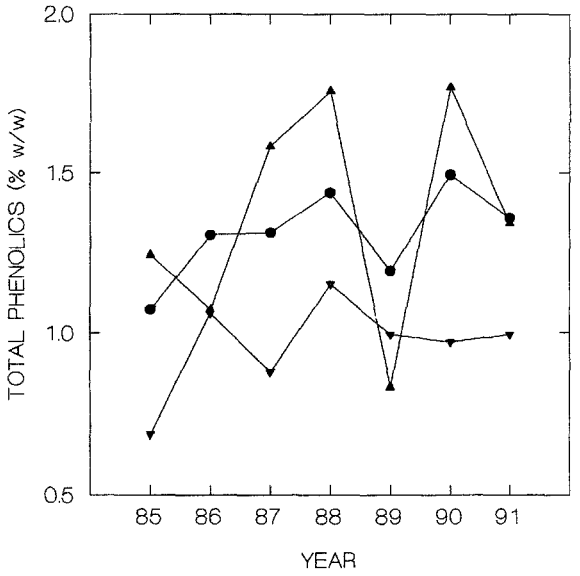

FIG. 1. Average total phenolics content (% w/w) plotted by year for *Andropogon hallii* (●), *A. Scoparius* (▲), and *Carex heliophila* (▼).

tinguished statistically. In 1986 and 1989, the previous pattern is absent. In 1986, *A. scoparius* contained significantly lower levels of phenolics than *A. hallii* while in 1989, *A. scoparius* had lower levels of phenolics than both *A. hallii* and *C. heliophila* ($P < 0.001$; Kruskal-Wallis ANOVA).

Figure 2 illustrates the seasonal change in total phenolics using data for *A. hallii* taken from all seven year's sampling. Data for the other species are more variable, but all three species exhibit a positive trend in phenolics content with time. The importance of Julian date as a source of variation in total phenolics levels is seen in both in Tables 1 and 2, where it appears as the covariate in these analyses.

Site-Specific Responses. In 1990 and 1991, we collected samples from valley, slope, and ridge locations along a dune transect (Table 2). The significance of the covariate and the effects of year and species are as expected from the above. The new insight established here is that the location in which the plants grow also effects their phenolic content (Table 2). However, compared to the other sources of variation, this seems to be a minor component of the variance relative to species, year, and Julian date.

Analyses of covariance were repeated on a species-by-species basis, and each showed the location effect to be a significant one. However, it was not the case that valley, slope, and ridge sites differed significantly from each other for

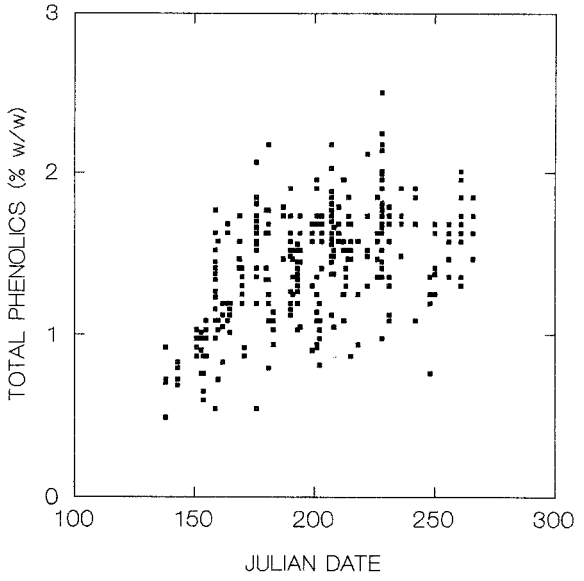

FIG. 2. Total phenolics contents (% w/w) of samples of *Andropogon hallii* collected during 1985–1991, plotted against the (Julian) date of collection.

TABLE 2. ANCOVA FOR TOTAL PHENOLICS CONTENT OF THREE GRAMINOID SPECIES COLLECTED ALONG TOPOGRAPHIC TRANSECT

Source	*df*	MS[a]	*P*
Location	2	0.593	0.001
Species	2	13.958	0.001
Year	1	6.907	0.001
Location * Species	4	0.360	0.001
Location * Year	2	0.087	0.362
Species * Year	2	0.845	0.001
Location * Year * Species	4	0.213	0.027
Julian date	1	4.215	0.001
Error	576	0.077	

[a] MS = mean squares.

each species. The following tests result from contrasts made subsequent to these within-species ANCOVAs. Each is a test comparing one location against the other two. For *A. hallii*, the samples from the ridge location have more phenolics than those from the other locations ($P < 0.001$); for *A. scoparius*, the slope samples have most phenolics ($P < 0.028$) and for *C. heliophila*, the slope and

ridge sites have more phenolics than samples collected from the valley location ($P < 0.001$).

UV(B) Absorbance. Scans of the UV absorbance of the extracts in the UV(B) range showed no sharp peaks of absorbance in any species, instead the spectra ranged from being relatively flat to cases where there was a decline in absorbance by about 50% from 280 nm to 320 nm (e.g., *Agropyron smithii*, *Bouteloua gracilis*). Those species with high total phenolics levels had high absorbances, as would be expected given the UV-absorbing nature of phenolics. Mean absorbances at 280 nm were: *Andropogon hallii*, 34.3 (0.26); *Andropogon scoparius*, 33.26 (0.41); and *Carex heliophila*, 27.2 (1.00). For the species with lower levels of phenolics, the absorbances were: *Agropyron smithii*, 14.6 (0.89); *Bouteloua gracilis*, 6.5 (0.37); *Calamovilfa longifolia*, 14.9 (1.03); and *Stipa comata*, 10.6 (0.69).

Chromatographic Analyses. Our goal in chromatographic analyses was to establish whether there were qualitative differences in phenolics between species, rather than to isolate and characterize specific phenolic metabolites. All seven species of graminoids from the site were analyzed (data presented below).

Unhydrolyzed aqueous extracts can be expected to contain substances as they are encountered in the plant, so we focused on results for these. The ether fractions from these extracts, chromatographed on TLC systems 1 and 2, were devoid of any UV-absorbing substances that reacted with Prussian blue reagents, providing no evidence for simple phenolics such as phenolic acids. Negative results with the vanillin reagent spray did not indicate any resorcinol or phloroglucinol derivatives in this extract. Analyses of the ethyl acetate fractions did reveal evidence for several phenolics. The forestal TLC system (6) revealed a substance that appeared bright yellow under UV at $R_f = 0.83$ in both *C. heliophila* and *C. longifolia*. This reacted with the Prussian blue reagents and is likely to be a flavonoid (e.g., flavonol). TLC using solvent systems 4 and 5 enabled the detection of several substances, all of which reacted positively with Prussian blue reagents and all of which appeared various shades of blue under UV light. In system 4, such substances were detected in *A. scoparius* ($R_f = 0.72$), *C. heliophila* ($R_f = 0.61$) and *C. longifolia* ($R_f = 0.66, 0.71, 0.83$). In system 5, substances were detected in *A. smithii*, ($R_f = 0.57$), *A. hallii* ($R_f = 0.66$), and *C. heliophila* ($R_f = 0.60$). These characteristics suggest that these phenolics are most likely to be phenylpropanoids. The amyl alcohol extracts were colorless, and the only fraction remaining for analysis was the aqueous one. In this fraction numerous substances with the above characteristics of phenyl propanoids were found. For TLC in system 5, these were detected in *A. smithii*, ($R_f = 0.30, 0.46, 0.54, 0.61$), *A. hallii* ($R_f = 0.46, 0.54, 0.61$), *A. scoparius* ($R_f = 0.46, 0.54, 0.61$), *C. heliophila* ($R_f = 0.61$) and *B. gracilis* ($R_f = 0.46$), *C. longifolia* ($R_f = 0.46, 0.54, 0.61$), *C. heliophila* ($R_f = 0.61$) and *S. comata* ($R_f = 0.48, 0.61$). For TLC in system 3, two likely phenyl propanoids were

detected, one at $R_f = 0.13$ in *C. longifolia* and one at $R_f = 0.64$ in *C. longifolia*, *C. heliophila*, and *S. comata*.

The analysis of the hydrolyzed samples was considerably more complex than the above. The main difference from the unhydrolyzed samples was that most substances were detected in the TLC analyses of the ether and ethyl acetate fractions, while the remaining aqueous fraction was essentially devoid of phenolics. None of the substances found in the aqueous fraction before hydrolysis remained after hydrolysis. In each TLC system used with the ether or ethyl acetate fractions, each species gave a chromatogram that typically had two to four substances that were blue, blue-green, or mauve under UV light or UV plus ammonia and that all reacted with the Prussian blue reagents. Many of these were common to several species, and their absence from these fractions generated from the unhydrolyzed extracts suggests that they are normally present as free glycosides or bound to the cell wall via *O*-glycosidic linkages. There was no evidence for the presence of substantial quantities of flavonoids in these species except for *A. hallii* and *A. scoparius*, which both yielded strongly orange-brown amyl alcohol fractions. TLC of these fractions in forestal produced a brown smear suggestive of phlobaphenes, but with a faint yellow band at an R_f indicative of the 3-deoxy anthocyanidin apigenin. There was no evidence of cyanidin or any other anthocyanidin in the amyl alcohol fraction of any species.

Additional samples of all seven species were tested for alkaloids, cyanogenisis, and the presence of saponins. The results for these tests were negative in every case.

DISCUSSION

The results indicating that these Sandhills graminoids have total phenolics contents of approximately 1% are within the expected range for graminoids, which typically have lower levels of phenolics than dicotyledonous plants. Waterman et al. (1983) report total phenolics contents of 1.38% and 1.23% for *Carex bequaertii* (Cyperaceae) and *Arundinaria alpina* (Gramineae), respectively. These species were placed at the extreme low end of the range of total phenolics contents measured in a study of leaves from 18 other, exclusively dicotyledonous, species sampled from a montane flora in Africa. At the upper limit of the range was a species with 17% total phenolics content. Most surveys of plant phenolics are concerned with dicotyledonous plants, where it is usual for the range of total phenolics contents to be between 0% and 20%, with few species containing 1% or less phenolic material (Gartlan et al., 1980; Janzen and Waterman, 1984; Marks et al., 1988). It is also typical for plants with high total phenolics contents to have either condensed or hydrolyzable tannins. The species in this study are typical graminoids in that they have the relatively low

total phenolics contents expected of species that do not possess tannins in any significant quantity.

Our results indicating between-years variation in phenolics have important implications, even though the absolute levels of phenolics are low. In biological terms, plant phenolics may still be important as guides to host-plant selection, such as by grasshoppers. Herbivores accepting or rejecting material based on threshold levels of phenolics can be expected to make different selections in different years given these results. In terms of the practical problems of plant analysis, interspecific comparisons of total phenolics made on the basis of a single year's sampling may not be replicable in subsequent years, even on a relative basis.

In the only other long-term study to examine between-years variation in a graminoid, Jonasson et al. (1986) also found significant between-years variation in the total phenolics content of *Eriophorum vaginatum* (Cyperaceae) in an eight-year study. In this species from Swedish Lappland, yearly mean total phenolics levels ranged between 3.9% and 5.4%. Unlike the species in the present study, *E. vaginatum* has substantially higher levels of phenolics, and it also contains condensed tannins (Jonasson et al., 1986). Indeed, their study indicates that virtually all the phenolics present were condensed tannins.

Jonasson et al. (1986) considered the likely factors causing between-years variation in their system. They could find no evidence that cyclic variation in microtine rodent (herbivore) population levels was important, while in our system there has not been any exceptional outbreak of grasshoppers during our study. Instead of herbivory, Jonasson et al. (1986) considered climate to be the dominant factor, observing high levels of phenolics in warm years. Plant growth is unlikely to be strongly limited by cool temperatures in the Nebraska Sandhills, but rainfall may be a limiting factor. In 1989, when phenolics levels were particularly low, rainfall was the lowest recorded since on-site recording began (1987). While the relationship between water deficit and phenolics metabolism may be nonlinear, Horner (1990) has shown a decline in tannin production at extreme water deficit. Further and longer-term studies might well uncover more definitive correlations between climatic effects and total phenolics production in the Sandhills system.

The results showing an increasing trend for total phenolics with time, during the growing season, are uncontroversial for dicotyledonous plants (Feeny, 1968; Mauffette and Oechel, 1989; Tempel, 1981). The only available data for a graminoid is from a second study of *E. vaginatum* by Chapin et al. (1986), working in the Alaskan Tundra. Here total phenolics levels for the leaves were lower than those from the Swedish site, with mean levels in the range 2.5–3.5%. No significant seasonal variation was seen during three years of study. We do find significant variation (Figure 2), but more studies will be required to obtain a general result for graminoids as distinct from dicots.

The results indicating variation between valley, slope, and ridge sites demonstrate significant spatial heterogeneity among conspecifics of all three species. There is evidence that plant–water relations vary along transects up sandhills (Barnes and Harrison, 1982) and that a plant water deficit is a factor in plant phenolics metabolism (Horner, 1990). This suggests the potential for drainage and topography to influence plant phenolic content, but further work, including replication on other dunes, is needed to establish this as a factor. A potential and unexplored confounding source of variation is spatial heterogeneity in soil mineral nutrients, particularly as soil nitrogen may be higher in valley sites (unpublished data).

The levels of phenolics in the Sandhills graminoids analyzed here do not reach the levels representative of plants with "quantitative" defenses against grazing (see above). This raises the question of other possible functions that they may have in these species. An early suggestion by Rhoades and Cates (1976) was that phenolics acted as a screen against UV light, but this suggestion was based in a study of creosote bush, where the phenolics are present as an extracellular resin on the surface of leaves. For these grasses, the phenolics are present internally in the leaf, most likely in cell vacuoles, which will make them less able to screen out UV light. There is also a fivefold range in the UV absorbances of the extractable phenolics found in this study. Furthermore, dicotyledonous plants may have an order of magnitude or more phenolics than the most phenol-rich Sandhills graminoid. Given these observations, it would seem that at least some of these graminoids are either poorly protected against UV relative to other species or that this function is accomplished some other way than by the possession of phenolics.

The particular kinds of phenolics present are unexceptional. The absence of free simple phenolics, as seen from the TLC analysis of the unhydrolyzed extract, is typical (Fahey and Jung, 1989). Hydrolyzable tannins have never been found in grasses or sedges, and the absence of condensed tannins is indicated here by the failure to find the anthocyanidins (cyanidin and delphinidin) in the amyl alcohol fractions of the hydrolyzed extract (Harborne, 1984). In addition to finding no chemical evidence for tannins, we also failed to find any evidence of protein-precipitating activity in simple qualitative tests made on aqueous extracts (Mole and Joern, unpublished observations). Most of the phenolics located on TLC plates were phenyl propanoids or their derivatives. Substances isolated from the unhydrolyzed extract in the remaining aqueous fraction were most likely to have been phenylpropanoid glycosides. The substantial generation of ether- and ethyl acetate-soluble phenolics after hydrolysis suggests the breakdown of these glycosides and, perhaps, the additional release of cell wall-bound phenolics by hydrolysis. The presence of such substances is usual for grasses, and in general they present no antinutritional problems to ruminants

(Fahey and Jung, 1989). Virtually nothing is known about the interaction of such substances with insects such as graminivorous grasshoppers.

There is sufficient chemical variety in the phenolics found in the unhydrolyzed extracts that an insect able to distinguish these from each other would have sufficient information to discriminate between these species without reference to other information: i.e., considering all the TLC analyses, each species contains a unique variety of phenolics in unhydrolyzed extracts.

Also typical for grasses and sedges is the lack of other kinds of allelochemicals such as alkaloids, saponins, and cyanogenic glycosides. We tested for cyanogenic glycosides early in the season when they are known to be present in the young growth of some grasses (Haskins and Gorz, 1988). Arapaho Prairie is likely to be a particularly nitrogen-poor system (Burke et al., 1991), and this may be a factor in their absence and the absence of alkaloids, all of which contain nitrogen. Several grass species have been found to contain endophytic alkaloids in recent years, but these seem to be more restricted to C_3 species found in relatively moist habitats in states east of Nebraska (Hemken and Bush, 1989). In addition, the species surveyed here are not in genera where indole alkaloids are to be expected (Corcuera, 1989), and so the failure to detect alkaloids comes as no real surprise.

In summary, the species on which this study has focused are the dominant graminoids at a Nebraska Sandhills prairie site. Their leaves have low levels of phenolics, which are variable among years and between species. There is also some evidence for seasonal and spatial heterogeneity in levels of total phenolics. The specific phenolics present do not include condensed tannins and are likely to be phenylpropanoids, particularly sugar-linked derivatives such as free glycosides or cell wall-bound phenolics. This and the absence of other common kinds of secondary metabolites indicate that these graminoids are unlikely to have significant chemical defenses, at least in terms of substances likely to be active against mammalian herbivores. In this they exemplify the situation thought typical for such species, prior to recent research drawing attention to exceptional toxicoses such as those for tall fescue or *Phlaris* alkaloids. We do not in anyway contradict this work, but do suggest that not all the grasses in grassland systems are full of high levels of secondary metabolites awaiting discovery.

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EFFECTS OF SOME LEAF-EMITTED VOLATILE COMPOUNDS ON APHID POPULATION INCREASE[1]

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Abstract—A role of some volatile compounds produced by plant tissues may be as defensive molecules against various pests, including arthropods. Volatile six-carbon compounds derived in plant tissue from polyunsaturated fatty acids via lipoxygenase/hydroperoxide lyase reduced tobacco aphid fecundity at certain concentrations when added to headspace vapor to which aphids were exposed. Both C_6 aldehydes and alcohols were effective, with the alcohols having greater activity. (Z)-3-Hexenyl acetate at levels in the headspace similar to those of the alcohols and aldehydes did not reduce aphid fecundity. A 6-hr exposure period to the C_6 aldehydes and alcohols was needed for maximum effect on the aphids feeding on tobacco leaves. Analysis of the direct versus indirect effects of these compounds indicates that the volatile aldehydes had both direct effects on aphid fecundity and indirect effects due to induced changes in the leaves upon which the aphids were feeding, while only indirect effects were observed for the alcohols. Tomato leaves have the capacity to produce volatile compounds at levels that impact aphid population increase, with the volatiles produced from crushed leaves having a much larger effect. The C_6 aldehydes and alcohols may be components of the fecundity reduction seen with tomato volatiles; however, volatile terpenes showed no effect. These results can be of significance for the genetic alteration of plants for improved aphid resistance.

Key Words—Aldehydes, alcohols, fatty acids, lipids, lipoxygenase, pest defense.

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INTRODUCTION

The tobacco aphid, *Myzus nicotianae* Blackman, closely related to the green peach aphid, *Myzus persicae* (Sulzer) (Blackman, 1987), is an important pest in the southeastern United States (Boiteau and Lowery, 1989; Koziol and Semtner, 1984). During the mid-1980s, a new red morph of the previously green tobacco aphid appeared in the Southeast. It is more difficult to control with chemical insecticides, particularly organophosphates (Boiteau and Lowery, 1989; Harlow and Lampert, 1990). The red morph of the tobacco aphid also develops more quickly to adulthood and has a higher fecundity than the green morph. Consequently, the red morph is a more serious pest, and more effective control methods for its management are needed.

One approach to enhancing control of aphids is through host-plant resistance, and one aspect of resistance may be the production of volatile compounds that are toxic or repellent to pests. Plants produce a wide array of volatile compounds. Some of these may be of significance in defense responses, particularly those induced by damage or injury to plant tissues (Lyr and Banasiak, 1983; Turlings et al., 1990; Zeringue and McCormick, 1989, 1990). Preliminary studies indicated that tomato leaves at certain stages of development produce sufficient levels of volatile compounds to reduce aphid populations.

The objectives of this study were: (1) to investigate the effects of the major fatty acid-derived volatiles produced by leaves on aphid population increase; (2) to examine which of the major volatile compounds emitted by plant tissues, such as tomato leaves, reduce aphid populations and to quantify the impact of dose and timing of applications of these materials on aphid reduction; and (3) to determine whether effects on aphids were direct or indirect via induction of changes in the leaves.

METHODS AND MATERIALS

Materials. Tomato leaves were obtained from *Lycopersicon esculentum* L. cv. Mountain Pride plants grown in containers in a greenhouse. Tobacco leaves were from *Nicotiana tabacum* L. cv. KY 14 plants also grown in a greenhouse. Tobacco aphids were reared on KY 14 tobacco plants raised in a greenhouse. Compounds used in these studies were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, except paraffin oil 335/350, which was purchased from Fisher Scientific Co. Compounds tested were: 2-carene, D-limonene, (*E*)-2-hexenal, hexanal, (*E*)-2-hexenol, (*Z*)-3-hexenol, hexanol and (*Z*)-3-hexenyl acetate.

Bioassay. The bioassay system was similar to that used by Hamilton-Kemp et al. (1991) to determine effects of tomato volatiles on pollen germination. Disks were cut from tobacco leaves or paper towels to fill the bottoms of 5-cm-

diameter glass Petri dishes, which were placed inside 9-cm-diameter glass Petri dishes (120 ml). Ten fourth instar aphid nymphs (just before adulthood) were placed on the tobacco leaf or paper towel disks. Although nymphs lacking wing pads were selected, about 10–20% of the aphids generally turned out to be alates.

Crushed or uncrushed tomato or tobacco leaves were placed inside the 9-cm dishes outside the 5-cm dishes and a glass cover was placed on the 9-cm dish, which was then sealed with parafilm for the duration of the experiment. The assemblies were placed under fluorescent lights in the laboratory at room temperature. Aphid progeny numbers were recorded after allowing the aphids to feed and reproduce on the tobacco leaf piece for six days.

Dilutions of test compounds were made (weight/weight) in 1,2-propanediol except the terpene hydrocarbons, 2-carene and D-limonene, which were diluted in paraffin oil. Ten microliters of these dilutions were placed on the surface of 1 ml water in a 1.4-cm-inner-diam. × 1.2-cm-high glass cup. The cups containing water and the test compounds were placed in the 9-cm glass Petri plates outside the 5-cm Petri plate and the assembly sealed as above. The levels of the volatile compounds used were based on the gas chromatographic determination of headspace analyses of tomato volatiles by Hamilton-Kemp et al. (1991), who showed that 10 μl of 100% of the C_6 alcohols gave similar vapor levels as 10 μl of 10% solutions in 1,2-propanediol of the C_6 aldehydes.

For determination of the actual levels of (*E*)-2-hexenal in the dose–response study, (*E*)-2-hexenal was added to the 1 ml water in the bioassay system (without leaves) in 1,2-propanediol at 0.1, 1, 10, 20, 30, and 40%. Headspace samples were withdrawn through septa fitted to holes in the glass Petri plate covers, at 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hr at 25°C, and the concentration of the (*E*)-2-hexenal in the headspace air was estimated as described by Hamilton-Kemp et al. (1991). (*E*)-2-hexenal reached a plateau in 1–2 hr and remained constant for the remainder of the 6-hr period (Figure 1). An average weighted by time was calculated for each of the concentrations of this compound. For example, a 15-min exposure had the concentration multiplied by 0.25, a 30-min exposure by 0.5, etc.

For comparison of the direct and indirect effects of (*E*)-2-hexenol and (*E*)-2-hexenal on aphid populations, the aphids and the leaf disks the aphids fed on were exposed to the test compounds both individually and in combination. For the individual exposure, aphids were placed on tobacco leaf disks, exposed to the test compounds for 6 hr, and then moved to unexposed leaf disks in assemblies that had not been exposed to the compounds and incubated for six days. Conversely, tobacco leaf disks were exposed to the test compounds for 6 hr, after which they were moved to fresh, unexposed assemblies, and aphids were placed on them and then incubated for six days. In some experiments, the level of a test compound [(*E*)-2-hexenal] was measured in the assembly to which a

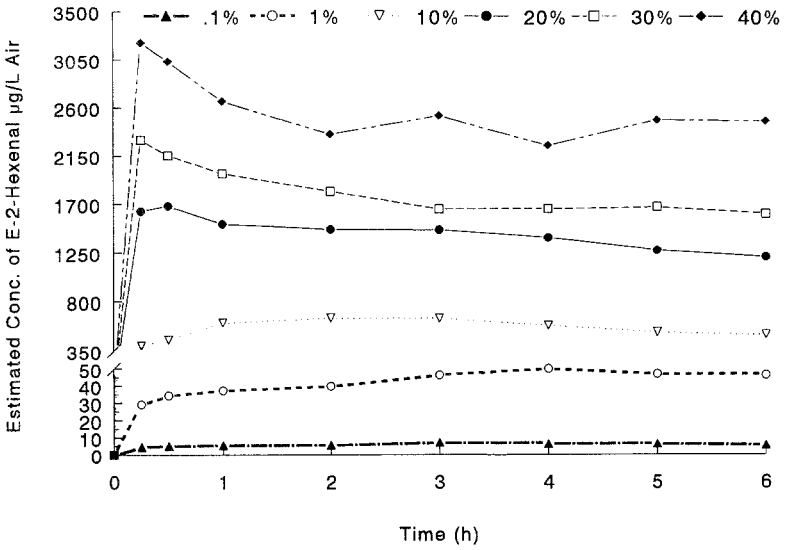

FIG. 1. Estimation of the concentration of (*E*)-2-hexenal in headspace air from different amounts added to the system over time. The percentages are of (*E*)-2-hexenol in the 10 µl 1,2-propanediol added to the 1 ml water in the assemblies as described in Methods and Materials.

previously exposed leaf disk was transferred, and the level was less than 10% of that with continuous exposure to this volatile. In the combination exposure, the aphids and leaf disks were continuously exposed to the test compounds. The aphids and/or leaf disks were exposed to (*E*)-2-hexenol at about 900 µg/liter or (*E*)-2-hexenal at about 450 µg/liter in the system for 6 hr at the beginning of the bioassay. As a test of the effects of leaf senescence promoting volatiles, leaves + aphids were exposed to methyl jasmonate at up to 83 mg/liter headspace (10 µl of pure compound) or ethylene at 0.2 ppm.

Each experiment was repeated at least two times with a minimum of three replications.

RESULTS

Volatile C_6 aldehydes and alcohols significantly reduced aphid population increase (Figure 2). Alcohols at headspace amounts similar to those of the aldehydes had relatively greater activity. Ethanol had much less effect than the six-carbon alcohols. (*Z*)-3-Hexenyl acetate as well as representatives of the terpene hydrocarbons found among the major volatiles produced by certain leaves

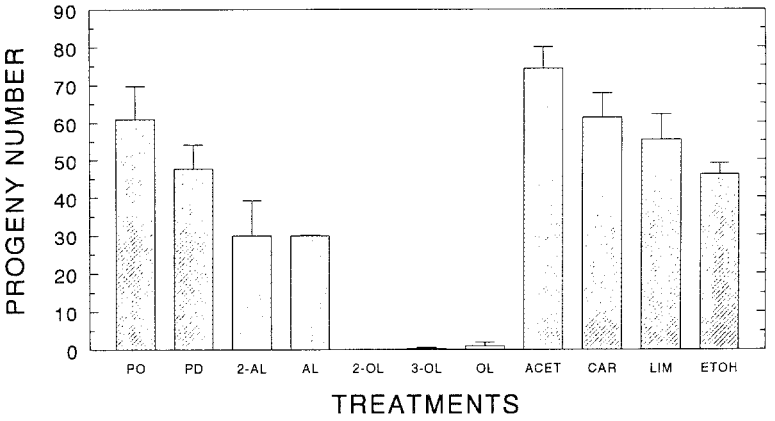

FIG. 2. Effects of the major volatile compounds emitted by tomato leaves on aphid population increase. ETOH (95% ethanol) was added neat for comparison to the volatile alcohols added. CAR (2-carene) and LIM (D-limonene) were diluted in paraffin oil (PO). Paraffin oil and water controls were always equivalent, so one or the other or both were included in the appropriate bioassays. 2-AL[(*E*)-2-hexenal], AL (hexanal), 2-OL [(*E*)-2-hexenol], 3-OL [(*Z*)-3-hexenol], OL (hexanol) and ACET [(*Z*)-3-hexenyl acetate] were diluted in 1,2-propanediol (PD). The compounds were added to the bioassay system to achieve a level of all compounds in the headspace of about 500–1000 μg/liter air. The percentages of these compounds in the carrier were: CAR, 10; LIM, 10; 2-AL; 10 AL, 10; 2-OL, 100; 3-OL, 100; OL, 100 and ACET, 10 (Hamilton-Kemp et al., 1991).

such as tomatoes, 2-carene and D-limonene, had little effect on aphid population increase.

The levels of volatile alcohols and aldehydes in the experimental design were important for determining the impact on aphid progeny production. Reducing the concentration of these compounds reduced their effect on aphid reproduction (Figure 3). Increased levels of the volatile aldehydes further reduced aphid progeny numbers, with the highest level tested resulting in an elimination of reproductive ability (Figures 3 and 4). Aphid reproduction decayed exponentially with increasing (*E*)-2-hexenal. The fitted regression for this result is $P = 44.7 * \exp\{-0.093[(E)\text{-2-hexenal}]\}$ (Figure 4).

The period of exposure of the aphids to the compounds tested was also important. For the experiments described above, aphids were continuously exposed to the test compounds for six days. We repeated the experiment with the volatile aldehydes and alcohols at the same levels as in Figure 2, but only exposed the aphids + tobacco leaf piece to the compounds for 24 hr (Figure 5), which was equivalent to continuous exposure for six days (Figure 2). We subsequently treated the aphids with a 1-hr pulse of these compounds and found

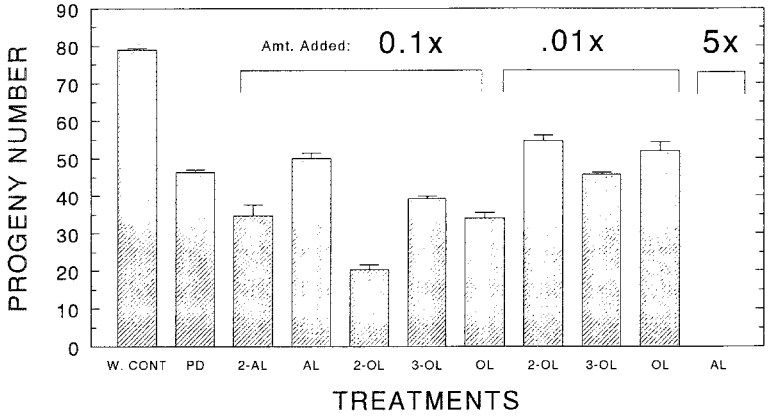

FIG. 3. Effects of different levels of the volatile aldehydes and alcohols on aphid population increase. The abbreviations for the compounds are as in the legend to Figure 2. The first five compounds, shown in the plot after the water and PD controls, were added at 1/10 the level tested in Figure 2 (labeled 0.1 in the Figure). The six-carbon alcohols were added at 1/100 the level in Figure 2 (labeled 0.01 in the figure). Hexanal was tested at five times the level in Figure 2 (labeled 5) and resulted in no progeny production.

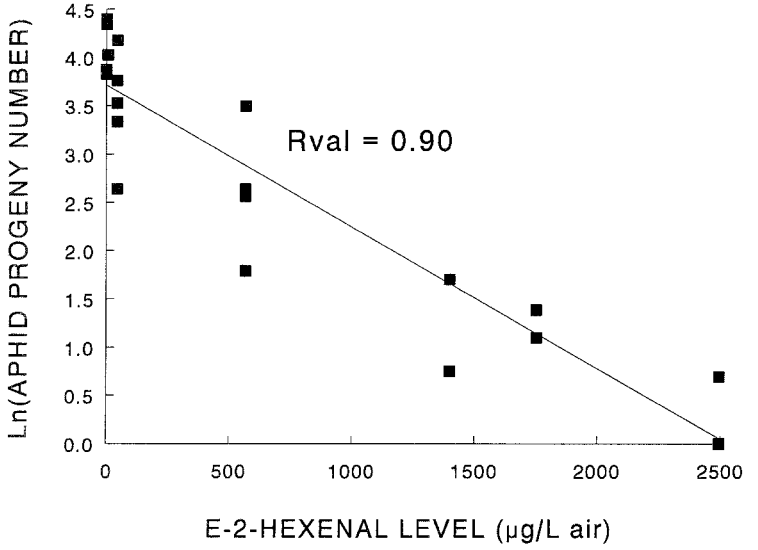

FIG. 4. (*E*)-2-Hexenal (2-AL) concentration effects on aphid population increase. Aphid progeny production was monitored in the presence of 2-AL at the same level as in Figure 2 and at two-, three-, and fourfold higher levels. The levels shown are the averages weighted over time (see Methods and Materials for details).

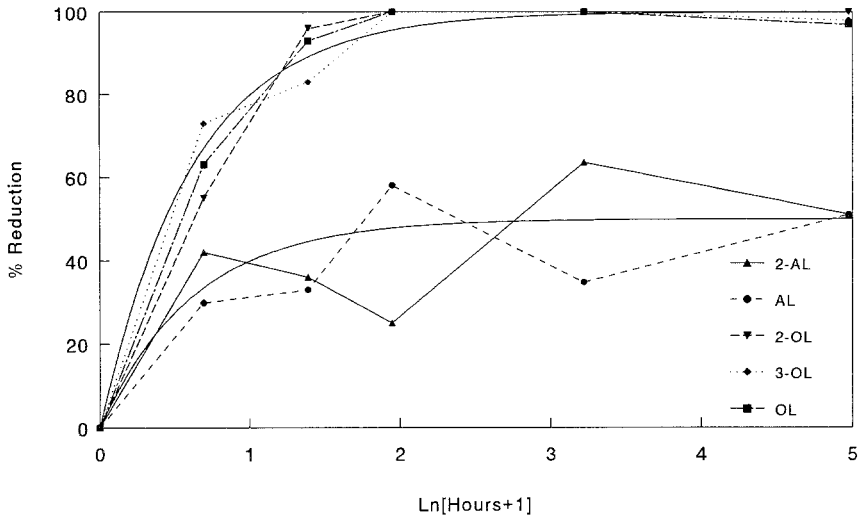

FIG. 5. Effects of time of exposure to volatile alcohols and aldehydes on aphid progeny production. The volatile aldehydes and alcohols were at the level tested in Figure 2, which is approximately 10× the level reached from crushed tomato leaves. Aphids on tobacco leaf disks were continuously exposed to the test compounds for 1, 3, 6, 24, and 144 hr.

a much lower impact. Treatment of aphids with a 6-hr pulse with these compounds was equivalent to the 24-hr treatment, and treatment for 3 hr had an intermediate effect (Figure 5).

Experiments were conducted to ascertain whether the effects of the volatile alcohols and aldehydes in this experimental design were direct or indirect. Treatment of the tobacco leaf alone with (*E*)-2-hexenol had the same large effect as treatment of the leaf + aphids (Table 1). Treatment of the aphids alone with this volatile alcohol had no effect on progeny production. Thus, it appears that some of the indirectly induced changes in the leaves had consequences on aphid reproduction with aphids fed only 6 hr on exposed leaves and then transferred to fresh, untreated leaves (Table 1). The results with one of the volatile aldehydes indicates both direct and indirect effects. About the same direct impact was seen on the aphids themselves and the tobacco leaf on which they fed (Table 1). Some yellowing of the tobacco leaves was observed after treatment with the highest levels of the alcohols and aldehydes. Experiments were therefore conducted to determine if the effects seen were due to induced yellowing and/or senescence. For this, tobacco leaves were exposed to levels of ethylene and methyl jasmonate that gave equivalent or slightly more yellowing, and the impact

TABLE 1. COMPARISON OF DIRECT AND INDIRECT EFFECTS OF A VOLATILE ALCOHOL (*E*)-2-HEXENOL AND ALDEHYDE (*E*)-2-HEXENAL ON APHID POPULATION INCREASE[a]

	Aphid progeny # after 6 days ($N \pm$ SE)		
		(*E*)-2-hexenal[b]	
Treatment	(*E*)-2-hexenol[b] 0.1×	0.1×	0.2×
Control (PD)	44 ± 7	54 ± 7	56 ± 4
Aphid + leaf 6 hr	6 ± 1	36 ± 5	19 ± 3
Aphid 6 hr on leaf	26 ± 4	41 ± 6	30 ± 3
Leaf 6 hr	5 ± 0.4	37 ± 2	11 ± 2
Aphid 6 hr	48 ± 8	39 ± 6	33 ± 5
Senescence induction controls			
Methyl jasmonate		38 ± 2	
Ethylene		47 ± 5	

[a]Control = only 1,2-propanediol added to the system. Aphid + leaf 6 hr = exposure to aphids on the tobacco leaf to the compounds for 6 hr, after which the tobacco leaf + aphids were transferred to another equivalent Petri plate assembly but without the compound (which is the equivalent of the 6-hr treatment in Figure 5). Aphid 6 hr on leaf = exposure of the tobacco leaf + aphids for 6 hr, after which only the aphids were transferred to fresh untreated tobacco leaves. Leaf 6 hr = exposure of a tobacco leaf to (*E*)-2-hexenol or (*E*)-2-hexenal for 6 hr, after which the leaf was transferred to a system without the volatile alcohol and untreated aphids placed on the leaf. Aphid = treatment of the aphids on damp paper towels with the compounds in the system for 6 hr, after which the aphids were transferred to assemblies without the compounds. Methyl jasmonate = continuous exposure of the leaf + aphids to methyl jasmonate. Ethylene = continuous exposure of the leaf + aphids to ethylene.

[b]Levels as in Figure 3. Data presented as Means ± standard error. See Methods and Materials for details.

on aphid reproduction monitored. Ethylene had no effect on aphid population increase and methyl jasmonate had only a small effect (Table 1).

Volatiles produced from uncrushed young tomato leaves reduced aphid population increase, but not so from uncrushed tobacco leaves, (Figure 6). Volatiles produced from crushed leaves of both tomato and tobacco reduced the increase in aphid progeny numbers, with the reduction being greater for crushed tomato leaves. Among the volatiles isolated from crushed tomato leaves, the C_6 compounds were active against aphids, but the terpene hydrocarbons were not (Figure 2). However, the levels of the active compounds were higher than those obtained from crushed tomato leaves. The 0.1× concentration for which some of the aldehydes and alcohols have some activity (Figure 3) represents a level that crushed tomato leaves are capable of producing (Hamilton-Kemp et al., 1991).

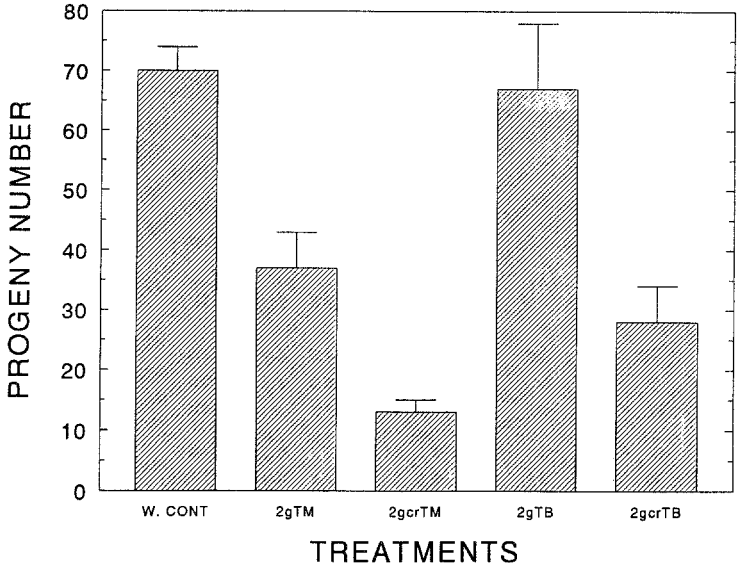

FIG. 6. Effect of leaf-emitted volatiles on aphid population increase during a six-day exposure. W. CONT = control with only water added to the system (Petri plate with tobacco leaf and aphids); 2gTM = 2 g of uncrushed tomato (cv. Mountain Pride) leaves added to the system; 2gcrTM = 2 g of crushed tomato leaves added; 2gTB = 2 g of uncrushed tobacco leaves added; 2gcrTB = 2 g of crushed tobacco leaves added. Data represent the means of 15 replications for control and tomato and nine for tobacco leaves ± standard errors.

DISCUSSION

It has long been suggested that the volatile aldehydes produced by plant tissues are of significance in plant defense against pests. Major et al. (1960) reported that a fungal growth inhibitor extracted from ginkgo leaves was (*E*)-2-hexenal. Lyr and Banasiak (1983) also reported that (*E*)-2-hexenal can be effective in control of a number of plant pests, including the green peach aphid. We found that volatiles from crushed tomato leaves inhibited hyphal growth of *Alternaria alternata* and *Botrytis cinerea*. Representative terpene hydrocarbons from crushed leaves were not inhibitory, but lipoxygenase pathway products such as (*E*)-2-hexenal and (*E*)-2-nonenal were inhibitors of hyphal growth (Hamilton-Kemp et al., 1992). Zeringue and McCormick (1989, 1990) reported that C_6–C_9 alkenals, particularly (*E*)-2-hexenal, produced by cotton leaves, reduced growth of the fungal pathogen *Aspergillus flavus*. They suggested that these compounds function as "gaseous phytoalexins." They found that volatile alcohols, including (*Z*)-3-hexenol, were much less effective. In the studies

reported here, both the volatile aldehydes and alcohols were effective in aphid control. However, the effect of at least one of the volatile alcohols was indirect, and (*E*)-2-hexenal had both direct and indirect effects. The indirect effects of these compounds on defense against pests have not been previously reported. The action of these compounds in inducing aphid resistance in the leaves is not due to promotion of senescence, because exposure of the leaves to levels of ethylene effective in promoting senescence had no effect. Gaseous compounds that have deleterious effects on plant leaves, such as the air pollutants SO_2 and NO_2, are reported to indirectly increase aphid performance on those leaves by altering host-plant chemistry (Brown et al., 1990). We propose that the volatile alcohols and, to some extent, the aldehydes promoted aphid resistance by altering host-plant chemistry such that defenses were enhanced. Methyl jasmonate, which has been shown to promote senescence in plant tissues when applied at high levels (Parthier, 1990), had a small deleterious effect on aphid population increase. It is now well documented that jasmonate [which interestingly is derived from another branch of the lipoxygenase pathway (Vick and Zimmerman, 1987; Hildebrand, 1989)] can induce production of protease inhibitors, which are important insecticidal defenses (Farmer and Ryan, 1990, 1992). Protease inhibitors are not expected to be useful for aphid defense since aphids can ingest free amino acids from the phloem fluids and do not need to rely on digestion of proteins. All the studies reported here are with detached leaf pieces, and it is not yet known how this would work with whole plant systems. However, it might be expected that the induced defenses are equal or higher in a whole plant system. Sams et al. (1975) reported highly significant correlations of results obtained with detached leaves and field evaluations in aphid resistance studies.

From these studies, it is clear that some plant tissues such as young tomato leaves can reduce aphid populations, particularly after leaf damage. We tested representatives of the major volatiles that Mountain Pride tomato leaves produced (Hamilton-Kemp et al., 1991). The volatile aldehydes and alcohols had their principal effect in curtailing aphid population growth. Hexanal is formed from linoleic acid, and (*Z*)-3- and (*E*)-2-hexenals are formed from linolenic acid via lipoxygenase and hydroperoxide lyase (Vick and Zimmerman, 1987; Hildebrand, 1989). The six-carbon alcohols are formed by reduction of the corresponding aldehydes by alcohol dehydrogenase, and (*Z*)-3-hexenyl acetate is formed from (*Z*)-3-hexenol, presumably by an esterase (Hatanaka et al., 1987). It is interesting that the esterified derivative of the volatile alcohol has no activity in the aphid bioassay. Two of the other three major volatiles that are derived from the terpenoid pathway, 2-carene and limonene, had little impact on aphid reproduction at levels up to 10-fold higher than that produced by the macerated tomato leaves. The impact of β-phellandrene is not known. The fact that there was a greater effect of the tomato volatiles after damage to the leaves suggests that the lipoxygenase pathway products were the principal active components

of the tomato volatiles, since these products increase upon damage (Buttery et al., 1987; Hamilton-Kemp et al., 1991).

The impacts of the volatile aldehydes and alcohols were quantitative. This is consistent with a horizontal resistance type mechanism for which there is a low probability that pests will develop resistance (Stipanovic, 1983). The combination of high production of volatile aldehydes and alcohols could be a pest defense system with the direct effects of the aldehydes and the indirect effects of both groups of compounds in inducing other defenses. It is well known that aldehydes have antibiological effects. Miglietta et al. (1987) reported that aerobic and anaerobic products of lipid peroxidation, such as hexanal and pentanal, are cytotoxic. Carbonyl compounds with α, β unsaturation such as (*E*)-2-hexenal are generally toxic to biological tissues and may be important in hypersensitive responses (Schauenstein et al., 1977; Feron et al. 1991; Croft et al., 1993). The results reported here indicate that the volatile alcohols have more of an indirect effect. In addition to the direct and indirect effects these volatile alcohols and aldehydes might have on herbivorous pests, they might also be useful in attracting parasitoids of these pests (Turlings et al., 1990) and perhaps predators as well.

The production of these compounds is usually wound-induced. It is generally thought that aphids cause minimal damage to plant cells except the actual phloem cells penetrated by the stylets when feeding. However, Spiller et al. (1985) reported that aphids probe for phloem sieve elements by both inter- and intracellular routes. Mesophyll cells may be penetrated, leading to irrevocable damage to chloroplast, plasmalemma, tonoplast, and mitochondrial membranes, presumably by physical or enzymatic action of aphid stylets and saliva (Spiller et al., 1985). It has not yet been clearly elucidated what the cellular and subcellular sites for formation of these compounds are. However, recent studies have shown that high levels of at least one of the key enzymes in this pathway, lipoxygenase, are found in bundle sheath and paraveinal mesophyll cells of soybean leaves (Tranbarger et al., 1991). These are some of the most likely cells to be damaged by aphid feeding. Further, phloem exudates from cucumber stems were found to have very high lipoxygenase activity (Avdiushko, personal communication).

It should be possible to engineer plants genetically for increased production of C_6 aldehydes and alcohols. As mentioned above, the biochemistry of the formation of these compounds is fairly well understood. Moreover, the substrates for the formation of these compounds are abundant components of most plant tissues. In leaves, the precursor of the unsaturated aldehydes and alcohols, linolenic acid, is often more than 1% of the dry weight. However, the mechanism of metabolic control of the conversion of polyunsaturated fatty acids into volatile oxidation products is not yet elucidated.

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SEQUESTRATION OF PYRROLIZIDINE ALKALOIDS IN SEVERAL ARCTIID MOTHS (LEPIDOPTERA: ARCTIIDAE)

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Abstract—Sequestration of dietary pyrrolizidine alkaloids (PA) by larvae and adults of six European arctiid moth species (*Spilosoma lubricipeda*, *Arctia caja*, *Phragmatobia fuliginosa*, *Callimorpha dominula*, *Diacrisia sannio*, and *Tyria jacobaeae*) was investigated for comparison with the well-studied Asian arctiid *Creatonotos transiens*. Larvae of all species metabolized free PA bases into the respective *N*-oxides. Only adults of *A. caja*, *P. fuliginosa*, and *S. lubricipeda*, but not their larvae, converted dietary 7(*S*)-heliotrine to 7(*R*)-heliotrine, a direct precursor of a male pheromone in some arctiids, 7(*R*)-hydroxydanaidal. The larval integument figures as the main storage site for resorbed alkaloids; only minor amounts were found in other tissues. In addition, a significant amount of alkaloid is deposited in the cocoon of *Arctia caja*; only traces of alkaloids could be found in the meconium and the exuviae of this species. A substantial part of the alkaloids fed was degraded to unknown, nonalkaloidal products.

Key Words—*Spilosoma lubricipeda*, *Arctia caja*, *Phragmatobia fuliginosa*, *Tyria jacobaeae*, *Diacrisia sannio*, *Callimorpha dominula*, pyrrolizidine alkaloids, Lepidoptera, Arctiidae, alkaloid sequestration, biotransformation, chemical defense.

INTRODUCTION

The sequestration of plant-derived, dietary allelochemicals (secondary compounds) figures as an important defense strategy in herbivorous arthropods,

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especially in Lepidoptera. The storage of cardenolides and alkaloids, such as pyrrolizidine alkaloids (PA) (Figure 1) and quinolizidine alkaloids (QA), in danaids, arctiids, pyralids, and aphids are well-known examples and have been examined in some detail (Brower et al., 1984; Boppré, 1990; Wink, 1992). Since the first discovery of PA sequestration in *Tyria jacobaeae* (Aplin et al.,

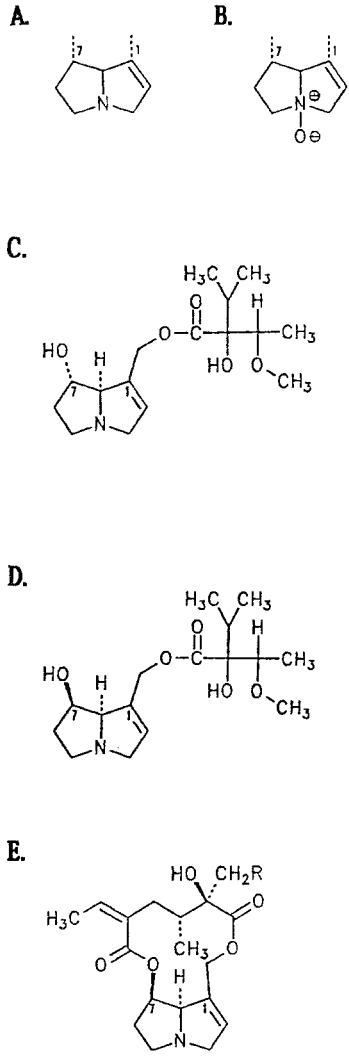

FIG. 1. Structures of pyrrolizidine alkaloids: (A) tertiary alkaloid, (B) alkaloid *N*-oxide, (C) 7(*S*)-heliotrine, (D) 7(*R*)-heliotrine, (E) senecionine.

1968), a number of other arctiids from Africa or the Malayan Archipelago were shown to possess a similar capability (Rothschild, 1985; Boppré, 1986, 1990). It has been assumed that the alkaloids taken up serve as chemical protectants against predators and that PAs have a defense function in all life stages and are even transferred from males to females during mating and from females to their eggs during oviposition (Eisner, 1980; cf. Boppré, 1986; Dussourd et al., 1988, 1989; Nickisch-Rosenegk et al., 1990b).

N-oxides of dietary PAs are probably resorbed by epithelial cells of the larval midgut by a carrier-mediated process, according to in vitro and in vivo bioassays using larvae of the south Asian arctiid *Creatonotos transiens* (Wink and Schneider, 1988; Nickisch-Rosenegk et al., 1990b). In *C. transiens*, the final site of alkaloid accumulation is the integument, where about 90% of the alkaloids are stored (Egelhaaf et al., 1989; Nickisch-Rosenegk et al., 1990b). Some male arctiids transform pyrrolizidine alkaloids into sex pheromones, which attract the female in short-distance courtship behavior (Conner et al., 1981; Wunderer et al., 1986). In *C. transiens* 7(*S*)-heliotrine, therefore, is metabolized by stereochemical inversion of the hydroxyl group into 7(*R*)-heliotrine, which serves as a direct precursor for its pheromone, 7(*R*)-hydroxydanaidal (Bell and Meinwald, 1986; Wink et al., 1988).

In this study we investigated whether the results obtained with the Asian *C. transiens* concerning alkaloid biotransformation and the site of alkaloid storage are also valid for other European arctiids, including the taxa *Spilosoma lubricipeda*, *Arctia caja*, *Phragmatobia fuliginosa*, *Diacrisia sannio*, *Callimorpha dominula*, and *Tyria jacobaeae*.

METHODS AND MATERIALS

Insect Species, Growing Conditions, Feeding Procedures. Adult female moths of *Spilosoma lubricipeda* L., *Arcia caja* L., *Callimorpha dominula* L., *Phragmatobia fuliginosa* L., *Diacrisia sannio* L., and *Tyria jacobaeae* L. were captured during summer by a light trap at the Max-Planck-Institut für Verhaltensphysiologie in Seewiesen, Bavaria, and were allowed to lay eggs. Hatched larvae were reared on suitable food material, which was changed daily: *Urtica dioica*, *Vincetoxicum hirundinaria*, *Atropa belladonna*, and *Taraxacum officinale* served as plant species free from PAs. *Tussilago farfara* and *Senecio fuchsii* contained PAs. Only the larvae of *Arctia caja* accepted artificial diet, as described in Bergomaz and Boppré (1986) but without preservatives (except sorbic acid) and antibiotics. Last-instar larvae of all species studied were kept singly in Petri dishes and were fed with a defined amount of the PA, 7(*S*)-heliotrine (Chemasea, Manuf. Pty Ltd., Sidney, Australia) (the alkaloid solution was adjusted

to pH 7.0) on leaf disks (0.5 × 0.5 cm; dose per disk was 1 mg of heliotrine). Larvae of *A. caja* were fed with a piece of diet (0.5 × 0.5 × 0.5 cm) prepared with a defined amount of alkaloid solution.

Only three of 10 larvae fed with leaf disks ate the sample completely, which made statistical treatment of the results difficult. Forty-eight hours after consumption of the alkaloid sample, all larvae were dissected into organ fractions [gut (including content), hemolymph, fat body, and integument]. Feces were collected in time intervals of 6 hr. Adult moths were collected at the day of eclosion. All samples were frozen immediately and stored at −20°C prior to alkaloid extraction.

Alkaloid Extraction and Gas–Liquid Chromatography (GLC). Techniques were carried out as described in Nickisch-Rosenegk et al. (1990b). Animals or tissues were ground in a mortar in 5–10 ml 0.5 M HCl. Zinc powder was added to reduce PA *N*-oxides. The homogenates were left standing for at least 5 hr at room temperature, occasionally mixed by shaking. For determination of *N*-oxides, homogenates were divided into two samples of equal volume. One was reduced with zinc (= total alkaloids, including *N*-oxides); the other was directly extracted (= free alkaloid bases). Then, they were made alkaline with 4 M NaOH and poured onto Chem elut columns (Analytichem, ICT, Frankfurt/M.) for solid-phase extraction with CH_2Cl_2 as an eluent. The eluate was evaporated in vacuo. Crude pyrrolizidine extracts were taken up in MeOH and analyzed by capillary GLC. The instrument used was a Varian 3300 gas chromatograph equipped with a nitrogen-specific detector. Column: DB1, 30 m × 0.3 mm, film thickness 1 μm. GLC conditions: oven: 150–300°C with 20°C/min, then 5 min isothermal. Injector: 250°C, split injection 1:20; detector: 300°C; carrier gas: helium, 90 kPa; makeup gas: nitrogen. 7(*S*)-Heliotrine or senecionine (kindly provided by R. Molyneux, USDA, Albany) were used as external standards for quantification (Spectra Physics Integrator SP 4270). 7(*S*)-Heliotrine and its metabolite 7(*R*)-heliotrine had been identified in previous studies (Wink et al., 1988; Egelhaaf et al., 1989) by GLC-MS.

RESULTS

Spilosoma lubricipeda. Between 26% and 53% (Table 1) of the ingested 7(*S*)-heliotrine was recovered either from the larvae or the larval feces of *S. lubricipeda* irrespective of whether the larvae were fed plant material with or without natural PAs. The rest of heliotrine (i.e., 47–74%) was metabolized to nonalkaloidal compounds that were not further investigated.

The major part of the heliotrine recovered (total amount = 100%) was excreted by the larvae (36–88%), whereas 12–64% was stored in the larval body

TABLE 1. LARVAE OF *Spilosoma lubricipeda* REARED ON PA-CONTAINING PLANTS *Senecio fuchsii* OR *Tussilago farfara* OR PA-FREE PLANTS *Urtica dioica*, *Vincetoxicum hirundinaria*, *Atropa belladonna*, *Taraxacum officinale*[a]

Food plant	Dose (mg)	Distribution of PA (μg) (N = 3–4)					Fate of PA's (dose = 100%)		
		Feces	Hemolymph	Gut	Integument	Σ PA	Storage	Excretion	Degradation
A.									
PA-free plants									
Urtica dioica[b]	2	240	116	38	264	258	20.9	12.0	67.1
Urtica dioica[b]	2	361	59	14	155	589	11.4	18.1	70.5
V. hirundinaria	2	549	30	0	134	713	8.2	27.5	64.3
Atropa belladonna	2	268	22	21	203	514	12.3	13.4	74.3
PA plants									
Tussilago farfara[b]	2	539	22	24	130	715	8.8	27.0	64.2
Senecio fuschsii[c]	2	496	0	18	50	564	3.4	24.8	71.8
B.		Distribution of PA (μg) [N-oxides (%)] (N = 2)							
T. officinale									
Male	3	1224 (17)	72 (100)	13 (100)	276 (82)	1585	12.0	40.8	47.2
Female	3	449 (16)	87 (100)	22 (100)	309 (100)	876	13.9	15.0	71.1

[a] In the last larval stage they were fed with pure 7(*S*)-heliotrine. After 48 hr, larvae were dissected into different organ fractions, which were extracted and analyzed by capillary GLC (total alkaloid including N-oxides). In the case of *Taraxacum officinale*-reared larvae the percentage of N-oxides was determinated separately.

[b] Alkaloid contents: < 100/g.

[c] Alkaloid content: < 40 μg/g.

(Table 1). Between 63% and 83% of the sequestered heliotrine was detected in the integument, whereas hemolymph and gut contained similar amounts up to 10%. Food plant-specific alkaloids (i.e., those of *Tussilago farfara* and *Senecio fuchsii*) were usually not detected. They were recorded in the sex organs of one male larva, but not in other tissues.

Although the free alkaloid base was applied, almost only heliotrine-*N*-oxide was detected in hemolymph and integument including fat body (Table 1B); thus a nearly complete biotransformation (82–100%) had taken place. No stereochemical inversion of 7(*S*)-heliotrine into 7(*R*)-heliotrine could be observed in the larvae. The only imago (female) analyzed contained 90% of the stored alkaloid as 7(*R*)-heliotrine.

Larvae of *S. lubricipeda* were reared on PA plants and PA-free species, prior to dosing. Table 1 implies that previous alkaloid experience had no substantial influence on alkaloid metabolism and sequestration.

Arctia caja. In a first set of experiments, larvae of *A. caja* were reared on PA-rich *Senecio jacobaea* plants, which produce PA diesters, such as senecionine and seneciphylline (Figure 1). The larvae were not treated with heliotrine or other pure PAs. The main site of alkaloid storage was the integument, which contained 64–76% of total alkaloids (Table 2). A substantial amount could be detected in the hemolymph (19–21%) and in the gut (3–8%). [In the last larval stage (L5), dietary alkaloids were resorbed completely, so that no alkaloids appeared in the feces.]

In order to examine the time course of PA uptake, radioactive (tritiated) heliotrine (1 mg "cold" heliotrine plus 10,000 cpm tritiated heliotrine) was fed to two L5 larvae of *A. caja*. Aliquots of 5 μl hemolymph were collected by a small hypodermic syringe at 10 minute intervals over a period of 1 hr (Figure

TABLE 2. DISTRIBUTION OF DIETARY ALKALOIDS (FROM PLANT ORIGIN ONLY) IN LARVAE OF *Arctia caja* REARED ON PA-CONTAINING PLANT *Senecio jacobaea*[a]

	Organ distribution of *Senecio*-alkaloids in larvae									
	Hemo-lymph		Gut		Fat body		Integument		Σ PA	
	M	F	M	F	M	F	M	F	M	F
Alkaloid content (μg/g)	110	104	44	19	34	18	340	419	528	560
Alkaloid (μg)/tissue	158	202	63	36	49	22	478	790	748	1050
Distribution (%)	21	19	8	3	7	2	64	76	100	100

[a]Larvae were dissected in the last larval instar (L5). Alkaloids per tissue were quantified by means of GLC. n = 3. M = male; F = female.

2). The animals continued feeding, hardly irritated by the sampling procedure. Within 10 min after ingestion, radioactivity appeared in the hemolymph, equivalent to 37% of the applied alkaloid. After 30 min, radioactivity could not be measured any longer. These results indicate an immediate uptake of alkaloids from the lumen of the gut and also a rapid transfer from hemolymph to other tissues, probably the integument.

In a second set of experiments, defined amounts of 7(*S*)-heliotrine were applied to larvae reared on PA-free *Taraxacum officinale* (Table 3). In this experiment, a high metabolic turnover of the dietary PA was determined, since only 10–20% of the initial dose was recovered from the animals (fourth and fifth instar) including their frass. Of the alkaloids sequestered, again the larval integument was the major site of accumulation (alkaloid content 71–92% of total alkaloids). Only minute amounts of 7(*S*)-heliotrine were converted by the larvae into 7(*R*)-heliotrine.

In order to study alkaloid metabolization and distribution in adult moths,

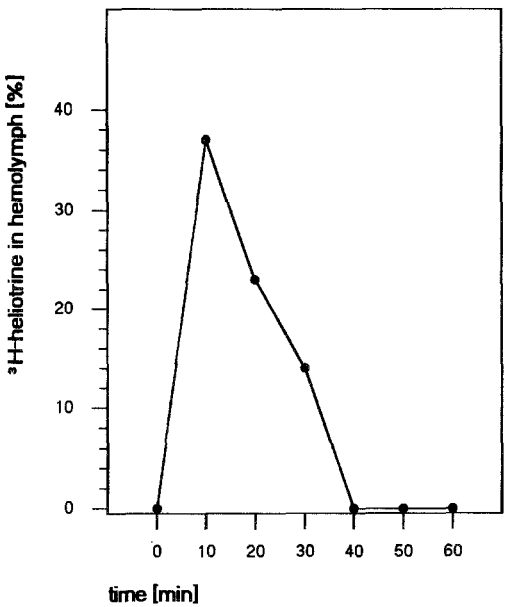

FIG. 2. Time course of [^{3}H]heliotrine in the hemolymph of two larvae of *Arctia caja* (mean values). Larvae were fed with both "cold" heliotrine (1 mg) and 10,000 cpm of tritiated heliotrine on a leaf disk. After ingestion of the sample within 5 min, volumes of 5 μl hemolymph were taken every 10 min from the middle of the lateral abdomen. Total radioactivity was calculated assuming a volume of 500 μl of total hemolymph.

TABLE 3. LOCALIZATION OF HELIOTRINE IN TISSUES OF *Arctia caja*, *Diacrisia sannio*, *Phragmatobia fuliginosa*, *Callimorpha dominula*, and *Tyria jacobaeae* (INDIVIDUALS)[a]

	PA dose (mg)	PA content/tissue (fraction) (μg)					Recovery (%)
		Feces	Hemolymph	Gut	Integument	Σ PA	
Arctia caja							
L5[b]	2.727	105	50	11	200	346	12.7
L5[c]	1.600	11	27	16	173	227	14.2
L4[c]	0.448	11	11	4	62	88	19.6
L4[c]	0.440	11	2	2	47	62	14.1
L3[c]	0.047	10			6	16	34.0
L3[c]	0.039	8			28	36	92.3
L3[c]	0.037	3			13	16	43.2
Diacrisia sannio[c]	1.260	82	4	12	152	250	19.8
Phragmatobia fuliginosa[c]	1.520	107	21	14	291	433	28.5
	1.269	32	25	4	164	225	17.7
Callimorpha dominula	1.000	20	0	34	125	179	17.9
	1.000	223			115	338	33.8
	0.500	121	0	37	91	249	49.8
Tyria jacobaeae	1.000	25			103	128	12.8
	2.000	45			148	193	9.7
	3.000	942			128	1070	35.7
	4.000	196			241	437	10.9

[a]Larvae were reared on *Taraxacum officinale*, except for *T. jacobaeae*, which was reared on *Tussilago farrara*. All animals were fed in the last larval stage with PA (if not otherwise stated). PA contents of fat body were included in the values of the integument.
[b]Dose = 3 × body weight.
[c]Dose = 2× body weight.

L5 larvae that had been kept continuously on a PA-free semisynthetic diet, were fed with doses of 1–3 mg 7(*S*)-heliotrine. After pupation, the freshly emerging adults were analyzed (Table 4). About 26–47% of the initial PA could be recovered, indicating a substantial degree of alkaloid degradation. Whereas only small amounts of heliotrine were recovered from the larval feces (<3%), 87–99% of heliotrine was detected in the bodies of the moths, 92–98% of these alkaloids were transformed to *N*-oxides and 20–30% of 7(*S*)-heliotrine was converted into 7(*R*)-heliotrine (Figure 3). No substantial difference between the sexes could be observed; i.e., the degree of stereochemical inversion was similar in males and females, which is in contrast to the situation in *Creatonotos transiens*, where male larvae converted twice as much heliotrine as the females (Nickisch-Rosenegk et al., 1990b). Only traces of alkaloids were localized in

TABLE 4. DISTRIBUTION OF DIETARY HELIOTRINE IN ADULT MOTHS OF *Artia caja*[a]

PA dose (mg)	Organ distribution of alkaloids (μg)/fractions												Recovery (%)	
	Adult body		Meconium		Pupal exuviae		Cocoon		Feces		Larval exuviae			
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
1	413	254	1	4	1	2	0	0	2	3	0	0	41.7	26.3
2	740	625	0	0	1	0	98	23	11	8	0	0	42.5	32.8
3	1271	1165	6	1	2	2	80	0	37	7	2	0	46.6	39.2

[a]The larvae were reared on a semisynthetic diet (Bergomaz and Boppré, 1986) up to the last larval stage (L5). After feeding heliotrine in L5 (1, 2, 3 mg on a piece of diet), the larvae were continuously supplied with alkaloid-free diet until pupation. All feces of each animal after application of heliotrine were collected. The emerged adults, their meconium, cocoons, and exuviae were also examined for alkaloid content by capillary GLC. $N = 3$. M = male; F = female.

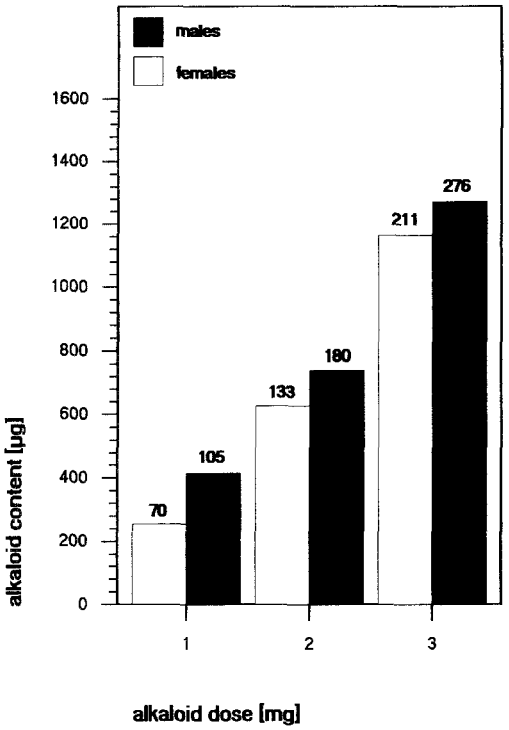

FIG. 3. Storage rates of heliotrine metabolites in male and female adults of *Arctia caja*. Columns include the amounts of 7(*R*)- and 7(*S*)-heliotrine. The amounts (in micrograms) of 7(*R*)-heliotrine are given at top of the columns ($N = 3$).

the meconium, the larval and the pupal exuvia, whereas cocoons contained substantial amounts of up to 100 µg of alkaloids (Table 4).

Phragmatobia fuliginosa. From 72% to 83% of the initial dose of heliotrine was metabolized by larvae into nonalkaloidal products (Table 3). 7(*S*)-Heliotrine was preferably stored in the integument. Neither 7(*R*)-heliotrine nor other PA metabolites were detected in this experiment. In one male and one female moth, which had been fed with 7(*S*)-heliotrine as larvae, significant amounts of 7(*R*)-heliotrine (40–50% of recovered alkaloids) were detected. The percentage of alkaloid *N*-oxides was not determined.

Tyria jacobaeae, Callimorpha dominula, Diacrisia sannio. Only a few larvae could be treated successfully with a defined amount of alkaloid; many others refused the alkaloid sample, at least partially. Alkaloid degradation was rather high in that 30–90% of the initial alkaloid was metabolized to nonalkaloidal compounds. From 55% to 90% of total alkaloids recovered from the

insects was stored in the body of the animals; 10–45% eliminated with the feces (Table 3).

DISCUSSION

Metabolism of Dietary PAs in Insects. Dietary PAs serve as precursors for the male pheromone 7(*R*)-hydroxydanaidal (Figure 1) in species of the genera *Creatonotos*, *Utetheisa*, *Phragmatobia*, and others (Conner et al., 1981; Schneider et al., 1982; Boppré and Schneider, 1985; Krasnoff et al., 1987). The hydroxyl group at C-7 of most PAs, present in plants, is in the *R* configuration, so that the PAs can be used directly as precursors. By stereochemical inversion of naturally occurring *S*-configured PAs, such as heliotrine, moths, such as *Creatonotos* (as an additional adaptation) are thus able to use both *R*- and *S*-configured PAs for pheromone production (Wink et al., 1988). In *Creatonotos*, even the larvae convert 7(*S*)-heliotrine into 7(*R*)-heliotrine (Nickisch-Rosenegk et al., 1990b). None of the arctiid larvae investigated in this study showed this capacity, but adult moths of *Arctia caja*, *Phragmatobia fuliginosa*, and *Spilosoma lubricipeda* contain significant amounts of 7(*R*)-heliotrine in their bodies. We assume, therefore, that in these species the transformation takes place during the pupal period. In addition, male moths of *Creatonotos* and *Phragmatobia fuliginosa* possess tubular abdominal scent organs (corema), which are inflatable and covered with hairs emitting the PA-derived pheromone 7(*R*)-hydroxydanaidal (Schneider et al., 1982; Krasnoff et al., 1987; Boppré, 1990; Egelhaaf et al., 1992). Other arctiids have hair brushes instead, i.e., *Arctia caja* or *Tyria jacobaeae*. Although *A. caja* and *Spilosoma lubricipeda* (with corema) are able to convert 7*S*-configured heliotrine into 7(*R*)-heliotrine they are not known to produce alkaloid-derived pheromones (cf. Birch et al., 1990). The significance of this metabolic process in the latter species remains to be elucidated.

Another aspect is the biotransformation of free PA bases into PA *N*-oxides by arctiid larvae. In many plants, PAs are mainly stored as *N*-oxides, whereas in others, the free bases are predominant (Hartmann et al., 1989; Vrieling, 1991). In *Creatonotos transiens*, nearly 100% of "free" PAs are converted into *N*-oxides within an interval of 2 hr after ingestion (Nickisch-Rosenegk et al., 1990b). This metabolic process seems to be a general phenomenon since it was also detectable in other species in this study (Table 1) and *N*-oxides have been reported to be present in insects by other authors, too (Rothschild et al., 1979). In some of our experiments, we found a high degree of free PAs in the larval frass (Table 1). This is probably due to the fact that a substantial proportion of dietary PAs is not completely resorbed by the larvae; i.e., these alkaloid molecules thus pass through the insect untransformed. Since PA *N*-oxides cannot

pass through biomembranes by simple diffusion, it might be easier for a PA-sequestering insect to control and concentrate these molecules in defined places, such as the integument. The free base would always diffuse according to concentration gradients.

A significant part of the resorbed PAs is metabolized into other, nonalkaloidal products, whose identity still needs to be established (Tables 1–4). Depending on the size of the larvae, uptake and storage capacity may be limiting. The surplus alkaloids are either eliminated or degraded, which would explain the varying detoxification rates and the different amounts of alkaloid in the feces of individual larvae.

The capacity to take up, process, and store PAs seems to be a constitutive feature. As shown for *Spilosoma lubricipeda* in our experiments, no significant difference in the storage capacity of the larvae was observed, whether they were reared on PA-containing plants or PA-free plants to which PA was added. In both feeding regimes (Table 1) mentioned, the compounds were taken up and stored in the integument at comparable rates. We assume that whenever the larvae of this and other species come in contact with PA plants in the wild, PA sequestration will be the consequence. This capacity does not depend on an earlier contact in younger larval stages. Probably, the midgut carrier system for PAs is constitutively expressed, since labeled PAs turned up in the hemolymph of *Arctia caja* larvae within 10 min, even though they had had no previous feeding experience with PAs (Figure 2).

Sequestration of Allelochemicals in Insect Integuments. In general, all the arctiids examined sequester dietary pyrrolizidine alkaloids, predominantly in their integuments. The deposition of defense chemicals in the integument may be a common strategy in insects: For example, quinolizidine alkaloids (QA) are stored in the larval integument of *Uresiphita reversalis*, a pyralid moth, which lives on alkaloid-containing legumes such as *Teline monspessulana*. The brightly colored ctenuchid moth (*Syntomeida epilais*), the monarch (*Danaus plexippus*), and the milkweed bug (*Oncopeltus fasciatus*) sequester cardiac glycosides from their larval food plant in their integuments (Parsons, 1965; Rothschild and Reichstein, 1976; Brower et al., 1984; Scudder et al., 1986; Wink et al., 1991; Wink, 1992; Wink and Schneider, 1988; Nickisch-Rosenegk et al., 1990a). Furthermore, cyanogenic glycosides are stored in special cuticular cavities by larvae of *Zygaena* (Franzl and Naumann, 1985; Franzl et al., 1988).

Adult *Danaus plexippus* contain the highest amounts of cardenolides in cuticle and wings (Brower et al., 1988). Therefore, certain avian predators of the monarch discard these tissues, swallowing only the contents of the thorax and abdomen, which are lower in cardenolide concentration. Brower et al. (1984) also found larval exuviae extremely rich in sequestered cardenolides. This is not the case with arctiids and PAs. In most of the larval and pupal exuviae examined, we detected no alkaloids or only trace amounts, whereas adult *Ure-*

siphyta reversalis lose all stored QAs at eclosion in the pupal exuviae and the cocoon (Wink et al., 1991). The meconium of *Creatonotos* contains up to 10% of the stored alkaloids, 90% were retained in the body (Nickisch-Rosenegk et al., 1990), and in *Arctia caja* the cocoon was found to contain PAs, but no alkaloids were found in the meconium.

It remains to be shown, for each insect species, which special function applies to the storage of various secondary plant substances in the integument: avoiding self-intoxication, defense strategy, or a combination of both.

PAs and Insects. Although a number of insect species including some in the Lepidoptera are attracted by decaying or flowering PA plants (Pliske, 1975; Boppré, 1986), they may not necessarily exploit dietary PAs for a particular function. Certain enzymatic and behavioral specializations are plausible in PA specialists such as *Utetheisa* or *Creatonotos* using PAs as chemical defense compounds in all life stages, as a pheromone precursor, or as a morphogen in the development of the coremata (in *Creatonotos*), which react to PAs as a dose-dependent growth factor (Schneider et al., 1982; Boppré and Schneider, 1985).

Several populations of *Tyria jacobaeae* exist that differ in their choice of food plants. In the Netherlands, the PA plant *Senecio jacobaea* is a preferred host plant, and the insects were shown to sequester PA (Vrieling, 1991). In the Bavarian Alps, we observed one population feeding on *Petasites paradoxus*, a plant that contains only trace amounts of PAs. As a consequence, *T. jacobaeae* larvae were PA-free. In close vicinity to the host plants, PA-rich species such as *Adenostyles glabra*, *Senecio fuchsii*, or *S. alpinus* occurred but were not accepted as larval food plants. Other alpine populations of *T. jacobaeae* feed on *Adenostyles glabra* but not on *Petasites* growing nearby; these larvae had accumulated substantial amounts of PA, such as seneciphylline. When larvae that had lived on *Petasites* were transferred (experimentally) to PA host plants, such as *Adenostyles*, PA sequestration was always observed (Wink unpublished). Another example for the arbitrary use of PA-producing or PA-free food plants is *Creatonotos transiens* itself. Most records of food plants, surprisingly, do not include PA plants (Boppré and Schneider, 1990), although under experimental conditions PA-containing food is clearly preferred (Nickisch-Rosenegk and Wink, 1993).

In conclusion, all taxa of the arctiid family studied so far seem to be able to store pyrrolizidine alkaloids (Rothschild, 1985; Boppré, 1990). Whether a particular function has not been elucidated yet or whether PA sequestration is an "evolutionary relict" in some species remains an open question.

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EVIDENCE FOR VOLATILE MALE-PRODUCED PHEROMONE IN BANANA WEEVIL *Cosmopolites sordidus*

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Abstract—Females of the banana weevil, *Cosmopolites sordidus*, were attracted to and made longer visits to live conspecific males, trapped volatiles from males, and dissected male hindguts in a still-air olfactometer. Male weevils were attracted to volatiles trapped from males and made longer visits to live males and volatiles from males. Live females, collected volatiles from females and female hindguts, elicited small or no behavioral responses from either sex. Electroantennogram (EAG) responses from both male and female antennae were elicited by collected volatiles from males and by dichloromethane extracts of male hindguts and bodies but not by surface washes of males. No significant EAG responses were given to equivalent material from females. It is therefore suggested that male banana weevils release an aggregation pheromone via their hindgut.

Key Words—Coleoptera, Curculionidae, *Cosmopolites*, hindgut, olfactometer, aggregation, EAG.

INTRODUCTION

The banana weevil, *Cosmopolites sordidus* (Germar) is a pest, particularly of cooking bananas (Sikora et al., 1989) and plantains (Jones, 1986), in all major banana-growing areas of the world (Ostmark, 1974; Waterhouse and Norris, 1987). The female weevil lays eggs in the rhizome of the plant. The larvae hatch and then feed and tunnel in the rhizome, weakening the plant, reducing

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bunch weight and, in serious cases, leading to snapping of the rhizome at ground level before the bunch is ripe. Adult weevils cause insignificant damage and feed mainly on rotting banana tissue.

Few studies have been carried out on the pheromones of the banana weevil, although Cuille (1950) reported on an "interattraction" between the sexes resulting from contact chemoreception. He found no evidence for any volatile pheromones. Here we report on studies carried out using a dual-choice still-air olfactometer and electroantennograms that indicated the release of a volatile pheromone by male *C. sordidus*.

METHODS AND MATERIALS

Insects. C. sordidus used were collected from split banana pseudostem traps in a banana plantation in Nairobi. Weevils (~120) were kept with pseudostem from the east African highland (AAA) cooking banana cultivar Githumo, which has been observed to be heavily attacked in the field, in 4.5-liter plastic jars at 24 ± 2°C, 12:12 light–dark photoperiod.

Collection of Volatiles and Extracts. Simultaneous volatile collections were made separately from the headspaces above 100 male and 100 female weevils on two occasions. The weevils had been kept separately according to sex for at least one week before the collection and were put in 5-liter glass flasks with damp cotton wool. Air was drawn through the flasks at 0.4 liters/min for 24 hr. Air was cleaned by passing through a column (8 × 2 cm ID) containing 7.1 g of Porapak Q (80–100 mesh Chrompack). Volatiles released by the weevils were trapped on a similar column. Volatiles on the trap columns were then eluted with 15 ml of dichloromethane (Aldrich, HPLC grade). Subsequently, further collections of volatiles were made separately from 50 male and 50 female weevils on four occasions. The weevils had been kept together and were sexed just prior to volatile collection. The weevils were placed in small glass containers (15 × 4 cm ID) with damp cotton wool. Air was drawn through the containers at 100 ml/min for 24 hr. Air was cleaned by passing through columns (20 × 3 mm ID) containing ~0.1 g of activated charcoal (80–100 mesh, Chrompack). Volatiles released by weevils were trapped on similar columns. Volatiles from the trap columns were eluted with 1 ml of dichloromethane.

Simultaneous collections of volatiles, for use as stimuli for EAG recordings, were made on three occasions from zero (as controls), one, three, and nine weevils of each sex. The weevils (which had been collected from the field within the previous three days) were put in glass tubes (10 cm × 8 mm ID) together with a damp piece of cotton wool. Air (~4 ml/min) was cleaned by passing through activated charcoal before entry, and the volatiles were trapped by a column of Porapak Q (5 cm × 3 mm ID). Collections were made between

1800 hr and 0900 hr (i.e., with 12 hr of scotophase and 3 hr of photophase). Volatiles were eluted from the columns with 1 ml of dichloromethane.

Surface washes and extracts of the hindgut and the remainder of the body were made for a group of 20 males and a group of 20 females using dichloromethane. Surface washes were made by dipping dry insects (previously rinsed with water to remove plant material), for about 5 sec in 5 ml of dichloromethane. Hindguts from the same insects were dissected and placed in 5 ml of dichloromethane, and the remainder of the body was treated similarly. After 24 hr, the dichloromethane solutions were filtered, and the extracts and washes were concentrated to 0.5 ml under a nitrogen stream. All of the dichloromethane solutions were stored in vials in the dark at $-20°C$.

Behavioral Bioassay. A dual-choice, still-air olfactometer (Budenberg et al., 1993, derived from that of Phillips and Burkholder, 1981) was used. It consisted of a ground glass disk (21 cm diam.) with two holes (13 mm diam.) drilled in it lying on a diameter 5 cm on either side of the center. The disk was moistened and placed so that the holes were above jars (10 cm high, 5 cm ID) containing the odor sources. A 10-cm-high Perspex ring (19 cm diam.) was then placed on top of the ground disk and covered with an intact glass disk. All bioassays involved testing a source of odor against a control.

Bioassays were conducted in a small fan-ventilated room ($25 \pm 1°C$, $\sim 80\%$ relative humidity). The only light was provided by a light box covered with a red filter (Kodak Wratten No. 70, transmitting only wavelengths greater than 640 nm). Weevils were removed from the pseudostem in the last hour of the photophase, and groups of five of the same sex were then put in small tubes with damp tissue paper. The sexes had been kept separately from those used in the first experiment. Bioassays were run between 1.5 and 7.5 hr after the onset of the scotophase. The same weevils were never bioassayed on consecutive days. Bioassays were started by putting five weevils approximately equidistant from the holes on the ground disk. The weevils walked slowly but continuously during the bioassay, mainly round the edge of the arena. They rarely fell into the jars. Continuous observations were made on two bioassays concurrently. Each visit to a hole, and its duration, was recorded. A visit was initiated when any part of a weevil came within 5 mm of the hole (judged by eye) and was terminated when all parts of the weevil left this area. In all the experiments observers of bioassays were unaware of the treatments applied to the jars, and therefore there was no chance of unconscious bias when judging the 5-mm distance. Each bioassay lasted for 10 min and glass disks were washed with soap and water after each run.

The first experiment used live weevils that had been kept with both sexes together as the treatment. Groups of 10 weevils of each sex were placed in separate jars with damp cotton wool. A wire mesh was placed at the top of the jar to prevent mixing of test and stimulus beetles. A thin piece of cotton wool

was put over the mesh to prevent any possible visual stimuli. The control jars were similar but without beetles.

In the second experiment, 50 beetle-day-equivalents of collected volatiles trapped from males and females on Porapak Q and activated-charcoal were applied to 5-cm-diam. filter papers and were tested against control papers treated with dichloromethane alone. The papers, when dry, were folded to fit into the top of jars with damp cotton wool in their base. The same filter paper was used in all the bioassays on a particular day, and therefore the concentration of volatiles was probably lower for bioassays carried out later in the day.

In the third experiment, hindguts were dissected from weevils, and 10 hindguts from the same sex were placed on pieces of filter paper (2 × 2 cm) moistened with Beadle's Ringer (7.5 g NaCl, 0.35 g KCl and 0.22 g $CaCl_2$ per 1000 ml water) solution. These papers were then placed inside jars with damp cotton wool in their bases. Control jars contained damp cotton wool and a piece of filter paper moistened with Beadle's Ringer solution.

Electroantennogram (EAG) Recordings. EAGs were recorded from isolated antennae. Clean antennae were obtained from *C. sordidus* that had been removed from pseudostem and placed with moist tissue paper overnight (to ensure that there was no banana material adhering to the antennae). Sensillae on the antennae of *C. sordidus* are concentrated on a cone that forms the end of the terminal, swollen, segment of the antenna. The flagellum was cut from the pedicel, and its proximal end was mounted in the reference electrode so that only the terminal segment was exposed. The recording electrode was inserted into a small hole that had been made in the cone using an electrolytically etched tungsten needle. Electrodes were made of extruded glass micropipets filled with Beadle's Ringer solution and were connected via chlorinated silver wires to the probe (amplifier), which connected directly to a PC-EAG interface card in a personal computer. This card contained a fast analog-to-digital conversion circuit with a resolution of 4096 units, and it sampled the signal at 100 Hz. The software then recorded and measured the size of the EAGs. The system was supplied by Syntech Ltd.

Volatile odors trapped from zero, one, three, and nine weevils of each sex were eluted directly from the traps with 1 ml of dichloromethane onto pieces of filter paper (6 × 1 cm), the dichloromethane was allowed to evaporate, and then the papers were put into Pasteur pipets. Extracts and washes from the weevils were applied to the filter paper in amounts of 10 and 100 μl (≡ 0.4 and 4 beetle equivalents, respectively). Stimulation was performed by a puff of air (0.45 liters/min for 0.2 sec) through the pipet into a humidified airstream (2.1 liters/min) blowing over the antenna. A reference stimulus (10 μl of 10% v/v dilution of nonanol in paraffin oil) was used after every two to four stimulations with test odors, and responses were calculated as the percentage of the response to the reference using linear interpolation. The reference gave responses of up to 1 mV for both male and female antennae.

Statistical Analysis. For each run of the bioassay, the number of excess visits to the treatment was calculated (i.e., the number of visits to the treatment hole less the number of visits to the control hole). These numbers were then tested for evidence of a departure from a balanced distribution about zero using the nonparametric signed-rank statistic. Comparisons using the Wilcoxon rank-sum test were also made between the numbers of excess visits by males and females to a particular odor source when experiments had been run simultaneously. The average durations of visits to treatments and controls were compared using t tests on log-transformed data. The transformation improved the fit of the distribution of the times to the normal distribution. In all cases, differences were deemed to be significant at $P < 0.05$.

The EAG data were analyzed by ANOVA according to the factorial design of the experiments, with each antenna as a split plot. The relative response was log-transformed to stabilize variance.

RESULTS

Female *C. sordidus* made significant numbers of excess visits to live males, male volatiles, and male hindguts, but not to the corresponding female stimuli (Table 1). Male *C. sordidus*, however, made significant excess visits only to male volatiles ($P < 0.001$ for Porapak-trapped, and $P < 0.005$ for charcoal-trapped), and to charcoal-trapped volatiles from females ($P < 0.05$). Similarly, females made significantly (either $P < 0.01$ or $P < 0.001$) longer visits to all the male stimuli, and males made longer visits to both live males and collected volatiles from males (Table 2). Females also made longer visits to live females ($P < 0.05$) and collections of volatiles from females ($P < 0.05$ and $P < 0.01$).

Females made significantly more excess visits to all odor sources of male origin than males did (live insects and Porapak-trapped volatiles, $P < 0.05$; hindguts $P < 0.01$; charcoal-trapped volatiles $P < 0.001$). There were no significant differences between males and females in the number of excess visits to female odor sources.

Collections of volatiles from males, but not those from females, evoked EAG responses from both male and female antennae (Figure 1, Table 3). Responses were a little stronger to collections from larger numbers of male weevils. Female antennae gave larger EAGs (main plot stratum, $P < 0.003$) than male antennae, but both sexes gave similar responses to the treatments (i.e., none of the interactions with other factors were significant). Extracts of hindguts and of bodies of males, but not those of females, evoked higher EAG responses than controls (Figure 2, Table 4). There was no significant response to the cuticle wash (by inspection of Figure 2). Larger volumes of the extracts elicited larger EAGs (effect of level significant). Both sexes gave similar responses to the treatments.

TABLE 1. RESPONSES OF MALE AND FEMALE *C. sordidus* TO VARIOUS ODOR STIMULI FROM CONSPECIFIC ORIGIN

	Test weevils			
	Males		Females	
Odor stimulus[a]	No. of reps	Mean No. of excess visits ± SE	No. of reps	Mean No. of excess visits ± SE
Live insects[b]				
10 males	56	0.30 ± 0.22	56	1.11 ± 0.29***
10 females	56	−0.02 ± 0.19	56	0.02 ± 0.20
Porapak-trapped volatiles[c]				
50 male b.d.e.	38	1.03 ± 0.29***[e]	38	2.18 ± 0.31***
50 female b.d.e.	38	0.16 ± 0.24	38	0.21 ± 0.27
Charcoal-trapped volatiles[c]				
50 male b.d.e.	40	0.58 ± 0.17**	40	2.00 ± 0.28***
50 female b.d.e.	40	0.30 ± 0.13*	40	0.20 ± 0.16
Hindguts[d]				
10 male	20	−0.05 ± 0.21	20	1.55 ± 0.49***
10 female	20	−0.45 ± 0.25	20	0.05 ± 0.37

[a] All tested against a control.
[b] Bioassays on seven days.
[c] Bioassays on four days, b.d.e. = beetle day equivalents.
[d] Bioassays on two days.
[e] $*P < 0.05$ $**P < 0.01$ $***P < 0.001$. Probability that the number of extra visits are distributed about zero, using the nonparametric signed rank statistic.

DISCUSSION

Observation of weevils in the bioassay showed that many of the visits recorded were a result of weevils orienting towards the source of odor, probably by tropotaxis. There was no suggestion of increased activity of weevils in bioassays containing active stimuli, and therefore it is reasonable to conclude that significant numbers of extra visits resulted from attraction of the weevils to the source of odor. Long visits were often due to weevils stepping into and climbing round the hole.

Female *C. sordidus* were attracted to conspecific males, their hindguts, and to volatiles from them, thereby providing evidence for a male-produced pheromone. Male *C. sordidus* were attracted to volatiles collected from males, and made longer visits to both live males and their collected volatiles. Therefore, there is evidence that this male-produced pheromone is an aggregation pheromone. Indeed, both sexes gave similar EAG responses to the pheromone (i.e.,

TABLE 2. MEAN DURATION (LOG_{10} NO. OF SEC) OF VISITS BY MALE AND FEMALE *C. sordidus* TO VARIOUS ODOR STIMULI OF CONSPECIFIC ORIGIN

	Test weevils			
	Males		Females	
Odor stimulus[a]	*N*	Mean ± SE	*N*	Mean ± SE
Live insects[b]				
10 males	71	1.45 ± 0.07*[c]	126	1.43 ± 0.05***
Control	54	1.18 ± 0.09	64	1.01 ± 0.07
10 females	53	1.24 ± 0.07	75	1.26 ± 0.05**
Control	54	1.14 ± 0.08	74	1.09 ± 0.06
Porapak-trapped volatiles[c]				
50 male b.d.e	80	1.11 ± 0.05**	126	1.30 ± 0.09**
Solvent	41	0.89 ± 0.06	43	0.99 ± 0.05
50 female b.d.e.	49	1.02 ± 0.04	61	1.14 ± 0.06*
Solvent	43	1.03 ± 0.05	53	0.94 ± 0.06
Charcoal-trapped volatiles[c]				
50 male b.d.e.	33	1.47 ± 0.07***	101	1.69 ± 0.04***
Solvent	10	0.91 ± 0.15	21	1.25 ± 0.11
50 female b.d.e.	31	1.16 ± 0.05	24	1.49 ± 0.10**
Solvent	19	1.11 ± 0.08	16	1.03 ± 0.11
Hindguts[d]				
10 male	19	1.04 ± 0.11	42	1.26 ± 0.08**
Control	20	0.78 ± 0.10	11	0.74 ± 0.11
10 female	8	0.57 ± 0.09	20	0.85 ± 0.10
Control	17	0.80 ± 0.10	19	0.87 ± 0.11

[a] All tested against a control.
[b] Bioassays on seven days.
[c] Bioassays on four days, b.d.e. = beetle day equivalents.
[d] Bioassays on two days.
[e] $*P < 0.05$ $**P < 0.01$ $***P < 0.001$. Probability that the visits to the treated and control holes are of different mean duration, *t* test.

male volatiles and extracts), providing corroborative evidence. However, females responded behaviorally to the pheromone significantly more strongly than males. This may have been due to the combined effect of the test males being habituated to the pheromone when kept in groups in the holding tubes and a less obvious concentration gradient of the pheromone in the assay chamber because of pheromone released by the test males themselves. However, lower responses by male beetles than females to their aggregation pheromone have been previously reported (e.g., for *Polygraphus rufipennis*; Bowers and Borden, 1990).

There was some evidence that females made longer visits to, but were not attracted by, live females and collections of volatiles from them and that male

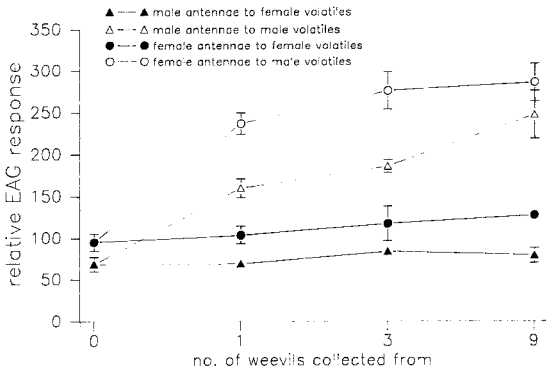

FIG. 1. EAG responses (means ± SE, as percentage of response to 10 μl 10% v/v dilution of nonanol in paraffin oil) by male and female banana weevils to volatiles trapped on Porapak Q from 0, 1, 3, 9 male and female conspecifics.

TABLE 3. ANOVA ANALYSIS FOR LOG-TRANSFORMED DATA OF RESPONSES FROM FIVE MALE AND FOUR FEMALE ANTENNAE TO COLLECTIONS MADE FROM 0, 1, 3, AND 9 WEEVILS OF EACH SEX (SEX-TREATMENT)[a]

Source of variation	*df*	ss	ms	vr	F pr
Main plot stratum (individual antennae)					
Sex of antenna	1	0.413	0.413	20.6	0.003
Residual	7	0.141	0.020		
Subplot stratum (within antennae)					
Sex-treatment	1	1.511	1.511	227.1	<0.001
Number	3	1.030	0.343	51.6	<0.001
Sex-treatment * number	3	0.516	0.172	25.8	<0.001
Residual[b]	56	0.373	0.007		

[a] *df* = degrees of freedom; ss = sums of squares; ms = mean square; vr = variance ratio; F pr = probability of a higher ratio under the hypothesis that the variance does not differ from the residual.
[b] None of the interactions within the subplot including the sex of antenna were significant, and these have therefore been included within the residual variance.

weevils made longer visits to female volatiles trapped on activated charcoal. It is possible that these results reflect reality, but the lack of consistency for the different stimuli and the lack of corroborative evidence from EAG results suggest that they do not. Sexing of weevils by external examination is only about 99% reliable, and therefore these results may reflect the presence of one or two males among the females from which the stimuli were derived.

Collections of volatiles made on Porapak Q and activated charcoal elicited

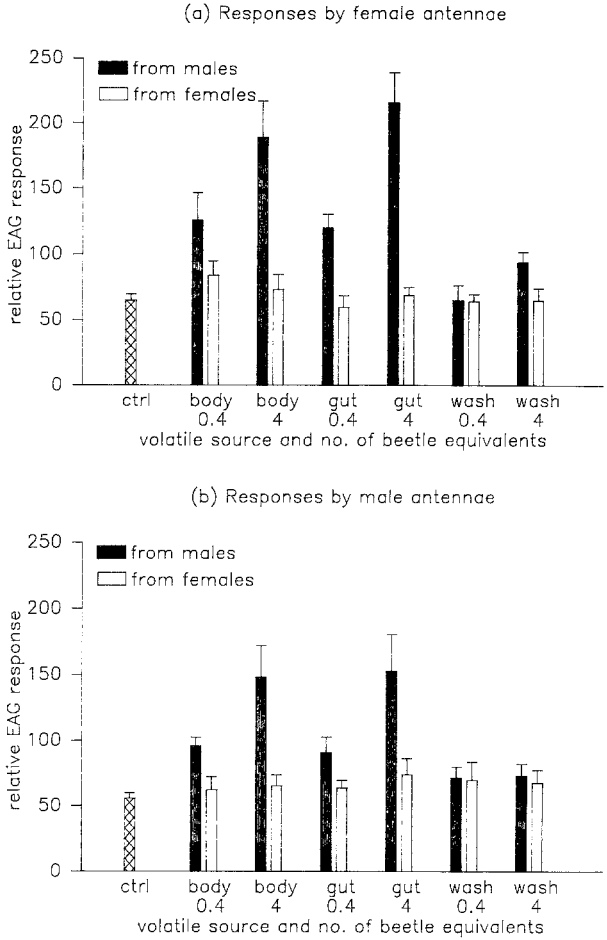

FIG. 2. EAG responses (means ± SE, as percentage of response to 10 μl of 10% v/v dilution of nonanol in paraffin oil) to body and hindgut extracts and to cuticle washes from male and female banana weevils: (a) female antennae; (b) male antennae. Control (ctrl) treated with dichloromethane alone.

similar responses, suggesting that both adsorbents were effective for trapping the component(s) of the pheromone and that the pheromone was produced both when males had had access to females and when they had not.

It is likely that the pheromone is released via the hindgut of the male, as hindguts were attractive on their own in the behavioral bioassay and an extract of male hindguts was stimulatory in EAG tests. Indeed, most beetles release their aggregation pheromones via the hindgut (Borden, 1985). Surface washes

TABLE 4. ANOVA ANALYSIS FOR LOG-TRANSFORMED DATA OF RESPONSES FROM TWO MALE AND TWO FEMALE ANTENNAE TO HINDGUT EXTRACT, BODY EXTRACT, AND CUTICLE WASH (TREATMENTS) FROM EACH SEX (SEX-TREATMENT) APPLIED IN VOLUMES OF 10 AND 100 μL (LEVELS)[a]

Source of variation	*df*	ss	ms	vr	F pr
Main plot stratum (individual antennae)					
Sex of antenna	1	0.038	0.038	0.8	0.451
Residual	2	0.087	0.043		
Subplot stratum (within antennae)					
Sex-treatment	1	0.577	0.577	95.3	<0.001
Treatment	2	0.203	0.102	16.8	<0.001
Level	1	0.113	0.113	18.7	<0.001
Sex-treatment * treatment	2	0.142	0.071	11.8	<0.001
Sex-treatment * level	1	0.067	0.067	11.1	0.002
Treatment * level	2	0.016	0.008	1.3	0.291
Sex-treatment * treatment * level	2	0.007	0.004	0.6	0.552
Residual[b]	33	0.200	0.006		

[a] *df* = degrees of freedom; ss = sums of squares; ms = mean square; vr = variance ratio; F pr = probability of a higher ratio under the hypothesis that the variance does not differ from the residual.
[b] None of the interactions within the subplot, including the sex of antenna were significant, and these have therefore been included within the residual variance.

of males did not elicit significant EAGs, but the extract of the bodies elicited responses as strong as those to the extract of the hindguts. This suggests that pheromone is also present elsewhere within the body apart from the hindgut. Other studies (Budenberg, unpublished) have shown that male weevils release much less pheromone after three days without food, suggesting that the pheromone is somehow derived from their food, as in many other beetles (Borden, 1985).

Female weevils gave higher relative EAGs than males when tested against the collections of volatiles from various numbers of conspecifics. This was true not only when the pheromone was present, but also in its absence (i.e., collections from zero weevils and from females). This is surprising and has not been consistently observed in other EAG investigations with the banana weevil.

Male-produced aggregation pheromones have been reported for many other species of weevil, e.g., the rice weevil *Sitophilus oryzae* (L.) (Phillips and Burkholder, 1981), the boll weevil *Anthonomus grandis* Boh (Tumlinson et al., 1969) the pea and bean weevil *Sitona lineatus* (L.), (Blight and Wadhams, 1987), the pine weevils *Pissodes* sp. (Booth et al., 1983), and the American palm weevil *Rhynchophorus palmarum* (L.) (Rochat et al., 1991), and are very common among bark beetles (Borden, 1985). Aggregation pheromones are gen-

erally used for both host-finding and bringing the sexes together (Borden, 1985), and it is likely that these are also its functions in the banana weevil.

The dual-choice still-air olfactometer used in these experiments is similar to the pitfall ones used in many other studies of weevil attraction (e.g., Phillips and Burkholder, 1981; Rochat et al., 1991). However, their results were based on the number of weevils that fell into odor sources, whereas we analyzed the numbers of visits to the odor sources. This, coupled with the behavioral observations of the weevils, has allowed the confirmation of an attractive response to the treatments, whereas studies using results from pitfall only confirm that the insects have a higher tendency to fall into, or be arrested in, the containers releasing odors.

Mass-trapping using split banana pseudostem and rhizome has been advocated as a control measure, with some reports of success (Wallace, 1938; Yaringano and van der Meer, 1975), although others report no success using the technique (Ostmark, 1974). If an efficient baited trap were to be designed, probably incorporating both plant volatiles (Budenberg et al., 1993) and the aggregation pheromone, it is possible that good control could be achieved using mass-trapping. Alternatively, the baits could be used for the selective application of pesticides or biocontrol agents, e.g., entomopathogenic fungi (Busoli et al., 1989) or nematodes (Treverrow et al., 1991).

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ATTRACTION OF PEA MOTH *Cydia nigricana* F. (LEPIDOPTERA: TORTRICIDAE) TO FEMALE SEX PHEROMONE (*E,E*)-8,10-DODECADIEN-1-YL ACETATE, IS INHIBITED BY GEOMETRIC ISOMERS *E,Z*, *Z,E*, AND *Z,Z*

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Abstract—Field attraction of *Cydia nigricana* males to synthetic female sex pheromone (*E,E*)-8,10-dodecadien-1-yl acetate, formulated on red rubber septa, declined continuously during two weeks. This was due to isomerization of (*E,E*)-8,10-dodecadien-1-yl acetate: eight days after application of purified *E,E* isomer, the proportion of *E,Z*; *Z,E*; and *Z,Z* isomers in rubber septa aged in the laboratory was 4%; a 5% addition of any one of these isomers to fresh lures of (*E,E*)-8,10-dodecadien-1-yl acetate significantly reduced male attraction. Stereospecific syntheses of (*E,Z*)-, (*Z,E*)-, and (*Z,Z*)-8,10-dodecadien-1-yl acetate are described. The pheromone gland of *Cydia nigricana* contains 0.8 ng/female of (*E,E*)-8,10-dodecadien-1-yl acetate, accompanied by three monounsaturated acetates, (*E*)-9-dodecen-1-yl acetate, (*Z*)-5-tetradecen-1-yl acetate, and (*Z*)-7-tetradecen-1-yl acetate (0.1 ng/female each). These compounds did not augment male trap catch when added to (*E,E*)-8,10-dodecadien-1-yl acetate.

Key Words—Sex pheromone, conjugated diene isomerization, attraction

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inhibitor, stereospecific synthesis, (*E,E*)-8,10-dodecadien-1-yl acetate, *Cydia nigricana*, Tortricidae, Lepidoptera.

INTRODUCTION

The pea moth *Cydia nigricana* F. is the most important insect pest of green peas (*Pisum sativum* L.) in Scandinavia and other parts of Europe. (*E,E*)-8,10-Dodecadien-1-yl acetate (*E*8,*E*10–12:Ac; *EE*) has been identified as the female sex pheromone (Greenway, 1984), but male attraction to synthetic *E*8,*E*10–12:Ac declines rapidly after only a few days in the field (Greenway and Wall, 1981; Wall et al., 1987a). A pheromone mimic, (*E*)-10-dodecen-1-yl acetate (*E*10–12:Ac) is currently used to monitor *C. nigricana* in pea crops (Macaulay et al., 1985; Wall et al., 1987b; Wall 1988). This compound is less attractive than fresh lures of *E*8,*E*10–12:Ac, but ensures constant attraction over the whole flight period (Greenway and Wall, 1981).

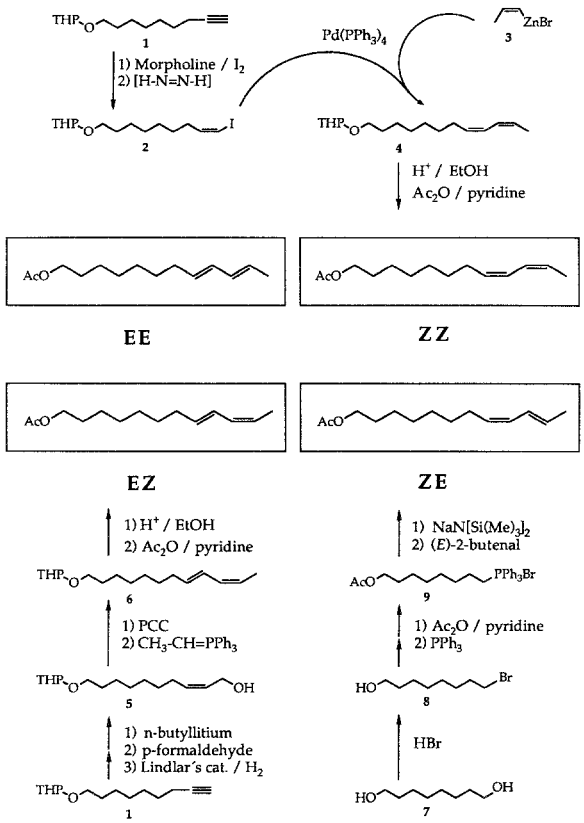
It has been proposed that isomerization or decomposition of *E*8,*E*10–12:Ac inhibits attraction of *C. nigricana* (Greenway and Wall, 1981; Davis et al., 1984; Wall et al., 1987a; Horák et al., 1989). We have reexamined the female sex pheromone production and tested the hypothesis that formation of the stereoisomers *E*8,*Z*10–12:Ac (*EZ*), *Z*8,*E*10–12:Ac (*ZE*), and *Z*8,*Z*10–12:Ac (*ZZ*) is responsible for reduced attractiveness of aged lures of *E*8,*E*10–12:Ac.

METHODS AND MATERIALS

Chemicals. EZ-, *ZE-*, and *Z*8,*Z*10–12:Ac were synthesized according to Scheme 1. *E*8,*E*10–12:Ac was purchased from S. Voerman, Institute for Pesticide Research, 6700 Wageningen, The Netherlands.

All reactions of air- and water-sensitive materials were carried out under N_2 or Ar. Liquid chromatography (LC) was done according to Baeckström et al. (1987). NMR spectra were recorded in $CDCl_3$ on Bruker WP 200 and AM 400 spectrometers. Mass spectra, ^1H and ^{13}C NMR, of the target 8,10-dodecadienyl acetates were in agreement with those reported by Ando et al. (1983, 1985).

(8E,10Z)-8,10-Dodecadien-1-yl Acetate (E8,Z10–12:Ac). 1-(2-Tetrahydropyranyloxy)-8-nonyne (Scheme 1, **1**) (2.91 g, 13 mmol) was elongated by treatment with *n*-butyllithium (12.2 ml, 1.6 M) and paraformaldehyde (585 mg, 19.5 mmol), and the triple bond semihydrogenated (200 ml H_2, 9 mmol) using Lindlar's catalyst ($Pd/BaSO_4$, 100 mg in 25 ml ethyl acetate), affording (*Z*)-1-(2-tetrahydropyranyloxy)-8-decen-10-ol **(5)** (2.16 g, 8.45 mmol, 65% yield). Oxidation of the hydroxy group by pyridinium chlorochromate (PCC) under conditions that isomerized the double bond (3 eq. PCC in methylene chloride,



SCHEME 1. Synthesis of *EZ*-, *ZE*-, and *Z*8,*Z*10–12:Ac.

buffered by sodium acetate), gave (*E*)-1-(2-tetrahydropyranyloxy)-8-decenal (790 mg, 3.11 mmol, 37%). This aldehyde was treated with ethylidenetriphenylphosphorane (2.5 eq.), generated by sodium hexamethyldisilazane, in a stereoselective Wittig reaction (Bestmann et al., 1978), affording 1-(2-tetrahydropyranyloxy)-(8*E*,10*Z*)-8,10-dodecadiene **(6)** (449 mg, 1.70 mmol, 55% yield) with 93% isomeric purity (4% *EE*, 3% *ZZ*), as determined by capillary GC. *E*8,*Z*10–12:Ac was obtained after deprotection and acetylation (327 mg, 1.46 mmol, 86% yield). The overall yield was 11%.

(8Z,10E)-8,10-Dodecadien-1-yl Acetate (Z8,E10–12:Ac). Monobromination of 1,8-octandiol **(7)** (3.65 g, 25 mmol) according to Rossi (1981), Babler and Invergo (1979), and Chapman et al. (1978) gave 8-bromo-1-octanol **(8)** (4.18 g, 20 mmol, 80%). Standard acetylation (Björkling et al., 1985) followed by treatment with 1 eq. triphenylphosphine at reflux yielded 8-acetoxy-1-octyl-

triphenyl-phosphonium bromide **(9)** (8.23 g, 16 mmol, 80%) (Bestmann et al., 1978). Compound **9** was deprotonated with sodiumhexamethyldisilazane and treated with 1.5 eq. (*E*)-2-butenal. This stereoselective Wittig reaction (Bestmann et al., 1978) afforded *Z*8,*E*10–12:Ac (1.85 g, 8.3 mmol, 52% yield) with 94% isomeric purity (4% *EE*, 2 *ZZ*). The overall yield was 33%.

(8Z,10Z)-8,10-Dodecadien-1-yl Acetate (Z8,Z10–12:Ac) was prepared according to Björkling et al., (1985). 1-(2-Tetrahydro-pyranyloxy)-8-nonyne **(1)** was converted to the corresponding (*Z*)-vinyl iodide **(2)** in two steps (yield 58%). Compound **2** (2.5 g, 7.1 mmol) was coupled with (*Z*)-1-propenylzinc monobromide **(3)** (24.8 mmol) via a palladium-catalyzed reaction to give 1-(2-tetrahydropyranoloxy)-(8*Z*,10*Z*)-8,10-dodecadiene **(4)** in 90% yield with >97.7% isomeric purity (0.3% *EE*, 2% *ZE*). No loss in isomeric purity was observed during deprotection and subsequent acetylation. *Z*8,*Z*10–12:Ac was obtained in 91% yield 1.3 g, 5.81 mmol). The overall yield was 48%.

Gas Chromatography and GC-Mass Spectrometry (GC and GC-MS). Identification of female gland components and synthetic chemicals by GC-MS with electron impact ionization (70 eV) was carried out on a Hewlett Packard 5970B instrument equipped with a 59970B computer system, and interfaced with a Hewlett Packard 5890 GC. Helium was used as carrier gas and analyses were done on a polar DB-Wax column (30 m × 0.25 mm ID, J&W Scientific, Folsom, California), programmed from 80°C (hold 2 min) at 10°/min to 230°C. Analysis of isomeric composition was done on a Hewlett Packard 5890 GC with flame ionization detection on a nonpolar SE-54 column (25 m, 0.32 mm ID, Kupper & Co., Bonaduz, Switzerland), programmed from 60°C (hold 2 min) at 10°/min to 100°C, and 1.5°/min to 230°C.

Purification of Chemicals. The four dienic acetates (*EE*-, *EZ*-, *ZE*-, and *Z*8,*Z*10–12:Ac) were purified by preparative HPLC on a Lichrosorb Si 60-7 column (25 cm × $\frac{3}{4}$ in. × 16 cm) coated with 20% $AgNO_3$ (Alltech). Toluene (Fluka, HPLC grade) was used as eluent at a speed of 5 ml/min. Compounds were detected using a Waters Differential Refractometer R401, the collected fractions were analyzed by capillary GC. After removal of toluene on a Rotavapor at room temperature, the residue was dissolved in some milliliters of hexane and the solution passed through a small column of silica gel 60 (Merck) and NaCl. Hexane was then removed under reduced pressure and the final products were analyzed by GC. Isomeric purity of the four isomers used for field trapping is shown in Table 1.

Extraction of Dispensers. Red rubber septa (Thomas Scientific) were purified prior to use with ethanol in a Soxhlet apparatus for three days, then washed with hexane and dried. Each septum was loaded with 100 μg purified *E*8,*E*10–12:Ac (Table 1) (1 μg/μl in hexane). The septa were placed in a wind tunnel at constant temperature, air flow, and artificial illumination (20°C, 30 cm/sec, 100 lux). After 0, 2, 4, 8, 16 and 32 days, the septa were extracted individually

TABLE 1. CHEMICAL PURITY OF 8,10-DODECADIENYL ACETATES USED FOR FIELD TRAPPING OF *C. nigricana* AFTER HPLC PURIFICATION AS DETERMINED BY CAPILLARY GC

Chemical composition	Test chemical (%)			
	EE	EZ	ZE	ZZ
*E*8,*E*10–12:Ac	99.4	0.5	0.3	[a]
*E*8,*Z*10–12:Ac	0.4	99.3	[a]	[a]
*Z*8,*E*10–12:Ac	0.2	0.21	99.5	0.4
*Z*8,*Z*10–12:Ac	[a]	[a]	0.2	99.6

[a] Below detection level (0.05%).

($N = 3$) with three portions of redistilled hexane (3 × 1 ml), and 100 μg of 12:Ac was added as internal standard. The samples were concentrated under a stream of nitrogen to a volume of ca. 100 μl; 5 μl of these extracts were analyzed by GC.

Preparation of Female Gland Extracts. C. nigricana were collected in pea fields near Höör (Scania, Sweden), the diapausing larvae were kept at 4°C for three months. They emerged under a 18:6 light–dark photoperiod at 22°C. Females started to emit pheromone ca. 5 hr after onset of the light period. Pheromone glands of calling females were extracted in batches of 10 ($N = 7$), 30 ($N = 4$), and 50 ($N = 1$) with 5 μl of hexane during 1–2 min. The solvent was immediately analyzed by GC or GC-MS.

Field Trapping. Hexane solutions of the test chemicals were applied to red rubber caps (Thomas Scientific). Tetra traps (Arn et al., 1979) were hung at 80 cm in pea fields and were checked every one to three days. Traps within a replicate were 5 m apart; they were set in straight rows perpendicular to the main wind direction. Replicates ($N = 10$) were spaced crosswind at >20 m and downwind at >150 m. The number of males caught was transformed to log ($x + 1$) and submitted to an analysis of variance, followed by a Tukey test ($P = 0.05$).

In 1991, we tested: (1) a dosage series of *E*8,*E*10–12:Ac and *E*10–12:Ac; (2) additions of *E*,*Z*-, *Z*,*E*-, *Z*8,*Z*10–12:Ac and *E*8,*E*10–12:OH (5 μg and 20 μg) to 100 μg purified *E*8,*E*10–12:Ac, at Heagård (Halland); in various other pea fields, (3) 10 pairs of monitoring traps, containing 100 μg *E*8,*E*10–12:Ac or *E*10–12:Ac were set at 1 m distance to each other; in 1992, (4 and 5) 1 μg and 10 μg of minor gland components were added to 10 μg *E*8,*E*10–12:Ac (Veberöd, Scania). Rubber septa and traps were replaced every three days during this trial.

RESULTS

Field Trapping with E*8,*E*10–12:Ac and* E*10–12:Ac.* Purified *E*8,*E*10–12:Ac (Table 1) attracted more *C. nigricana* males than *E*10–12:Ac. Over three days, pairs of monitoring traps baited with 100 μg *E*8,*E*10–12:Ac or 100 μg of *E*10–12:Ac caught 19.7 ± 15.6 and 3.1 ± 2.9 males, respectively ($N = 10$). Trap catch with a dosage series of *E*8,*E*10–12:Ac and *E*10–12:Ac is shown in Figure 1. During the first three days, fresh lures of 10 μg dienic acetate caught 20 males, and 1000 μg of the monoenic acetate caught four males. From July 21 on, attraction to the diene declined rapidly, and from July 27 on, 100 μg and 1000 μg *E*10–12:Ac caught significantly more males than all four doses of dienic acetate (Figure 1).

Field Trapping with Geometrical Isomers of E*8,*E*10–12:Ac.* In a second test, addition of 5% of the geometrical isomers *EZ*, *ZE*, or *ZZ* to 100 μg *E*8,*E*10–12:Ac reduced male attraction significantly; addition of 20% of these isomers completely shut down trap catch (Figure 2). Addition of *E*8,*E*10–12:OH to *E*8,*E*10–12:Ac reduced trap catch (Figure 2) (Greenway et al., 1982), but a 20% addition of the alcohol did not inhibit attraction entirely.

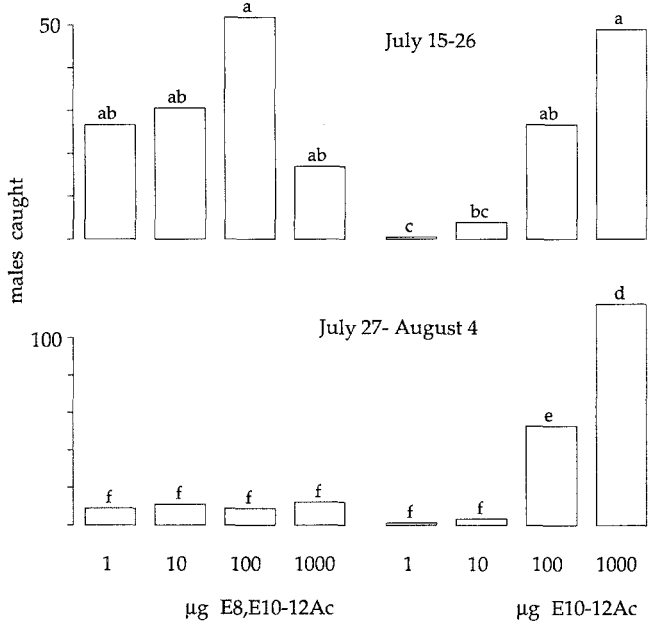


FIG. 1. Field attraction of *C. nigricana* males to four doses of sex pheromone *E*8,*E*10–12:Ac and pheromone mimic *E*10–12:Ac (Heagård 1991; $N = 10$; Tukey test, $P = 0.05$).

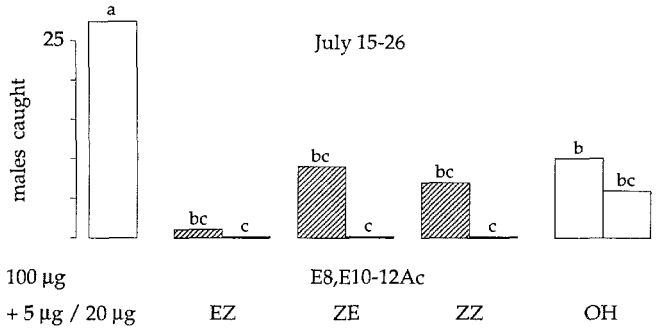


FIG. 2. Field attraction of *C. nigricana* males to 100 µg of sex pheromone *E*8,*E*10-12:Ac, blended with 5 or 20 µg of *EZ*-, *ZE*-, *Z*8,*Z*10-12:Ac or *E*8,*E*10-12:OH (Heagård 1991; (N = 10; Tukey test, P = 0.05).

The range of attraction of *C. nigricana* males to 100 µg of *E*8,*E*10-12:Ac is up to 500 m (Wall and Perry, 1980, 1987). In our tests, traps within one replicate were hung 5 m apart. During the first 12 days, 52 males were attracted to the 100 µg *E*8,*E*10-12:Ac source along with various doses of diene and monoene (Figure 1), and 27 males were attracted to this lure, when close to traps with the other isomers (Figure 2). This indicates that the nonpheromonal isomers also reduced male attraction to separate baits of *E*8,*E*10-12:Ac.

Extraction of Rubber Septa. Extraction of rubber septa with an initial load of 100 µg HPLC-purified *E*8,*E*10-12:Ac (Table 1) showed that, after two days, the *EZ* isomer was present at 1% and the *ZE* and *ZZ* isomer at 0.5% (Figure 3). After eight days, these isomers amounted to 4% of *E*8,*E*10-12:Ac (2.4% *EZ*, 1.2% *ZE*, and 0.4% *ZZ*); the release rate of *E*8,*E*10-12:Ac was rather constant over 16 days. This demonstrates that deterioration of *E*8,*E*10-12:Ac lures in the field (Figure 1) was due to isomerization.

The septa used for extraction (Figure 3) were aged in a wind tunnel at 20°C and 100 Lux. Three other septa and a vial with *E*8,*E*10-12:Ac in hexanic solution (1 µg/µl) were exposed to sunlight, but were protected from UV light by glass. After 32 days, isomeric composition of the hexanic solution had not changed, but the rubber septa contained 11% *EZ*, 6.7% *ZE*, and 2.2% *ZZ*. It has been shown that the rate of isomerization of *E*8,*E*10-12:Ac can be even faster in the field (25.1% *EZ*, 14.2% *ZE*, and 4.2% *ZZ* after four weeks; Davis et al., 1984) and also depends on the type of rubber used as dispenser (Brown and McDonough, 1986; Horák et al., 1989; McDonough et al., 1990).

Chemical Analysis. The pheromone-related compounds identified from female gland extracts are listed in Table 2; *E*8,*E*10-12:Ac has been reported previously (Greenway, 1984). The geometrical isomers *EZ*, *ZE*, and *ZZ* were not detected at >0.05% of *EE*. The monoenic dodecenyl (*E*9-12:Ac) and

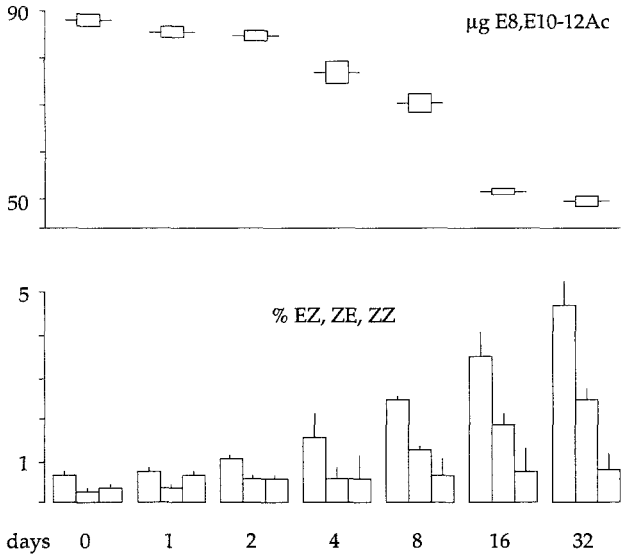


FIG. 3. Amounts of *E*8,*E*10–12:Ac (µg; top) and *EZ*, *ZE*, *ZZ* isomers (relative to *EE*; bottom) extracted from red rubber septa exposed in a wind tunnel 0 to 32 days after application of 100 µg purified *E*8,*E*10–12:Ac (see Table 1) (mean values ± SD; $N = 3$).

TABLE 2. PHEROMONE-RELATED COMPOUNDS IDENTIFIED IN *C. nigricana* FEMALES BY GC AND GC-MS

Compound	Short form	ng/female
Dodecyl acetate	12:Ac	2.5
*E*9-Dodecenyl acetate	*E*9–12:Ac	0.1
*E*8,*E*10-Dodecadienyl acetate	*E*8,*E*10–12:Ac	0.8
*E*8,*Z*10-Dodecadienyl acetate	*E*8,*Z*10–12:Ac	[a]
*Z*8,*E*10-Dodecadienyl acetate	*Z*8,*E*10–12:Ac	[a]
*Z*8,*Z*10-Dodecadienyl acetate	*Z*8,*Z*10–12:Ac	[a]
Tetradecyl acetate	14:Ac	1.7
*Z*5-Tetradecenyl acetate	*Z*5–14:Ac	0.1
*Z*7-Tetradecenyl acetate	*Z*7–14:Ac	0.1
Hexadecylacetate	16:Ac	1.5
Octadecylacetate	18:Ac	0.3

[a]Below detection level as determined by GC (0.05% of *E*8,*E*10–12:Ac).

tetradecenyl acetates (*Z*5–14 : Ac, *Z*7–14 : Ac) were present in all extracts (Table 2). Their mass spectra and retention times, both on the polar DB-Wax and the nonpolar SE-54 column, matched those of synthetic compounds; ratios of characteristic mass spectral fragments were in accordance with Lanne et al. (1985).

Field Trapping with Female Gland Components. The monoenic acetates identified from female glands were added individually at 10% and 100% to 10 μg *E*8,*E*10–12 : Ac (Table 3). Addition of *Z*5–14 : Ac or *Z*7–14 : Ac did not modify trap catch significantly, but addition of 100% *E*9–12 : Ac decreased attraction. Blends including two or three of the monoenic acetates at 10% were not significantly different from *E*8,*E*10–12 : Ac alone (Table 4).

Males were observed to display their typical wing-fanning behavior close (0–15 cm) to calling females or sources of synthetic *E*8,*E*10–12 : Ac. Addition of 10% *E*9–12 : Ac to *E*8,*E*10–12 : Ac did not modify orientation or close-range

TABLE 3. FIELD ATTRACTION OF *C. nigricana* MALES TO 10 μg OF *E*8,*E*10–12 : Ac BLENDED WITH FEMALE GLAND COMPONENTS[a]

Compound	μg/trap									
*E*8,*E*10–12 : Ac	10	10	10	10	10	10	10	10	10	10
*E*9–12 : Ac			1	10						1
*Z*5–14 : Ac					1	10				1
*Z*7–14 : Ac							1	10		1
12 : Ac									30	30
14 : Ac									20	20
16 : Ac									10	10
Males caught	227 a[b]	204 a	295 a	63 b	277 a	303 a	254 a	280 a	185 a	189 a

[a]Veberöd 1992; $N = 10$.
[b]Tukey test, $P = 0.05$.

TABLE 4. FIELD ATTRACTION OF *C. nigricana* MALES TO 10 μg OF *E*8,*E*10–12 : Ac BLENDED WITH MONOENIC ACETATES IDENTIFIED FROM FEMALE GLANDS[a]

Compound	μg/trap					
*E*8,*E*10–12 : Ac	10	10	10	10	10	10
*E*9–12 : Ac			1	1		1
*Z*5–14 : Ac			1		1	1
*Z*7–14 : Ac				1	1	1
Males caught	300 a	296 a	312 a	373 a	277 a	298 a

[a]Veberöd 1992; $N = 10$.
[b]Tukey test, $P = 0.05$.

behaviors conspicuously, but males rarely landed in the vicinity (<30 cm) of 1:1 blends of *E*8,*E*10–12:Ac and *E*9–12:Ac.

DISCUSSION

The female sex pheromone of *C. nigricana* is *E*8,*E*10–12:Ac. Other components identified from the female gland did not contribute to male attraction in the field, but detailed behavioral studies are needed before a synergistic effect of the minor acetates can be excluded.

Occurrence of *E*9–12:Ac, along with the saturated acetates (Table 2) compares to *Cydia pomonella*, where the analogous alcohols *E*9–12:OH, 12:OH, 14:OH, 16:OH, and 18:OH (Einhorn et al., 1984; Arn et al., 1985) are biosynthetic precursors of codlemone, *E*8,*E*10–12:OH (Löfstedt and Bengtsson, 1988).

The presence of two tetradecenyl acetates in *C. nigricana*, *Z*5–14:Ac and *Z*7–14:Ac, is remarkable. These two compounds are commonly found in species belonging to the phylogenetically older (Bradley et al., 1973) subfamily Archipinae, but are not known from the Olethreutinae (Arn et al., 1992).

The geometric isomers of *E*8,*E*10–12:Ac (*EZ*, *ZE*, and *Z*8,*Z*10–12:Ac) were not detected in female gland extracts (Table 2), and they strongly inhibit male attraction to *E*8,*E*10–12:Ac (Figure 2). Deterioration of rubber septa baited with synthetic *E*8,*E*10–12:Ac (Figure 1; Greenway and Wall, 1981) is due to formation of these isomers (Figures 2 and 3).

As in *C. nigricana*, small amounts of *E*8,*Z*10–12:Ac reduce attraction of *C. caryana* to *E*8,*E*10–12:Ac, but males of *C. ingrata* and *C. latiferreana* are attracted to blends of these two isomers. Females of *C. toreuta* produce only *E*8,*Z*10–12:Ac (Davis et al., 1984; Chisholm et al., 1985; Katovich et al., 1989; McDonough et al., 1990). This indicates that specificity of the pheromonal signal in these species may depend largely on isomeric composition, rather than on additional minor gland compounds.

Dienic acetates and alcohols are pheromone components in many other tortricids and are used for monitoring and mating disruption, for instance against grapevine moth *Lobesia botrana*. Males of *L. botrana* have been observed to fly straighter and more rapidly towards calling females than towards an optimized blend of *E*7,*Z*9–12:Ac, *E*7,*Z*9–12:OH, and *Z*9–12:Ac (Witzgall and Arn, 1990). Such differences in the chemistry of synthetic pheromone and of calling females may be attributed to missing components, as well as to isomeric impurities. Our results with *C. nigricana* emphasize that in order to mimic the female emission of dienic pheromone components with artificial dispensers, the problem of isomerization must be solved.

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RADAR DETECTION OF DRONES RESPONDING TO HONEYBEE QUEEN PHEROMONE[1]

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Abstract—The response of honey bee (*Apis mellifera* L.) drones to queen pheromone(s) (either natural from a mated queen, or synthetic from a lure) was recorded using an X-band, ground-based radar. The distribution of drones (insect targets on the radar screen) changed from a scattered distribution to a line concentration (downwind) when the pheromone was released. Displacement within the line concentration was toward the pheromone. This response was seen as far as 800 ± 15 m downwind from a lure with 10 mg of synthetic 9-oxodec-*trans*-2-enoic acid (9-ODA) and as far as 420 ± 15 m from a mated queen. These studies demonstrate that queen pheromone can be detected by drones at much greater distances than previously believed and illustrate how X-band radar may be used to establish the distances at which insects of similar or larger size respond to pheromones.

Key Words—*Apis mellifera*, honeybee, Hymenoptera, Apidae, remote sensing, drone behavior, radar detection.

INTRODUCTION

The distances over which individual insects can respond to the pheromone output of other individuals has been the subject of considerable speculation and theoretical treatment (see the review by Murlis et al., 1992). Indeed, although at least 1000 pheromones have been described and over 250 formulations have

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been deployed to monitor or control various species (Ridgway et al., 1990), the distances over which target insects can be attracted is usually unknown. Little is known about the downwind concentrations or the response-limiting sensitivity of the target insects and their searching response to the dynamics of wind-borne pheromone plumes.

Various models have been proposed to describe the efficiency of anemotactic searching behavior for distant (Murlis and Jones, 1981; Sabelis and Shippers, 1984) and close (Dusenberry, 1990) orientation of insects to pheromone sources. Perry and Wall (1984) have described a mathematical model of the response of the pea moth (*Cydia nigricana*) to pheromone-baited traps based on data indicating that these moths respond from 500 m (or possibly 1000 m; see Wall and Perry, 1987). Elkington and Cardé (1988) provide an insightful discussion comparing their results of gypsy moth (*Lymantria dispar*) pheromone response distances (80 m) within three types of canopy to those of the pea moth over open fields.

In these and other studies, models and interpretations are inferred rather than based on direct observations of the paths taken by individuals responding to point sources of the pheromones. Although long-distance tracking of individual insects tagged with retroreflective tags or laser-emitting diodes may be possible, this methodology is not fully developed. Until such technology is available, a modified X-band radar offers an alternative (Loper et al., 1987). Such a radar can quickly scan a large area (1000 ha/sec for honeybee-sized insects) before and after the release of target insects and pheromone sources. Under conditions of minimal background clutter (from bushes, trees, or uneven ground surfaces), the responses of targets to wind-blown pheromones can be recorded. At low target densities, individuals can be "tracked" for hundreds of meters.

Mating in honeybees (*Apis mellifera*) occurs in midair (Gary, 1963; Koeniger, 1986). Free-flying drones respond in large numbers to caged virgin and mated queens, whole queen extract, and synthetic "queen substance" if these are suspended in (or upwind of) drone flight paths or congregation areas (Gary, 1963; Ruttner and Ruttner, 1972; Loper et al., 1987, 1992). Queen substance is secreted from the queen's mandibular gland and its composition is well known (Barbier and Lederer, 1960; Callow and Johnston, 1960; Slessor et al., 1988).

In this study, X-band radar was used to observe the distances at which drones responded to a single mated queen and a lure impregnated with a 100 queen equivalents of (*E*)-9-keto-2-decenoic acid (9-ODA), the principal queen pheromone component.

METHODS AND MATERIALS

Assessment of drone activity was made using an X-band marine radar operating at 9.4 GHz, with a pulse length of 50 nsec and pulse repetition rate of 3400 Hz. The unit has a 1.22-m-diameter, parabolic antenna, which forms

a pencil-shaped beam 1.65° wide. This beam was rotated about a vertical axis at a 1-, $1\frac{1}{2}$-, or 2-sec rotation rate. Distance and direction of individual insects was displayed on a short-persistence cathode ray tube (CRT). The narrow beam width and short pulse length provided a target resolution of 15 m. In other words, two or more insects within 15 m are seen as a single dot on the radar screen. Verification of visual on-the-ground sightings of drones near a pheromone source correlated with radar reception was made in real time by voice communication over two-way portable radios. Pictures of the radar screen during each dish rotation were obtained with a 16 mm movie camera mounted over the CRT. Single-frame color photographs were also taken with a 35 mm camera mounted over the CRT. The movie film was projected using normal and stop-action modes to facilitate measurement of the response of the drones to the pheromone source downwind of the queen or lure.

Location. Most of the observations were made during March and April 1987 and 1990 on the Willcox Dry Lake (WDL), which extends approximately 14 × 14 km near Willcox, Arizona. The dry lake bed is flat (1219 m above sea level) and without vegetation (i.e., no background clutter to interfere with radar observations). Drone source colonies ($N = 11$) were moved to the WDL study site 4.8 km from the lake "shore" the day after radar observations were begun. The radar was used to scan the area before, during, and after drone flight was observed at the colony entrances. Additional observations were made at a grassland site (April 1987) near Sonoita, Arizona, in an area with low, rolling hills (approx. 1500 m above sea level). As at the WDL site, the 11 drone source colonies were brought to the site one day after radar observations had shown no honeybee and very few other insect targets present. Air temperature and wind speed and direction measurements were automatically recorded using a mechanical weather station (Meteorological Research Inc., model 1072) (1987) or an Omnidata (Omnidata International Inc., model ELS24-GP, Logan, Utah 84321) weather recording system (1990).

Pheromone Sources. In 1987 (April 14), 10 mg of synthetic 9-ODA was applied to a wick as a lure and suspended from a pole at a height of 3.5 m at the WDL site. The lure exposed a 0.8-cm length (approx. 25 mm^2 surface) of tubular knitted cotton of the type used in laboratory alcohol burners. Butler and Fairey (1964) reported that a queen elevated near an apiary was as attractive to drones as 100 μg of 9-ODA. This agrees with the data of Pain et al. (1967) and Butler and Paton (1962), who reported 108 μg and 130 μg of 9-ODA per queen, respectively. Our 10-mg lure was loaded with possibly 100 times more 9-ODA than a single queen would emit; however, we did not establish its rate of emission. On April 15–17, at the grassland site, a kite was used to elevate the lure approximately 6 m above the ground. In 1990 (April 12–20) a kite was used to elevate a mature, mated queen (in a queen cage) at 3–4 m elevation.

RESULTS

Worker bee flight was primarily to water and pollen sources (provided on the lake bed) and organized into distinct pathways. Drone flight was broadly scattered and usually higher than that of workers, and it was not organized into distinct flight paths or congregation areas as we had seen elsewhere (Loper et al., 1992). Drones were easily distinguished by both sight and sound by the observer near the lure.

Playback of the movies from both locations showed a scattered and apparently unorganized distribution of drones prior to pheromone release (Figure 1). As soon as a queen or queen pheromone was elevated, a linear concentration of drones formed downwind. During one trial at WDL (April 14, 1987) the pheromone source (10 mg 9-ODA on a wick) was opened at 1523 hr (MST) and suspended from a pole 3 m above ground. Drones could be heard and seen immediately in the near and downwind vicinity of the odor source. The radar showed an undulating "plume" of drones that increased in length extending downwind of the pheromone source. By 1607 hr, drones were moving upwind toward the pheromone from as far as 800 $\pm$ 15 m downwind. The wind was from the northwest, and the pheromone source was 885 m and 280° azimuth from the radar (temperature = 33°C, wind = 10–14 km/hr). The radar detected drones at a greater distance downwind; however, their distribution and behavior did not suggest a pheromone response beyond 800 m.

A similar response to the same pheromone source was obtained at the grassland site on April 17, 1987 (Figure 2). In this case, the radar detected a well-defined plume of drones downwind from the pheromone source, which was suspended from a kite 10–12 m above ground between 1619 and 1632 hr (temperature = 31°C, wind 14 km/hr). The wind was from the west and the pheromone source was at 345° azimuth and ~350 m from the radar. As the pheromone source was moved westward in a straight line, drone activity appeared on the radar screen as a whited-out area extending ~250 m downwind with small clusters and individual drones following from as far as 530 m. The high density (average spacing < 15 m) of drones prevented plotting of individual drone trajectories within the plumes.

A mature mated queen was used as the source of pheromone for additional observations on the dry lake in 1990. On April 14, 1990, a caged queen was suspended from a kite 3–4 m above ground (temperature = 31.5°C, wind = 4.8–14.5 km/hr) between 1520 and 1550 hr. Films of the radar screen indicated drones were orienting upwind toward the queen from distances of 190–420 m. As with the synthetic pheromone, a clear upwind direction of drone movement toward the pheromone source was seen. During brief periods (1–30 sec) when no drones were sighted near the queen, the radar detected clusters and or a plume downwind.

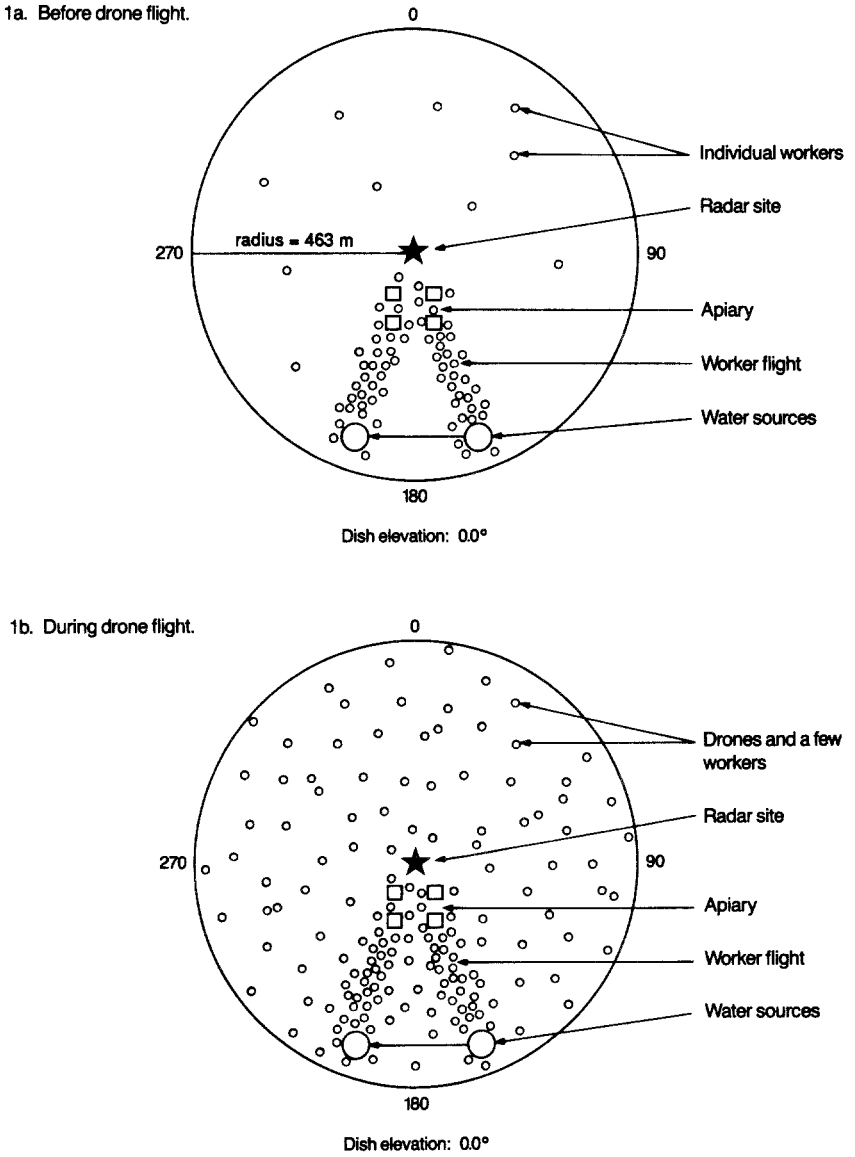

FIG. 1. Representation of radar screen prior to pheromone release: (a) before drone flight time; (b) during drone flight time.

Because of turbulence, pheromone plumes are known to waft in varying degrees downwind of the source. Most often, the radar showed a cluster of drones in the close vicinity of the queen (within 40–70 m), with smaller clusters and individual drones farther downwind (Figure 3a). At other times, evidence for wafting was seen when the trail of drones was laterally displaced as the plume extended downwind (Figure 3b). The dimensions of the drone clusters

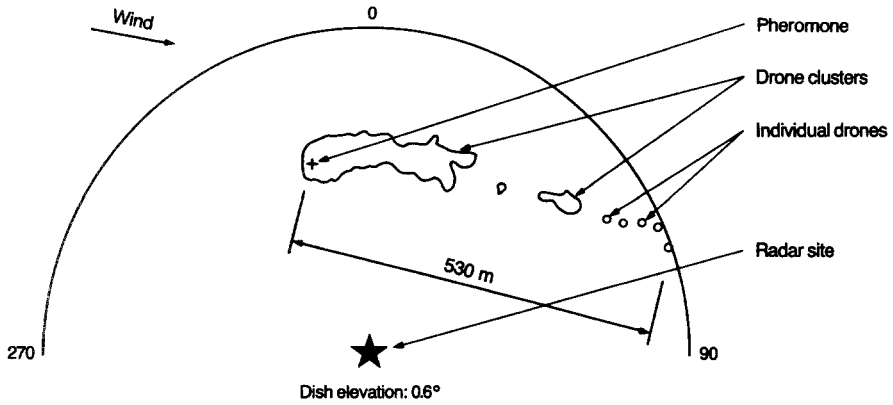

FIG. 2. Representation of radar screen showing length (m) and direction (azimuth) of plume of honeybee drones downwind of pheromone source (9-ODA) that was tethered below a kite at the left edge of the plume. Grassland site near Sonoita, Arizona (1623 hr MST) April 17, 1987.

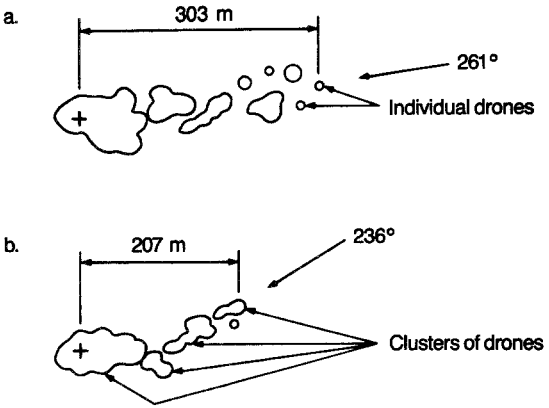

FIG. 3. Length (m) and direction (azimuth) of plumes of honey bee drones responding to a caged queen as detected by radar. April 14, 1990: (a) 1545:28 hr MST, straight downwind plume; (b) 1544:00 hr MST, Wafting plume.

changed with time (Figure 4); 5 min after queen exposure (1539 hr), a narrow, short (45 × 191 m) cluster and plume was seen (Figure 4a); after 10 min, the plume extended to 328 m (Figure 4b); after 10.5 min, to 421 m (Figure 4c). After 12 min, the plume was 360 m long (Figure 4d) and the cluster around the queen was more compact (area of queen cluster ≃ 8300 m^2). The duration of some downwind drone clusters and their relatively slow toward-queen movement suggests rapid maneuvering within the cluster (random or oriented flight would produce different distributions). Immediately after the sequence shown in Figure 4d, the queen was lowered, wrapped in aluminum foil, and pocketed. Just 62 sec later, the radar image (Figure 5) showed that the downwind response had terminated and the cluster nearly tripled in size to a nearly circular distribution (≃170 m diameter, encompassing 23,000 m^2), from which drones began to

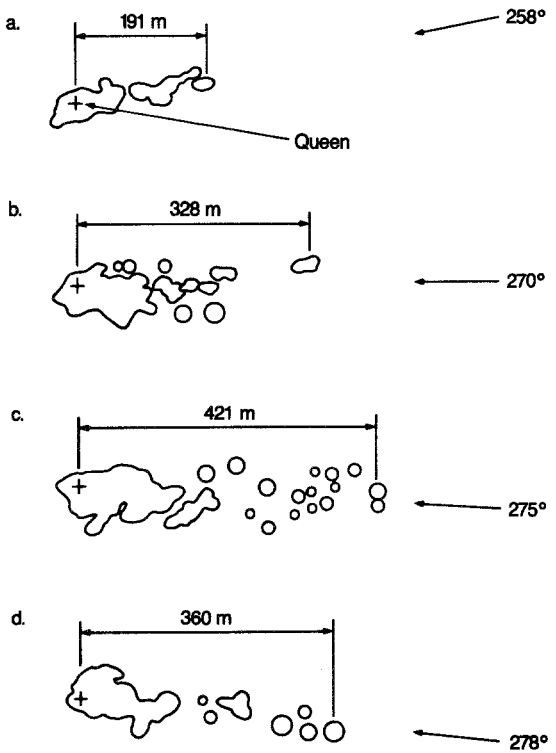

FIG. 4. Length (m) and direction (azimuth) of plumes of honeybee drones responding to a caged queen as detected by radar. Queen exposed at 1539:20 hr MST, April 14, 1990: (a) 5 min after queen exposure; (b) 10 min after queen exposure; (c) 10.5 min after queen exposure; (d) 12 min after queen exposure.

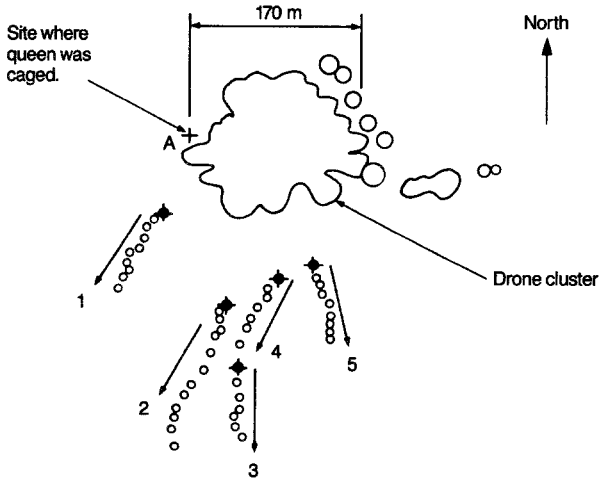

FIG. 5. Area of drone cluster nearly tripled in size (to 23,000 m^2) within 62 sec (1552:13 hr MST) after caged queen was removed from site A. Tracks (1–5) are drones returning towards apiary after the queen was removed.

return towards the apiary. These drones (tracks 1–5, Figure 5) appeared to use some nearby landmarks (vehicle tracks) for orientation.

DISCUSSION

The maximum distance that an insect can perceive a pheromone signal has been estimated to be 500–1000 m (Wall and Perry, 1987), depending on the strength of the pheromone source, the physical chemistry of the components, and the physical ambient conditions such as windspeed and temperature. Although there are numerous estimates of the maximum response distance to pheromones and theories on how the above-listed factors may interact, most, if not all, are based on inferential evidence. Direct observations of the paths taken by distant individuals to reach pheromone sources appear to be lacking in the insect literature. Arn (1990) emphasized that "we cannot be professionals in this field before we can visualize the dispersal of the disruptant chemical." While radar cannot visualize the chemical, our preliminary observations suggest that radar can record honeybee drones responding to the odors of a single queen from as far as 400 m and from a concentrated synthetic queen substance component (9-ODA) from as far as 800 m.

Although orientation by drones to sources of queen pheromones is well known (Gary, 1963; Taylor, 1984), the distances over which such responses

occurred were thought to be no more than 60 m (Butler and Fairey, 1964). Our observations indicate that flying drones respond to pheromones well beyond the limits of sight and hearing of a human observer. This observation is relevant, since we anticipate that in the future attempts will be made to bioassay the several components of the queen pheromone to determine which are the most effective in long-distance attraction and/or the release of copulatory behavior by drones. Radar can determine if drones are responding to components at distances of more than 60 m (beyond human observation).

The radar used in this study is capable of accurately detecting honeybees and other insects with cross-sectional areas approximating 1 cm^2 up to 1.0 km away. Honeybee drones are maximally 2 cm^2 in cross-sectional area and were easily detected at 1.5 km; the signal strength is inversely proportional to the fourth power (Wolf et al., 1993). This entomological radar unit is currently being used to study individual insects of medium to large size while foraging and responding to pheromones.

Acknowledgments—We acknowledge the expert assistance of John Edwards, Richard Berdel, and Robert Roselle and the cooperation of the Land Management Department, U.S. Army Electronic Proving Ground, Ft. Huachuca, Sierra Vista, Arizona, in scheduling the experiments on the Willcox Dry Lake. The assistance of Lenard Hines in obtaining permission from the Brophy family to use the grassland site on the San Ignacio del Babocomari Land Grant is gratefully acknowledged. Gordon Waller furnished the drone source colonies and virgin queens for these studies.

A videotape ($\frac{1}{2}$-in. VHS) with sequences showing the drone response to pheromone sources is available upon request from the senior author.

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SIMULATION AND EQUATION MODELS OF INSECT POPULATION CONTROL BY PHEROMONE-BAITED TRAPS

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Abstract—A spatial–temporal model for personal computers is developed that simulates trapping of an insect population based on trap and population parameters that can be varied independently. The model allows individual "insects" to move forward at any step size with right or left turns within any specified angle taken at random. The x and y axes of the area within which insects move can be varied as well as the number of insects, their flight speed, and the duration of the control period. In addition, the number of pheromone-baited traps, their placement in a grid or at random (with a variable degree of spacing), and their effective catch radius (proportional to pheromone release rate) can also be varied. Simulations showed that catch was similar regardless of whether traps were placed in a grid or practically at random (random placement but no traps were allowed to overlap in their effective catch radii). Iterative equations were developed for computer that can rapidly obtain values that correspond to the mean results from the slower simulation model. Based on a set of input parameters, the equations determine the percentage of the population that should be caught during a specified time, the time required to catch a specified proportion of the insects, and the number of traps necessary to catch the population proportion in the time period. The effects of varying the number of insects, flight speed, trap radius, and number of traps on the percent control or time to catch all insects are presented. Population control of the bark beetle *Ips typographus* was simulated using realistic pheromone trap and population parameters. A discussion of insect and bark beetle (Coleoptera: Scolytidae) population control using pheromone traps is presented.

Key Words—Semiochemical, pheromone, pest, biological control, insect trap, personal computer program, Scolytidae, Coleoptera, mass trapping.

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INTRODUCTION

Basic research in chemical ecology begins with the observation of natural phenomena regarding an organism's movements (behavior) in response to chemical stimuli released by other organisms of the same or other species (or different phylogeny). The natural phenomena are often utilized in a bioassay for isolation and identification of the chemical stimuli (semiochemicals). These semiochemical mechanisms are not only of inherent interest but also important from economic and applied viewpoints. In fact, much of the impetus for research on pheromones, allomones, and kairomones is due to their promise for use in an integrated system of pest management and control (Silverstein, 1981). The synthetic semiochemicals can be used for monitoring populations of insects and in direct control (by interfering with natural information-bearing chemicals leading to reduced ecological fitness and reproduction).

Pheromones have been used in the field to disrupt mate finding in moths (Hodges et al., 1984; Zvirgzdins et al., 1984; Flint and Merkle, 1984; Campion et al., 1989), beetles (Villavaso and McGovern, 1981; Villavaso, 1982), and flies (Jones et al., 1982). In most cases, relatively large quantities of pheromone (consisting of several pheromone components) are more or less evenly distributed throughout the field to adapt sensory receptors or habituate behavioral response (confusion) or to exhaust the individuals in orientation attempts (wild-goose chases). The best successes so far have involved straight-chain hydrocarbons of moths.

Bark beetles that colonize forest trees may present problems for disruption techniques for several reasons; one is that their pheromone components, usually oxygenated monoterpenes, are more volatile than moth straight-chain hydrocarbons (Byers, 1989). More important perhaps is that compared to moths even larger quantities are expected to be required for disruption of bark beetles since these individuals generally release higher rates (nanograms to milligrams per hour) of pheromone components than moths (picograms to nanograms per hour) (Browne et al., 1979; Schlyter et al., 1987; Birgersson and Bergström, 1989; Byers et al., 1990a,b; Ramaswamy and Cardé, 1984; Du et al., 1987). Furthermore, even higher quantities of synthetic pheromone are required to compete with pest bark beetles that typically call in large aggregations on their host tree. Possibly because of these reasons, as well as that both sexes are attracted by pheromone, several attempts to control bark beetles have used the mass trapping method. This method employs traps, either sticky-screen (Browne, 1978) or cylinder with holes or barriers (Bakke, 1989), baited with synthetic pheromone components to catch adults. Traps releasing pheromone components have been used in control programs to lure other pest insects such as moths to their death (Haniotakis et al., 1991; Sternlicht et al., 1990).

Previous theoretical attempts at determining the effectiveness of pheromone

mass trapping have used population dynamic models (Knipling and McGuire, 1966; Roelofs et al., 1970; Beroza and Knipling, 1972; Nakasuji and Fujita, 1980; Nakamura, 1982; Barclay, 1984, 1988; Fisher et al., 1985; Barclay and Li, 1991). These models are mathematically complex and make several assumptions about population survival and mating rates as well as attraction rates to pheromone traps, which limits their application. There have been no models where "insects" are moved in "real" time and space in relation to traps of specific dimensions and positions.

The first objective of the present study was to develop a simulation model where any number of individual insects can move within any size area at any specific speed and duration. Traps of any size radius, number, and placement may "catch" the individuals by intercepting them during flight. By varying these parameters, one can determine the number of traps required for effective control based on realistic assumptions or actual data about the insect species, its pheromone, and population level. The simulation model led to the development of iterative equations that rapidly derive the same results as the simulation model, although without indicating the statistical variation. The second objective was to use the model and the iterative equations to estimate the trapping efficiency of pheromone-baited traps during control of the bark beetle *Ips typographus*.

METHODS AND MATERIALS

A computer program for personal computers that simulates insect movement and their catch on traps was developed with the QuickBASIC programming language (version 4.5, Microsoft). The source code (TRAP-SIM.BAS) was compiled to a binary, machine-coded, executable file, by the Microsoft compiler version 4.50 and overlay linker version 3.69 to obtain maximum speed for simulations. IBM-compatible personal computers with EGA/VGA graphics can execute the simulation program (a math coprocessor is recommended). The operation of TRAP-SIM.BAS is diagrammed in Figure 1 and the arrows between boxes indicate program flow.

The model is similar to a mate-finding model published earlier (Byers, 1991) except that each trap (formerly a female) can catch any number of insects. The user enters the x and y sides of the area, the number of insects, flight speed, trap radius, and duration of the control period. Realistic movement of insects is achieved by using polar coordinates in which the angle of directional movement is changed randomly at each step at most equal to the angle of maximum turn (AMT), which can be either right or left (chosen at random) from the previous direction (Figure 2). The step size and AMT can be varied in the model but have little effect on the results (Byers, 1991). When insects impact the area's

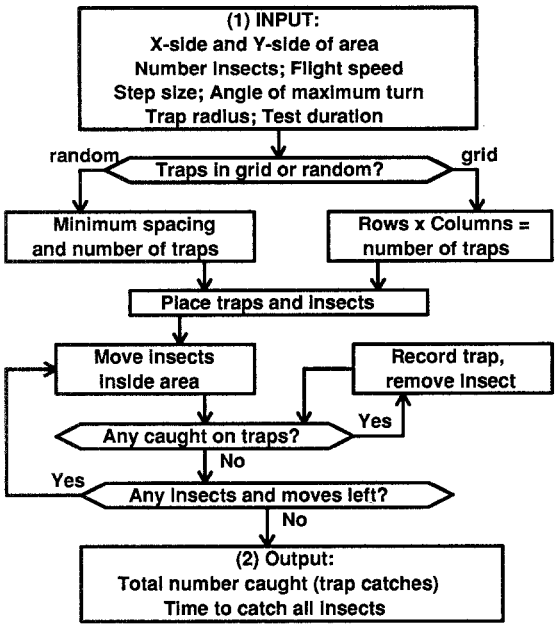

FIG. 1. Relationships of program parts of TRAP-SIM.EXE, a simulation model for insect movement inside a bounded area where any number of traps can intercept insects during the simulation period.

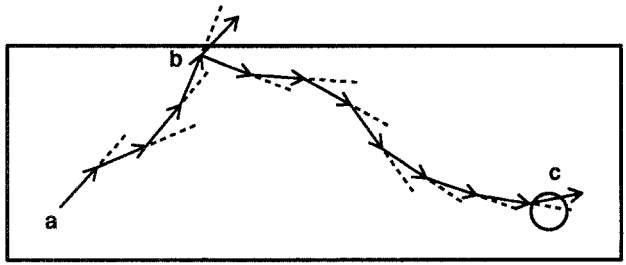

FIG. 2. Animal movement begins at (a) at a random angle for a uniform step (of any specified size). The next step is either to the left or right of the previous direction in an angular amount of less than the AMT (angle of maximum turn). The AMT is chosen by the user (0–180°), but the deviation (right or left) and magnitude (<AMT) are chosen at random. If an animal attempts to move outside the area's boundaries, e.g., at (b), then another angle of up to 360° is chosen at random. Animals are caught by circular traps when within the radius or even when intersecting the circle regardless of the step size (c).

boundaries, they rebound at a random angle (Figure 2). Initial angular directions and positions of individuals are also chosen at random.

Placement of a certain number of traps in the model can be done in a grid of any row × column arrangement with automatic even spacing (Figure 1). Alternatively, traps can be placed in a random pattern with at least a specified amount of minimum spacing between traps (Byers, 1984, 1992). The "insects" move a step at a time (usually 5 m) up to the number of moves determined by the test duration and the speed of flight, or until caught by a trap. Insects are removed from the simulation if caught, and the percentage of the initial population caught at the end of the test, or the time observed to catch all the insects, is recorded at the end (Figure 1).

While the simulation model can be used with any insect where an average flight or walking speed can be determined, in this paper the focus will be on bark beetles (Coleoptera: Scolytidae), specifically *Ips typographus* L., the major pest of Norway spruce, *Picea abies* (L) Karst., in Europe (Austarå et al., 1984). In order to determine the effect of a model parameter on the catch, one can hold most other important parameters constant while varying the parameter of interest. It is more realistic and interesting to choose parameters that are as close to the expected natural values as possible. Byers et al. (1989) presented a method for comparing relative attraction distances of different insect species to pheromones called the "effective attraction radius" (*EAR*). The *EAR* for a pheromone-baited trap is the radius that would be needed by an imaginary spherical passive trap in order to intercept as many insects as that actually caught on the pheromone trap (Byers et al., 1989).

$$EAR = \sqrt{\frac{AL}{P\pi}} \tag{1}$$

The *EAR* is found by comparing the catches (P) on passive traps of known dimension (L is trap silhouette area) to the catch on pheromone-baited traps (A). For the synthetic two-component pheromone blend of *I. typographus* (2-methyl-3-buten-2-ol released at 50 mg/day and [1(*S*),4(*S*),5(*S*)-*cis*-verbenol at 1 mg/day], the *EAR* estimation was about 2 m (Byers et al., 1989). This three-dimensional theoretical distance was approximated as a two-dimensional (effective catch radius) in the model. The flight speed of *I. typographus* in nature was also estimated at about 2 m/sec (Byers et al., 1989).

The relationship between the number of moves and the cumulative catch for 200 beetles in a 1-km^2 area (a very low population density) was obtained by simulation. The model parameters are reported in Figure 3. The effects of increasing the number of insects, trap radius, flight speed, and number of traps also were determined on the percent control in 8 hr or the hours required to catch all insects. Based on the results from the simulation model, iterative

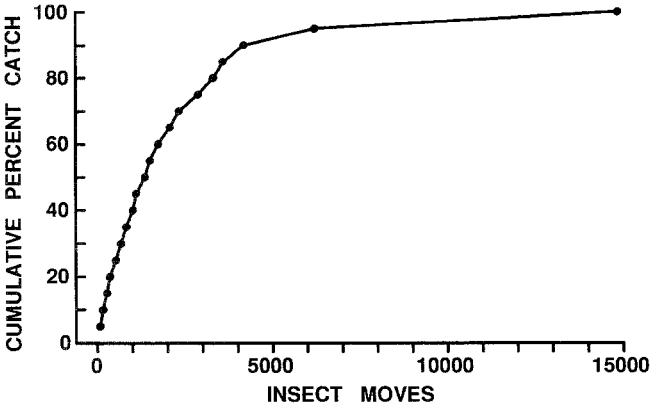

FIG. 3. Relationship between the number of insect moves and the cumulative percentage of catch obtained in the simulation model. Model parameters were: 1000 × 1000-m (1-km^2) area, 200 insects, 2 m/sec flight speed, 5 m step size (move), 30° angle of maximum turn (AMT), 25 traps (5 × 5 grid, 142.8 m spacing), 2-m trap radius, and 8 hr test duration.

equations were derived that can construct the same relationships, but rapidly give theoretical mean results.

RESULTS

The simulation model (Figures 1 and 2) found that the cumulative percent catch of the beetles increased rapidly during the initial moves, but it took increasingly longer (more moves) to catch the last of the beetles (Figure 3). This result can be expected since initially there are many more beetles available to be caught by the 25 traps, but in the later part of the test period there are relatively few beetles (the density is much less) so the probability of catch by all the traps is much less. However, the probability of any particular insect being caught is constant over the whole period.

Surprisingly, the turn angle taken at random by each insect at each move (less than a maximum angle of turn = AMT) has very little effect on the rate of catch. An AMT of 30° simulates insect flight reasonably well, but use of 5° for a more straight flight produces almost the same effects on trap catch. For instance, using parameters as in Figure 3 but a trap radius of 0.25 m, the percent control for an AMT = 5° (nearly straight path) was 47.04 ± 2.60% (±95% CL, N = 12), while it was 52.00 ± 2.18% for an AMT = 30° (used in most simulations) or 43.92 ± 1.99% for an AMT = 180° (purely random). The iterative equation (discussed later, Figure 8) predicts 51.5% control in 8 hr. The

step size also has little effect on the catch rate as shown earlier (Byers, 1991). Purely random path angles (Brownian motion) produce slightly less catch, since the insects cover relatively less new ground due to more likely back-stepping over areas they had just been in.

Another intriguing finding was that the placement of traps, whether distributed in a highly regular grid arrangement or at random (but not overlapping), had no apparent effect on the rate of catch. Using the same parameters as above, 49 traps of 2-m radius were placed in either a 7 × 7 grid (125-m spacing) or at random with a spacing of at least 4 m between centers (minimum allowed distance was 4 m). It was found that the average time to catch all insects was not significantly different between the two placements, 3.97 ± 0.36 hr and 4.03 ± 0.68 hr (±95% CL, $N = 10$), respectively. The iterative equations (Figure 8) predict a time of 4.17 hr for 100% control.

Increasing the numbers (and densities) of insects in the initial population has a logarithmic effect on the number of hours needed to catch all the insects (Figure 4). Thus, for 25, 2-m radius traps in a 1-km^2 area, the time required to catch all beetles changes relatively little as the number of beetles is increased beyond several hundred. Increasing the radius of the trap or the flight speed has the same effect on the catch (percent control) of 200 beetles in a period of 8 hr and parameters as above (Figure 5A and B). Again, increases in flight speed or trap radius have greater effects on catch at lower speeds and smaller radii. As the flight speed or trap radius is increased still more, it has proportionally less

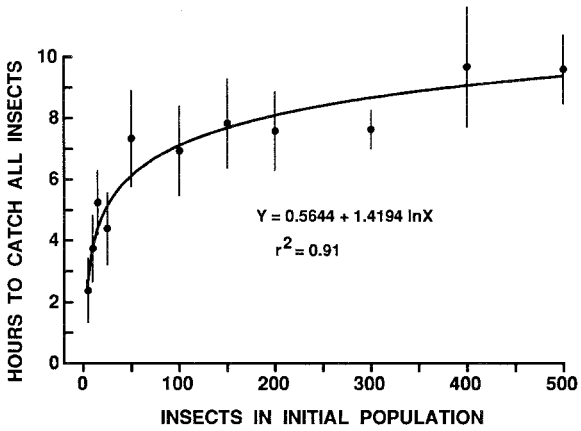

FIG. 4. Relationship between the number of insects in the initial population before control and the number of hours to catch all these insects as found with the simulation model. Model parameters were as in Figure 3 except that the number of insects was varied and the test duration was made long enough to catch all insects. Vertical bars represent 95% confidence limits ($N = 4$).

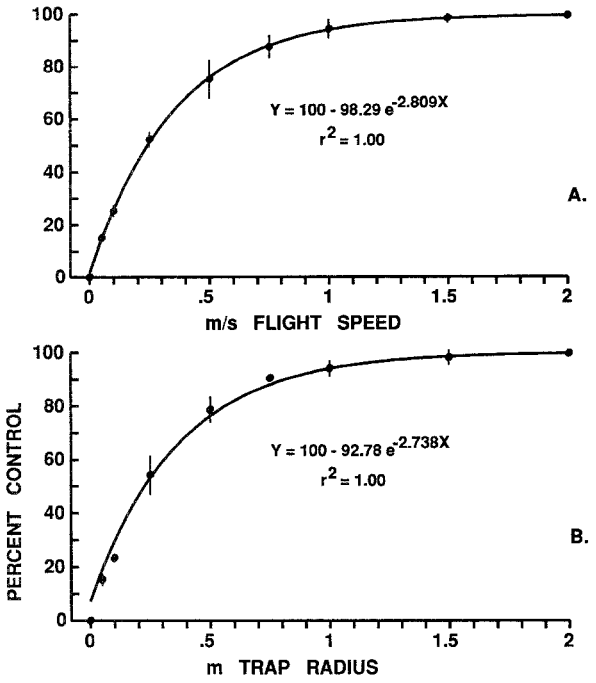

FIG. 5. (A) Relationship between flight speed (m/s) and percent control of the insect population as found with the simulation model. Model parameters were as in Figure 3 except for variation of flight speed. (B) Effect of trap radius (m) on percent control of the population as found with the simulation model, model parameters as in Figure 3 except for variation of trap radius. Vertical bars represent 95% confidence limits (N = 4).

effect on the time to catch all insects, even though less time is needed to catch them at these larger speeds or radii (Figure 6A and B). In the model, the number of traps of 2-m radius can be increased from 1 to 25 (Figure 7A), causing the percent control or catch to increase less and less rapidly as the trap number is increased. When the number of traps is increased still further, from 25 to 400/ km^2, then the time to catch all insects decreases but less and less rapidly with an increase in number of traps (Figure 7B).

The results of the simulations above gave insight for the development of iterative formulas that calculate the expected time required to catch a given proportion of the insects in a certain area based on trap radius, number of traps, number of insects, and average speed of flight. Intuitively, the time between catches of insect on traps would be inversely proportional to trap diameter, i.e., a larger trap would likely intercept insects sooner than a smaller trap. Further-

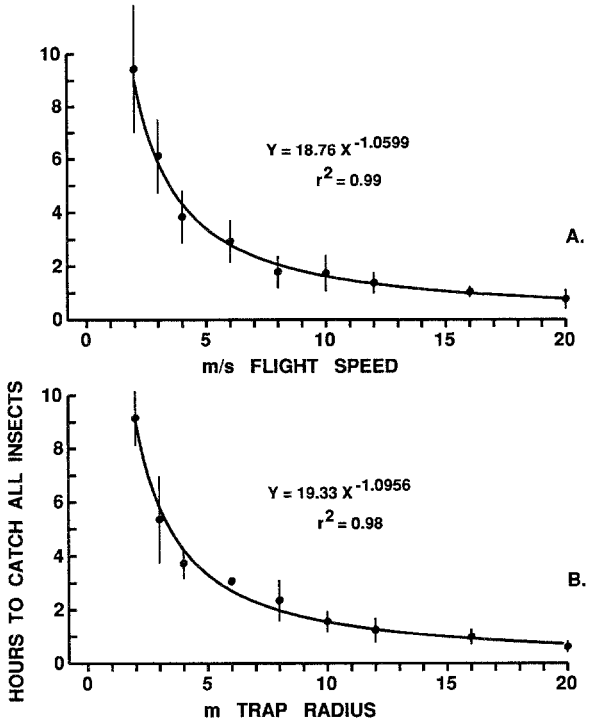

FIG. 6. (A) Relationship between flight speed (m/s) and the number of hours to catch all insects in the area as found with the simulation model. Model parameters were as in Figure 3 except for variation of flight speed and test duration was indefinite. (B) Effect of trap radius (m) on time required to catch all insects in the area as found with the simulation model, model parameters as in Figure 3 except for variation of trap radius and indefinite test duration. Vertical bars represent 95% confidence limits ($N = 4$).

more, a greater insect flight speed, greater number of traps, or greater density of insects would decrease the expected time between catches. Therefore, let R = radius of the trap in meters, K = number of traps, T = time in seconds, S = flight speed in meters per second, N = number of initial insects, and A = area in square meters, then the time needed to catch the first insect is: $T = A/(2RSKN)$. The total time to catch any proportion P of insects is given by the equation:

$$T = \sum_{C=0}^{NP-1} \frac{A}{2RSK(N - C)} \tag{2}$$

Thus, if A = 1,000,000 m^2 (i.e., 1 km^2), there are 25 traps each of 2-m radius, beetles fly at 2 m/s, and there are 70,000 beetles initially, then the expected

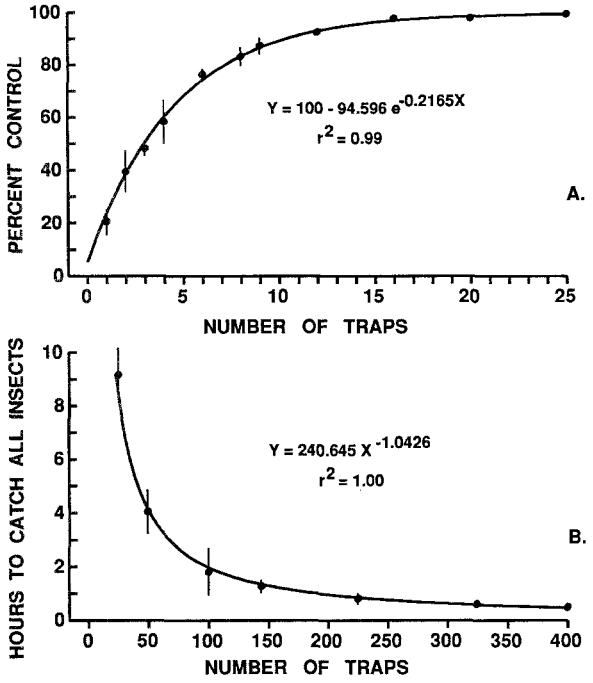

FIG. 7. (A) Relationship between number of traps and percent control of the insect population as found with the simulation model. Model parameters were as in Figure 3 except for variation of number of traps and grid rows and columns. (B) Effect of number of traps on time required to catch all insects in the area as found with the simulation model, model parameters as in Figure 3 except for variation of trap number, rows and columns. Vertical bars represent 95% confidence limits ($N = 4$).

time to catch 95% ($P = 0.95$) of the beetles is equal to 14,978 s (4.16 hr). A BASIC program that calculates the expected time from equation 2 and others below is shown in Figure 8.

When one wants to determine the expected proportion of the population that will be caught (percent control) in a specified time period based on the above parameters, then a repetitive calculation is done. Here the expected times (from equation 2) to catch successive insects are subtracted sequentially from the specified time until no time is left. The number of insects caught divided by the initial population estimate multiplied by 100 is then the percent control expected in the specified time period. The BASIC program in Figure 8 demonstrates the algorithm (lines 140–170). For example, Byers et al. (1989) used sticky trap screens to estimate that 9000 *I. typographus* were flying during a few hours in a 1-km^2 area. If control of *I. typographus* at this density is imple-

```
10 CLS : COLOR 15: PRINT "TRAPS NEEDED TO TRAPOUT PROPORTION OF INSECTS"
20 PRINT "IN TIME ALLOTTED - (C) 1992 by John A. Byers": PRINT : COLOR 11
30 INPUT "NUMBER OF INSECTS IN AREA"; N: N = INT(N): IF N <= 0 THEN 30
40 INPUT "RADIUS OF TRAP (In m)"; R: IF R <= 0 THEN 40
50 INPUT "SPEED OF INSECT (In m/s)"; S: IF S <= 0 THEN 50
60 INPUT "NUMBER OF TRAPS"; K: K = INT(K): IF K <= 0 THEN 60
70 INPUT "ENTER %CONTROL ( <=100% )"; P: IF P <= 0 OR P > 100 THEN 70
80 INPUT "ENTER DURATION OF TRAPOUT (In s)"; TIME: IF TIME <= 0 THEN 80
90 INPUT "AREA OF TEST (In m2)"; A: IF A <= 0 THEN 90
100 P = P / 100: TT = TIME: COLOR 15: PRINT
110 PRINT "WORKING...": PRINT : FOR C = 0 TO N * P - 1
120 T = T + A / (2 * R * S * K * (N - C)): NEXT: C = 0
130 PRINT "TIME TO CATCH"; P * 100; "% ="; T; "s OR"; T / 3600; "h"
140 IF C = N THEN K = 0: GOTO 170
150 TIME = TIME - (A / (2 * R * S * K * (N - C)))
160 C = C + 1: IF TIME > 0 THEN 140 ELSE K = 0
170 PRINT C / N * 100; "% CAUGHT IN TIME ="; TT; "s OR"; TT / 3600; "h"
180 FOR C = 0 TO N * P - 1: K = K + A / (2 * R * S * TT * (N - C)): NEXT
190 PRINT K; "TRAPS NEEDED TO CATCH"; P * 100; "% IN"; TT; "s ALLOTTED"
```

FIG. 8. BASIC listing of program using iterative equations (see text) to predict (1) the time required to catch a specified percentage of the insect population, (2) the population percentage caught within a specified time period, and (3) the number of traps required to catch a specified percentage of the population within a specified time period. The predictions (1–3) are dependent on the number of insects in the initial population, the effective radius of the trap, the average flight speed of the insect, the number of traps, the desired level of control, the duration of the trapping period, and the size of the control area.

mented for 48 hr (172,800 s) using 10 pheromone traps with an effective radius of 2 m in a 4-km^2 area containing (4 × 9000) beetles flying at 2 m/s then 96.8% of the population would be trapped out.

Another calculation of interest is the expected number of traps of a certain dimension that are required to trap a certain percentage of the population in a given time period. Using the parameters described in equation 2, the number of traps can be found from the following equation:

$$K = \sum_{C=0}^{NP-1} \frac{A}{2RST(N - C)} \tag{3}$$

The BASIC program (Figure 8) calculated that in a trapping period of 20 hr five or six traps (actually 5.2 traps) each with a radius of 2 m would be needed to achieve 95% control of 9000 beetles (flying at 2 m/s) in an area of 1,000,000 m^2. Even if there were 100 times more beetles (900,000) flying in the area, only 5.2 traps would still be needed for 95% control in 20 hr (since the number of traps needed is logarithmically related to the number of insects). If 95% control is to be achieved in one tenth the time (2 hr) then 10 times the number of traps are needed (52 traps).

The iterative equations (Figure 8) also were used to validate the statistical regressions obtained from the simulation model. In Figure 4, a logarithmic relationship was found between population density and time to catch all insects ($r^2 = 0.91$), and the iterative equations also gave results that were a perfect logarithmic relation ($r^2 = 1.00$). The iterative equations also gave results that perfectly fit the asymptotic exponential form shown in Figures 5A and 5B and in Figure 7A. The negative power curves in Figures 6A, 6B, and 7B were validated with results from the iterative equations, which fit these curves perfectly.

DISCUSSION

The personal computer video monitor allows viewing of the individual "insects" as they wander about within the simulated area. Traps and their diameter are also represented on the screen and when insects are caught, they turn color from white to red and cease moving. The model is primarily useful for demonstrating the principles of insect mass trapping since the iterative equations yield essentially the same results and require much less computing time. The simulation model does allow an estimation of the statistical variation that could result in nature under similar conditions, while the iterative equations give exact-mean answers. The chemical ecologist and forest pest manager will find the iterative equations more useful in practice since several parameters of interest can be tried rapidly.

The simulation model revealed relationships that are not always intuitive. For example, it might seem that an increase in flight speed ought to cause a linear decrease in the time needed to catch all insects. The problem is that the entire population (or a specific proportion) must be caught, and this time decreases as a power function. Another example is that the percent of the population caught in a certain time period (percent control) with an increase in trap radius might seem to be linearly related, but this implies that more than 100% control could be achieved, which is impossible. Almost no matter how large the trap is made, there will always be some simulations where not all the beetles were caught in the time period due to pure chance.

Pheromone plumes are composed of packets of more concentrated pheromone molecules (cf. Baker, 1986). The precise dimensions of the packets are not known but it can be assumed that they differ under different wind and weather conditions. Gaussian surfaces can represent to some degree of accuracy the time-averaged dimensions of a pheromone plume (cf. Elkinton and Cardé, 1984; Byers, 1987). A plume extends indefinitely but its detection by the insect depends on a threshold concentration, and furthermore, the probability of orienting to the pheromone source (baited trap) depends on where the insect enters the plume.

These complications for use in models may be largely avoided with the concept of the effective attraction radius (*EAR*, see equation 1) of a semiochemical release rate for an insect. In order to use the *EAR* for the present models, one must determine it for the particular pest species. In *I. typographus*, as mentioned above, this radius was about 2 m for a specific release rate of synthetic pheromone components. This radius is three-dimensional, but for the model it was considered as two-dimensional (i.e., a cylinder). Thus, the *EAR* is probably a lower estimate of the effective catch radius that would be most appropriate for the simulation model. A review of these concepts of attraction radius is given by Schlyter (1992).

It should be stressed that the simulation model was instrumental in deriving the equations (2 and 3 and Figure 8) that iteratively calculate the same results as the simulation model. The simulation model can handle up to about 15,000 insects but becomes prohibitively slow. The iterative equations are much faster than the simulation, but the equations still must iterate once for each insect in the population. However, for populations of even one million insects, it takes only 1–2 min on IBM-compatible personal computers (486 or 386 + math coprocessor) to calculate expected catches or required traps (from Figure 8) for any set of model parameters.

The first major attempt to control bark beetle populations using pheromone-baited traps was done in 1970 in California (Bedard et al., 1979; Wood, 1980; DeMars et al., 1980). Large (1 × 2 m) sticky screens baited with *exo*-brevicomin and frontalin, pheromone components of the western pine beetle, *Dendroctonus brevicomis* (Silverstein et al., 1968; Kinzer et al., 1969), plus the host monoterpene, myrcene (Bedard et al., 1969), were placed in ponderosa pine forests at Bass Lake, California. In four plots of 1.3 km^2 each, 66 pheromone traps were deployed in a grid of about 161 m spacing. Over a million beetles were caught, and the test appeared to be successful since the number of trees killed by the beetle declined to 10% of the pretreatment level for several years (Bedard et al., 1979; Wood, 1980; DeMars et al., 1980).

Norway and Sweden have extensive forests, and in the 1970s a major outbreak of the European spruce engraver, *I. typographus*, devastated many areas (Austarå et al., 1984). Since the pheromone of this beetle had recently been identified as a mixture of 2-methyl-3-buten-2-ol and 1(*S*),4(*S*),5(*S*)-*cis*-verbenol (Bakke et al., 1977), an extensive mass-trapping control program was initiated in 1979 and may have led to the decline of the outbreak after 1980 (Bakke, 1985, 1988, 1989; Vité, 1989). Several other European studies have reported successful control of bark beetles with the intensive use of pheromone-baited traps (Vrkoc, 1989; Richter, 1991).

These pioneering studies of mass trapping using pheromones did have some deficiencies. Many of these studies lacked appropriate controls or check plots, so it is not possible to determine the success of the control program. Further-

more, a combination of experience and intuition led to subjective estimates as to the level of trapping and the pheromone release rate most appropriate for control of the population. Certainly these questions are complex, and it is not surprising that they were not solved entirely successfully back then. However, the models presented here may help establish a rational basis for future control efforts using pheromone traps based on estimates of the population size (density), average flight speed, expected control duration, effective trap catch radius, and number of traps deployed.

Weber (1987) was critical of pheromone trapping of bark beetles for control since he calculated that enough beetles would remain untrapped to then colonize susceptible hosts and replenish the population due to an absence of competition. This assessment seems overly pessimistic since trapping experiments with different traps and pheromone dosages were not done. Furthermore, the complementary effects of other forest management practices, such as removal of slash and infested trees to reduce populations, were not considered. The consequences of population reduction to densities below the threshold required to overcome tree resistance by means of a mass attack were also not considered (Berryman and Stenseth, 1989; Berryman et al., 1989). In contrast, the present models indicate that insect populations can potentially be drastically reduced with a surprisingly small number of traps with an effective radius that seems smaller than that one might intuitively expect for pheromone baits. However, whether this population reduction is sufficient to affect natural matings and population levels over several generations is still an open question.

In many past control programs using pheromone trapping there has been the problem of finding control areas to determine whether the treatment has been effective. However, several monitor traps placed inside the control area (or even the control traps themselves) will indicate the population density and the progress of the control program. If no more insects are being caught, then obviously the control is a success, unless the flight period is over. This can be determined by monitor traps in untreated areas some distance away but still within the same general biotope and climatic regime. Usually only one beetle or pair of bark beetles begins attack of a tree, and at this time pheromone release is relatively low compared to a few days later when thousands of beetles participate in the mass attack. Thus, it seems advantageous to initiate mass trapping before beetles swarm in the spring and have time to build aggregations that can compete with traps for attraction of dispersing beetles. In moths, reproduction can occur despite the high trapping efficiency suggested by the model because male moths may mate with females before being trapped (Roelofs et al., 1970).

There are several variables that can influence the situation in nature so that trapping of the population does not follow the predictions based on the simulation model or iterative equations. For example, the "flight" speed used in the models (2 m/s; Byers et al., 1989) may be more than the speed observed for

flying or wind-blown insects since they often stop to rest or feed. Moreover, in the case of bark beetles, there can be host volatiles that attract the beetles during their swarming flight or trees under colonization where aggregation pheromones are released (Byers, 1989). Several studies have indicated that as the density of calling female moths increases, the catches on pheromone-baited traps increase relatively less or may decline, probably due to competition between the natural and synthetic sources (Raulston et al., 1979; Nakamura, 1982; Witz et al., 1992). Traps can also be overloaded with catch, and synthetic pheromone release rates can diminish, which will cause catches in nature to differ from model predictions. Pheromone release rates can decrease (and the effective pheromone trap radius) due to compound degradation and in other cases due to exponential decline from substrates (e.g., rubber septa). Methyl decadienoate, a pheromone component of the bark beetle *Pityogenes chalcographus*, is especially sensitive to sunlight and attraction rates can be halved in a few hours unless the compound is shaded. In the models, the shapes of pheromone plumes emanating from traps have been transformed to the *EAR*, which also reduces the correspondence between reality (some type of Gaussian time-averaged plume depending on the wind) and the models (a circle).

In spite of these assumptions, the iterative equations should prove useful to pest control managers in agriculture and forestry. Models are useful to define problems, organize thoughts, understand the system, identify areas to investigate, communicate understanding, make predictions, generate hypotheses, and act as standards for comparison (Worner, 1991). The model here allows one to balance resources (number of traps per area) with expected populations and parameters of the pest insect to obtain a cost-effective deployment of pheromone traps as a first approximation for experiments and control treatments.

The programs TRAP-SIM.EXE (Figure 1) and TRAPOUT.EXE (Figure 8) can be obtained from the author (send a formatted disk and mailer for IBM-compatible computers). Donations of $5 or 35 Swedish Kronor for shipping would be appreciated.

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CAFFEOYLTARTRONIC ACID FROM CATNIP (*Nepeta cataria*): A PRECURSOR FOR CATECHOL IN LUBBER GRASSHOPPER (*Romalea guttata*) DEFENSIVE SECRETIONS

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Abstract—Adults of the lubber grasshopper (*Romalea guttata*) secrete increased amounts of catechol from their defensive glands when fed diets containing only catnip leaves (*Nepeta cataria*). Model compound bioassays showed that these insects were able to sequester and biomagnify simple phenols, such as catechol and hydroquinone, in their defense gland secretions. Excessive catechol secretions from caffeic acid-fortified diets indicated met-

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abolic pathways exist to perform efficiently more complex biochemical conversions. Reverse-phase HPLC of methanol extracts of catnip revealed only one major caffeoyl-polyphenol as a possible precursor for the observed elevated catechol secretions, when this plant is fed to lubbers. The compound was shown to be caffeoyltartronic acid (CTA). During analysis of CTA by probe-MS or gas chromatography (of its silylated derivative), CTA decomposed by loss of carbon dioxide to form caffeoylglycolic acid (CGA), making identification by these methods ambiguous. Only fast atom bombardment mass spectrometry (FAB-MS, negative mode) gave a true molecular weight. Groundivy (*Glecoma hederacea*), a relative of catnip, was also shown to contain CTA. The mung bean (*Phaseolus radiatus* = *Vigna radiata*), a species totally unrelated to catnip, is the only other reported plant source of CTA. Catnip leaves were found to contain about twice as much CTA as mung bean leaves.

Key Words—Catnip, *Nepeta cataria*, caffeoyltartronic acid, lubber grasshopper, *Romalea guttata*, catechol secretions.

INTRODUCTION

Many insects sequester plant allelochemicals, and these compounds often constitute key elements in the chemical arsenals of these herbivores (Rothschild, 1972; Blum, 1981). Plant natural products or their metabolites are frequently incorporated into the defensive secretions of insects (Duffey, 1980; Jones et al., 1989; Blum et al., 1987, 1990) but, for the most part, the metabolic pathways for these altered plant constituents are unknown. Recent investigations on the chemistry of the deterrent exudate produced in the metathoracic glands of a generalist herbivore, the lubber grasshopper (*Romalea guttata*), demonstrate that while some compounds in this secretion may be biotransformed from obvious precursors, the origins of others do not appear to be as readily apparent (Blum et al., 1990). Notwithstanding the metabolic eclecticism that characterizes these phytochemicals, it is evident that the deterrent value of the secretions (towards ants; Blum et al., 1990) may reflect both the quantitative and qualitative properties of selected plant compounds.

R. guttata is an excellent model for studying the chemistry of plant-derived defensive secretions because these grasshoppers discard the glands, plus their secretions, when they molt. These insects, with their new, empty glands, can be fed either selected plant species or candidate compounds. Indeed, the chemistry of the subsequent secretions reflects the ingested allelochemicals that have been sequestered and/or metabolized before or after storage in the defensive glands (Jones et al., 1987; Blum et al., 1990).

The concentrations of catechol and hydroquinone, two major glandular constituents, are subject to great perturbations when grasshoppers are fed on different diets. For example, concentrations of these two phenolics in defensive gland secretions were depressed when lubbers were fed only lettuce or an arti-

ficial diet lacking fresh plant material, as compared to exudates produced by insects feeding on a natural polyphagous diet (Jones et al., 1989). When adults were fed exclusively wild onion (*Allium canadense*), phenolic concentrations were about 37× lower than those found in the secretions of wild-fed grasshoppers (Jones et al., 1989). On the other hand, when the sole food plant was catnip (*Nepeta cataria*), both the concentrations of catechol and hydroquinone and their ratio were the same in secretions as they were in exudates produced by field-collected lubbers that had fed on polyphagous diets (Blum et al., 1990). Furthermore, when catnip was added to a natural diet, the concentration of catechol increased about fourfold.

The ability of ingested catnip to stimulate the production of high levels of catechol in the defensive secretions of grasshoppers strongly suggests that this plant species is providing a precursor for this phenolic. In this report, we examine the polyphenolic chemistry of *N. cataria* in order to identify the phenolic precursor and, in addition, examine the effects of some ingested polyphenolics on catechol concentrations in the defensive secretions.

METHODS AND MATERIALS

All solvents were analyzed reagent grade. Catnip and mung beans were grown in pots in a greenhouse.

HPLC Analyses. Methanol extracts of catnip or mung bean were analyzed on a Nucleosil C-18 reverse-phase column; linear solvent gradient from 20% MeOH/H_2O to 90% MeOH/H_2O in 35 min (0.1% H_3PO_4 in both solvents, pH 2.5). A Waters MicroBondapak C-18 column—linear solvent gradient from 13% MeOH/H_2O/0.08 M KH_2PO_4 (adjusted to pH 4.4 with H_3PO_4) to 50% MeOH/H_2O in 35 min—was also used. Column effluent was monitored at 340 nm. Quantitation was done by the internal standard method using 7-hydroxycoumarin. Newly emerged, 25-cm-long catnip leaves and 14-day-old mung bean plants (25 cm leaves) were analyzed.

GC Analyses. Gas chromatographic analyses were performed with an immobilized SE-54 (30 m × 0.3 mm ID) capillary column; injector 250°C, detector 350°C, temperature program 100–300°C at 8°/min. Analyses of caffeoyltartronic acid and caffeoylglycolic acid were performed on trimethylsilylated derivatives (TMS) prepared from BSTFA/DMF (1 : 1) and heated at 75°C for 30 min. Preparation of the TMS derivative of tartronic acid, from the hydrolysis of CTA, required initial treatment with trimethylchlorosilane followed by BSTFA and pyridine (Petersson, 1972).

Analysis of Defensive Exudates. Exudates from lubber grasshoppers fed catnip, natural, or artificial (lettuce + oatmeal) diets fortified with selected chemicals (adsorbed onto oatmeal) were analyzed as described previously (Blum et al., 1990).

Isolation of Caffeoyltartronic Acid (Caffeoylhydroxypropanedioic Acid). Mature catnip (*Nepeta cataria*) leaves (225 g) were removed and slurried in a Waring blender with 5 liters of MeOH. The extract was filtered and reduced in volume on a rotary evaporator to approximately 150 ml. The resulting water solution was extracted with methylene chloride (3 × 100 ml) to remove chlorophyll and lipids, and the water was adjusted to pH 2.5 with H_3PO_4.

The water extract was submitted to preparative reverse-phase chromatography (in two equal batches). The packing material from a Waters PrepPAK 500 C-18 cartridge was repacked into a 2.5-cm-ID × 28-cm chromatographic column (Fisher and Porter Co., Vineland, New Jersey). This column was operated by slight (10 psi) nitrogen pressure. The Waters reverse-phase material was also packed into a 1.25-cm-ID × 109-cm Cheminert LC column (Valco Instruments Co., Inc., Houston, Texas). This column was run under gradient elution conditions using an Isco model 2360 gradient programmer and model 2350 pump (Isco, Lincoln, Nebraska). Half of the water extract (75 ml) was placed on the column and eluted with water (500 ml) and then MeOH/H_2O (1:1, 250 ml) under nitrogen pressure. The MeOH/H_2O eluents contained the compound of interest and were combined from two runs and evaporated to dryness. The residue was dissolved in MeOH and deposited, by evaporation, onto 20 g of silicic acid (SA, Mallinkrodt, 100 mesh, washed with MeOH and activated at 155°C for 16 hr). This material was placed onto the top of an 80-g SA column, packed in ethyl acetate (EtOAc), and then eluted with 500 ml of EtOAc.

The EtOAc was evaporated to dryness, and the residue was dissolved in water and chromatographed on the gradient reverse-phase column utilizing a 100% water to 100% MeOH linear gradient over 400 min. Both solvents contained 0.1% H_3PO_4 and 8-ml fractions were collected. Fractions containing the compound were combined and evaporated to give a syrupy liquid from which crystals of caffeoyltartronic acid separated on cooling in a refrigerator. The crystals were pressed onto filter paper to remove solvent. Caffeoyltartronic acid turned pink just before its melting point (123–125°C), with gas evolution. Hydrolysis in acid gave caffeic acid, with a UV spectrum identical to authentic standard and with retention time correlation by HPLC and GC (silylated derivative). Base hydrolysis gave tartronic acid; GC retention time (silylated derivative) correlated with authentic standard (Sigma Chemical Co., St. Louis, Missouri).

Mass Spectrometric Analysis. Negative ion FAB-MS *m/z*: 281 [M−H], 237[M−H−CO_2], 179[M−CH−$(CO_2H)_2$], 162[M−H−O−CH−$(CO_2H)_2$], 119 [$C_3H_3O_5$]. Probe EI-MS *m/z* (abundance): 238 (70) [M−CO_2, molecular ion not seen], 163 (55) [M−CO_2−O−CH_2−CO_2H, caffeoyl acylium ion], 134 (100) [M−CO_2−CH−$(CO_2H)_2$, dihydroxyphenylacetylene], 45 (99) $[HCO_2]^+$. GC-EI-MS of TMS derivative (GC conditions as above) *m/z* (abundance): 454 (66) [M−CO_2TMS+H, molecular ion not seen], 439 (5) [454-

CH_3], 307 (24) [M−O−CH−$(CO_2TMS)_2$], 219 (100) [307-$(CH_3)_4Si$], 191 (9) [219-CO]. GC-CI-MS of TMS derivative indicated TMS molecular weight of 454.

NMR Analysis. ^1H NMR data were obtained in D_2O at room temperature with a Bruker AC300 spectrometer; δ: 4.70s CH; 5.31d, 6.59d CH=CH; 6.09s, 5.96s three ring protons.

Synthesis of Caffeoylglycolic Acid. Caffeoylglycolic acid was synthesized by a reported method (Kolodynska and Wieniawski, 1967) from *O,O*-bis(carbomethoxy)caffeoyl chloride and glycolic acid. The crude product was purified by reverse-phase chromatography, using the gradient system described above for CTA. Caffeoylglycolic acid was also prepared from *O,O*-bis(acetoxy)caffeoyl chloride in the same manner. Caffeoylglycolic acid was recrystallized from water containing a small amount of methanol; mp 183–185°C, with no evolution of gas (lit.: 170–171°C; Kolodynska and Wieniawski, 1967). Probe EI-MS *m/z* (abundance): 238 (18) [M^+], 163 (40) [M−O−CH_2−CO_2H, caffeoyl acylium ion], 134 (100) [M−H−CO_2−CH_2−CO_2H, dihydroxyphenylacetylene]. GC-MS of TMS derivative (GC conditions as above) *m/z* (abundance): 454 (100) [M^+], 439 (7) [454-CH_3], 309 (73), 307 (62) [M−O−CH_2−(CO_2TMS)], 219 (79) [307-$(CH_3)_4Si$], 191 (9) [219-CO].

RESULTS

Levels of Catechol and Hydroquinone in Lubber Defensive Exudates. Lubber grasshoppers were fed wild diets fortified with selected phenolics and catnip (Table 1). Diets spiked with catechol and hydroquinone resulted in 47- and 25-fold increases, respectively (over controls), in the amount of these compounds found in defensive secretions. Diets fortified with catnip were found to significantly increase (22-fold) the levels of catechol in the exudates. When the diet was fortified with *p*-benzoquinone, hydroquinone was found in the secretions in a 16-fold increase over controls. This suggests that the former compound was reduced to hydroquinone prior to secretion.

Isolation of Major Catnip Polyphenol. Reverse-phase HPLC of the methanol extract of catnip leaves revealed only one major polyphenol (peak B, Figure 1a). The unknown polyphenolic compound was observed to undergo a considerable shift in HPLC retention time with a reduction in eluting solvent pH from 4.4 to 2.5 (peak B, Figure 1b), indicating a free carboxyl group in the molecule. This fact was utilized in the development of an isolation scheme consisting of a combination of preparative reverse-phase and silicic acid column chromatography. Acidic solvents ensured protonation of the unknown polyphenol. This ensured sufficient retention on the column to prevent coelution with void-volume components. Phosphoric acid was found to be the acid of choice because it

TABLE 1. RELATIVE LEVELS OF CATECHOL AND HYDROQUINONE IN ADULT *R. guttata* DEFENSIVE GLAND SECRETIONS AFTER FEEDING ON DIETS FORTIFIED WITH CANDIDATE COMPOUNDS OR PLANT SPECIES

Diet ingredient[a]	Catechol[b]	Hydroquinone[b]
p-Benzoquinone	0.2	15.8
Hydroquinone	1.1	25.0
p-Hydroxybenzoate	0.6	0.5
Salicyl alcohol	0.9	0.5
Catechol	46.6	0.6
Caffeic acid	11.4	0.4
p-Coumaric acid	0.1	0.2
Nepeta sp. + wild diet	21.6	0.3
Wild diet	5.8	0.2
Control diet 1 (lettuce)	1.1	3.5
Control diet 2 (lettuce + oatmeal)	1.0	1.0

[a] Diets fortified at 0.2% level for each compound. Compounds adsorbed onto oatmeal.
[b] Relative to control diet 2.

appeared to aid in stabilizing the compound during isolation. Substitution of acetic or hydrochloric acids for phosphoric acid resulted in complete decomposition of the compound upon evaporation of the solvent.

Identification of Major Catnip Polyphenol. Ultraviolet spectra (Figure 1) and the longer HPLC retention time in pH 2.5 solvent versus pH 4.4 indicated that the compound was a low-molecular-weight hydroxy acid esterified with either caffeic acid (3,4-dihydroxycinnamic acid) or ferulic acid (3-methoxy-4-hydroxycinnamic acid). Mass spectral analyses of the isolated compound in the direct probe mode (both EI and CI) indicated an apparent molecular weight of 238 and exhibited an ion of *m/z* 163 for the caffeoyl acylium ion. The compound formed a TMS derivative that again gave an apparent molecular ion of *m/z* 454 and 219 (a diagnostic caffeoyl acylium-*O*,*O*-bridged $Si(CH_3)_2$ ion; Morita, 1972; Horvat and Senter, 1980). The UV and mass spectral data indicated the compound was caffeoylglycolic acid (Figure 2). Caffeoylglycolic acid was synthesized and its TMS derivative was shown to coelute with the TMS derivative of the catnip unknown. Although the mass spectra of caffeoylglycolic acid and its TMS derivative were similar to the natural product, differences were observed in ion abundances including the presence of a *m/z* 309 ion in the TMS spectra of caffeoylglycolic acid that was absent in the natural product. Further, the melting points of the two compounds did not agree and they did not coelute on HPLC. Fast-atom bombardment mass spectrometry (FAB-MS), operating in the negative ion mode, revealed that the molecular weight of the catnip compound was actually 282 and not 238, as indicated from probe and GC-MS studies.

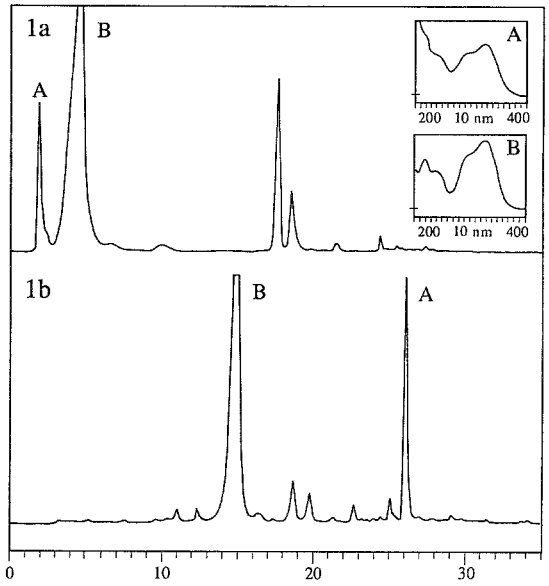
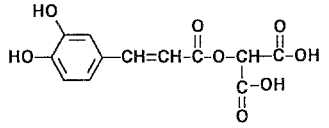
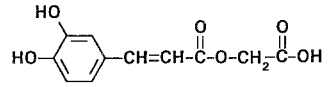


FIG. 1. HPLC chromatograms of catnip (*Nepeta cataria*) leaf methanol extract: (a) pH 4.4 buffered solvent system (see Methods and Materials); (b) pH 2.5 solvent system. UV spectra A and B: caffeoyl type.



Caffeoyltartronic Acid



Caffeoylglycolic Acid

FIG. 2. Structure of the major catnip polyphenol, caffeoyltartronic acid (caffeoylhydroxypropandioic acid) compared to caffeoylglycolic acid.

NMR data showed the molecule contained a single aliphatic proton in addition to the caffeoyl protons. Hydrolysis of the compound liberated caffeic acid and tartronic acid, indicating the structure to be caffeoyltartronic acid (caffeoylhydroxypropandioic acid, Figure 2).

Quantitation of Caffeoyltartronic Acid in Catnip and Mung Beans. Catnip and mung bean leaves were analyzed by HPLC for their caffeoyltartronic acid levels and the results are given in Table 2 as percent fresh weight.

DISCUSSION

Adults of the generalist herbivore, *Romalea guttata*, can be induced to become specialists by feeding them exclusively catnip (*Nepeta cataria*) leaves or they can be fed artificial diets fortified with catnip (Blum et al., 1990). Adult grasshoppers, feeding only on catnip, secrete inordinately large amounts of catechol from their defensive glands compared to those fed control diets very similar to the selection of plants that they feed on under field conditions. The ability of lubbers to sequester, transform, and secrete phenolics was investigated with diets containing selected phenolics and catnip (Table 1). Catechol and hydroquinone were readily sequestered, biomagnified, and then secreted unaltered from the defensive glands in relatively high concentrations. *p*-Benzoquinone, however, was found to be reduced efficiently to hydroquinone by the lubbers before secretion. This is not unexpected since these insects undoubtedly have evolved a potpourri of defensive mechanisms for metabolizing toxic allelochemicals in their unusually wide range of host plants. Caffeic acid was also found to be readily converted to catechol.

Lubber grasshoppers were also very efficient in producing and sequestering unusually large amounts of catechol that are generated from compounds contained in ingested catnip leaves. Since catnip contains no free catechol, the observed increased production and secretion of this substance in the glandular exudates must have been due to autogenous production from an ingested catnip constituent. In light of the facile conversion of caffeic acid to catechol by the lubbers (11-fold excess over controls), polyphenolics are the most likely candidate precursors in catnip. Analysis of catnip methanol leaf extracts by high-performance liquid chromatography, revealed only one major polyphenol (Figure 1). Initial UV data indicated that a caffeoyl moiety was present in the molecule.

The isolation and identification of the major polyphenolic in catnip proved difficult due, in part, to its instability to certain acids and heat. Acid was required to keep the polyphenol protonated so that separation could be achieved by reverse-phase preparative chromatography, but it was shown that only phosphoric acid, as compared to acetic or hydrochloric acids, appeared to impart a certain degree

TABLE 2. LEVELS OF CAFFEOYLTARTRONIC ACID IN CATNIP AND MUNG BEANS

Plant	Amount (% Fresh Weight)
Catnip	2.59
Mung Bean	1.41

of stability to phenolic compounds containing caffeoyl groups. Consequently, it should be considered in other isolation schemes of these types of compounds. Thermal instability of the isolated catnip polyphenol and its TMS derivative further complicated identification efforts and led to an initial belief that the compound was caffeoylglycolic acid. FAB-MS, NMR, and hydrolysis studies proved the true identity of the catnip polyphenol to be caffeoyltartronic acid (caffeoylhydroxypropanedioic acid). It is not unreasonable to believe that the lubber grasshopper, a generalist herbivore with an eclectic diet, is eminently capable of producing catechol from this precursor, as a prelude to sequestering this simple phenolic. Further, since caffeoyl esters (e.g., caffeoylquinic acid, chlorogenic acid) are widespread in the plant kingdom and can occur in relatively large amounts (Sondheimer, 1964), one would expect that the lubber's polyphagous diet should provide it with eminently sufficient catechol defensive gland precursors.

Mung beans (*Phaseolus radiatus* = *Vigna radiata*) constitute the only other reported plant species that contains caffeoyltartronic acid (Strack et al., 1985). Analyses of catnip and mung beans showed that catnip produces twice as much caffeoyltartronic acid as mung beans (fresh weight, Table 2). Interestingly, both catnip and mung beans produce caffeoyltartronic acid as their major polyphenol even though they are in different and unrelated families. However, we have found ground ivy (*Glecoma hederacea*), like catnip, a species in the family Labiatae, also produces the compound, but it is only one of several major phenolics in this species.

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CHEMICAL LABELING OF NORTHERN PIKE (*Esox lucius*) BY THE ALARM PHEROMONE OF FATHEAD MINNOWS (*Pimephales promelas*)

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Abstract—In previous experiments, chemical stimuli from northern pike (*Esox lucius*) elicited fright responses from pike-naive fathead minnows (*Pimephales promelas*) only if the pike had recently eaten conspecific minnows. We used a behavioral assay to determine if the fright response is the result of the incorporation of the minnow alarm pheromone into the chemical signature of the pike. Because the alarm substance cells (epidermal club cells) of fathead minnows are seasonally lost by males, we used chemical stimuli from pike that had eaten breeding male minnows as a control stimulus. In independent laboratory and field experiments, pike-naive minnows exhibited fright reactions (e.g., increased shelter use, avoidance) when exposed to water from tanks containing pike that had eaten nonbreeding fatheads (with alarm substance cells), but not to water from tanks containing pike that had eaten breeding male fatheads (without alarm substance cells). These data indicate that the fathead minnow alarm pheromone chemically labels northern pike as dangerous to pike-naive receivers.

Key Words—Predator recognition, alarm pheromone, alarm substance, alarm substance cells, club cells, Schreckstoff, fish behavior, fathead minnow, northern pike, *Esox lucius*, *Pimephales promelas*.

INTRODUCTION

Predator recognition by naive prey may be enhanced by chemical cues associated with the predator's diet. For example, Keefe (1992) demonstrated that naive

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brook trout distinguished between predatory Atlantic salmon fed goldfish and salmon fed mealworms, and Crowl and Covich (1990) showed that snails can discriminate between the chemical stimuli of crayfish actively foraging on snails versus crayfish foraging on spinach. Recently, Mathis and Smith (1993a) documented a more specific diet-related response. In that study, pike-naive fathead minnows (*Pimephales promelas*) exhibited a fright response following exposure to water from a tank containing northern pike (*Esox lucius*) that had eaten conspecific minnows, but not to water from a tank containing pike that had eaten heterospecific fish prey (swordtails: *Xiphophorous helleri*).

Fathead minnows and other members of the Superorder Ostariophysi possess a chemical alarm pheromone that warns receivers of nearby predation on conspecifics. The alarm pheromone (alarm substance or AS) is released from epidermal club cells (alarm substance cells) when mechanical damage to the skin occurs (see reviews in Smith, 1986, 1992). Mathis and Smith (1993a) hypothesized that the pike in their study were excreting minnow alarm substance as a by-product of digestion, leading to a fright response to the residual alarm substance by receiving minnows. Our goal in this study was to test the hypothesis that residual alarm substance is responsible for the fright reaction of pike-naive fathead minnows to chemical stimuli from pike that have eaten conspecific minnows.

Production of alarm substance cells by fathead minnows is inhibited by androgens, and males in breeding condition exhibit greatly reduced numbers of alarm substance cells (Smith, 1973, 1974). To determine the role of the alarm substance in diet-related cues for predator recognition by fathead minnows, we compared the response of pike-naive minnows to chemical stimuli from pike that had eaten breeding male minnows (without AS) with their response to chemical stimuli from pike that had eaten nonbreeding minnows (with AS). We examined fright responses of fathead minnows under both laboratory and field conditions.

METHODS AND MATERIALS

Experiment I: Laboratory Experiment

Collection and Maintenance of Study Animals. Fathead minnows were collected from Marshy Creek in central Saskatchewan. Marshy Creek drains into Redberry Lake, a large saline lake that is within an enclosed evaporation basin with no piscivorous fish species. Mathis and Smith (1993a) and Mathis et al. (1993) demonstrated that fathead minnows from this population do not exhibit fright responses to chemical stimuli from northern pike that were fed swordtails. Minnows were maintained in the laboratory in a 300-liter artificial stream tank

at approximately 15°C on a 14:10 light–dark photoperiod and were fed daily with commercial fish food.

Northern pike were collected from Pike Lake in south-central Saskatchewan. Pike were also maintained in an artificial stream tank (300-liter) at the same temperature and photoperiod as the minnows and were fed once every five days with fathead minnows until the start of the experiment.

Stimulus Preparation. Two pike ($\bar{X}$ fork length $\pm$ 1 SD = 22.3 cm $\pm$ 1.06) were randomly assigned to either AS [i.e., diet of fathead minnows in nonbreeding condition (with alarm substance)] or no AS [i.e., diet of male fathead minnows in breeding condition (without alarm substance)] treatments and were placed into separate halves of a 150-liter aquarium. For prey minnows, "breeding condition" was defined as the exhibition of tubercles on the snout and a dorsal mucous pad (Smith and Murphy, 1974), while "nonbreeding condition" was defined as the absence of these two characteristics. The protocol for collection of the stimulus water was identical to that of Mathis and Smith (1993a). For each of three feedings immediately prior to collection of the stimulus water, pike were fed prey fish from their assigned diet condition (volume of prey = approximately 4–5 ml, measured by displacement in water). After 1 hr, each test pike was removed from the tank and rinsed with dechlorinated tap water to remove any residue of the prey fishes from the pike's skin.

The chambers used for final collection of the stimulus water were small (26 × 8 × 8 cm) and contained only 1200 ml of water, so that the stimulus could be as concentrated as possible. Preliminary observations suggested that the pike's rate of digestion (i.e., time to production of first feces) was slowed when the pike were placed into the collection chambers, presumably due to restricted activity in the small chambers. To lessen this effect, the pair of pike were first transferred into separate halves of a 150-liter aquarium that contained clean water (to provide an additional rinse) where their movement was less restricted. After approximately 16 hr, test pike were transferred to the stimulus collection chambers. The chambers were well-aerated but contained no filtration system to ensure that the active chemical components were not removed from the water. After three days, the pike were removed from the chambers and the water from the chambers was frozen in 60-ml units in plastic bags.

To determine if identity of individual pike influenced the behavior of stimulus-receivers, each pike was subsequently fed the opposite diet from that in its original treatment condition, following the same protocol as above, and second stimulus solutions were collected. Pike I was fed nonbreeding fathead minnows first and pike II was fed breeding male fathead minnows first.

Testing Protocol. The testing protocol was also identical to that used by Mathis and Smith (1993a). Testing tanks were 37-liter aquaria filled with tap water that had been filtered through charcoal and sodium zeolite to remove chlorine and ammonia (part of the chemical treatment system applied to local

municipal water supplies). The bottom of each testing tank was covered with a shallow layer of sodium zeolite chips to remove ammonia from the water. An airstone located in the center of the back wall of each tank provided aeration.

Each tank contained a shelter consisting of a ceramic tile (9.8 × 20.0 cm) mounted on three cylindrical glass legs (5.5 cm long). Because large fathead minnows often aggressively defend shelters from smaller minnows (personal observations), a partial divider (1.5 × 9.8 cm) was placed along the center of the tile. This divider decreased the effect of aggression by a dominant minnow so that more than one individual at a time could occupy the shelter.

Stimulus water was introduced into the tank via plastic Tygon tubing that was tied to the airline so that the end of the tubing was approximately 1 cm from the surface of the airstone. The inflow of air from the airstone caused the stimulus water to be rapidly dispersed throughout the tank. In trials with vegetable dyes, the dye was uniformly distributed throughout the tank in approximately 13 sec.

Pike-naive fathead minnows from Marshy Creek were arbitrarily removed from the holding tank and were randomly placed into testing tanks so that each tank held four minnows and was treated as a single replicate. Treatment conditions were randomly assigned to each tank with 10 tanks in each treatment group for each pike. Minnows remained in the tanks for four days prior to testing on the same photoperiod and feeding schedule as before. Trials were conducted between 1050 and 1510 hr at a mean water temperature of 25°C.

During each trial, an observer was positioned approximately 1.5 m in front of each tank. Immediately prior to the beginning of the trial, 60 ml of tank water was drawn through the stimulus-introduction tube by a syringe and was discarded to remove any stagnant water that might have collected in the tubing. An additional 60 ml of tank water was then drawn into the syringe and saved, and a second syringe was filled with 60 ml of the pike stimulus water. The minnows were observed undisturbed for 8 min to determine baseline levels of activity. At the end of 8 min, 30 ml of the pike stimulus water was slowly (approximately 1 ml/sec) introduced into the tank. Observations were resumed immediately after the introduction of the 30 ml. After 4 min of observation, the remaining 30 ml of the stimulus water was injected into the tank immediately followed by the injection of 60 ml of tank water to ensure that none of the pike stimulus remained in the stimulus-introduction tube. Observations continued for an additional 4 min (total observation time = 8 min prestimulus, 8 min poststimulus).

During both pre- and poststimulus observation periods, activity levels were quantified at 15-sec intervals by recording the number of minnows that were beneath the shelter. During the poststimulus period, we also recorded whether or not minnows responded to the introduction of the stimulus by swimming to the surface and "nipping" at the surface of the water. We interpreted nipping

behavior as a feeding response because it closely resembled the behavior of the minnows during routine daily feeding. Incidences of dashing (i.e., very rapid, apparently disoriented swimming) were also recorded during the poststimulus period. Dashing is a common response of fathead minnows to conspecific alarm pheromone (Lawrence and Smith, 1989).

An index of activity for each group of minnows was calculated as mean poststimulus minus mean prestimulus activity. Activity indices were analyzed with a nonparametric two-factor analysis of variance (Zar, 1984) with pike identity (i.e., pike I and II) and pike diet (i.e., nonbreeding fatheads versus breeding male fatheads) as the factors considered in the analysis. Presence versus absence data for both feeding responses and dashing were analyzed with either the chi-square test or with the Fisher exact probability test (Siegel, 1956).

Experiment II: Field Experiment

The purpose of this experiment was to determine whether pike-naive fathead minnows in natural habitats avoid areas that are marked with water from chambers containing pike that had eaten nonbreeding fathead minnows (with AS). The experiment was conducted in Oscar Creek in central Saskatchewan in September 1992. Oscar Creek is similar to Marshy Creek in that it drains into Redberry Lake and has no piscivorous fish species. Fathead minnows occur in large numbers at the study site, along with several other species of small fishes (brook stickleback, *Culaea inconstans*; finescale dace, *Phoxinus neogaeus*; pearl dace, *Margariscus margarita*) (personal observation).

The stimulus water that was prepared in experiment I (see above) was also used in this experiment. Because the results of experiment I (and also the results of Mathis and Smith, 1993a) demonstrated that the identity of individual pike does not have a significant effect on the response of the minnows, stimulus water from pikes I and II were mixed together for each treatment in this experiment. Therefore, there were two treatment groups: (1) water from chambers containing pike that were fed nonbreeding fathead minnows (with AS) and (2) water from chambers containing pike that were fed breeding male fathead minnows (without AS).

We used the methods of Mathis and Smith (1992) to test whether minnows avoided areas marked with water from the AS treatment. To provide marked areas, we saturated cellulose sponges (5.0 × 2.0 × 1.5 cm) with the stimulus solutions and placed them into minnow traps. Each sponge was saturated with approximately 20 ml of pike stimulus water, and the saturated sponges were placed in a freezer at approximately −20°C. Sponges were removed from the freezer on the day of testing and were kept on ice until the beginning of the experiment. Traps (Gee's Improved minnow traps) consisted of roughly cylindrical wire enclosures (43 cm length × 22 cm diameter) with a funnel located

at each end leading into the trap. Funnel entrances were approximately 2.5 cm in diameter. Two sponges from the same treatment (either AS or no AS) were placed into each trap so that one sponge was approximately 2 cm in front of each trap entrance. The two sponges were held in place with wire. Twenty-two traps contained sponges saturated with water from chambers that had housed pike that had eaten nonbreeding fathead minnows (with AS) and 22 traps contained sponges saturated with water from chambers that had housed pike that had eaten breeding male fathead minnows (without AS).

Pairs of AS and no AS traps were simultaneously placed into the water along the shoreline so that traps from the two treatment groups spent exactly the same amount of time in the water. Successive traps were separated by approximately 10 m. The linear order of placement of AS and no AS traps was determined randomly with the proviso that no more than two traps in a row could be of the same treatment condition. Each trap remained in the water for approximately 4 hr, and pairs of AS and no AS traps were removed simultaneously from the water. Fishes from each trap were preserved in 10% formalin. For each individual, species and either fork length (fathead minnows, finescale dace, and pearl dace) or total length (brook stickleback) were recorded.

Statistical comparisons of AS versus no AS traps were made using the Mann-Whitney U test (Siegel, 1956). Statistical tests were one-tailed because the results of experiment I provided a strong rationale for predicting the direction of the difference between treatments in this experiment.

RESULTS

Experiment I: Laboratory Experiment

Groups of minnows exposed to stimulus water from pike that were fed nonbreeding fathead minnows (with AS) tended to increase their use of shelter (average increase of 33%) while minnows exposed to stimulus water from pike that were fed breeding male fatheads (without AS) tended to decrease their use of shelter (average decrease of 10%) (Figure 1). A nonparametric two-way ANOVA indicated that pike diet exerted a significant effect ($H = 18.97$, $df = 1$, $P < 0.001$) while identity of individual pike did not ($H = 0.85$, $df = 1$, $P > 0.25$). There was no significant interaction effect between pike diet and pike identity ($H = 1.48$, $df = 1$, $P > 0.10$).

Because the two-way ANOVA indicated no significant effect of pike identity on activity of minnows, data for both pike were combined for the statistical analysis of feeding responses (nipping at the surface of the water) and dashing. A significantly greater proportion of trials in which groups of minnows were exposed to stimulus water from pike that had been fed breeding male fatheads (no AS treatment) resulted in feeding responses by at least one minnow in

comparison to minnows in the AS treatment ($\chi^2 = 14.4$, $df = 1$, $P < 0.001$) (Table 1). Dashing was only observed in five of the 40 trials. All five instances of dashing were observed in response to stimulus water from pike fed nonbreeding fathead minnows (with AS) (Fisher exact probability test, $P = 0.02$, one-tailed).

Experiment II: Field Experiment

Ninety-one percent of the 2868 fishes that were caught in the 44 traps were fathead minnows. Significantly fewer fathead minnows were found in traps marked with water from chambers containing pike that were fed nonbreeding

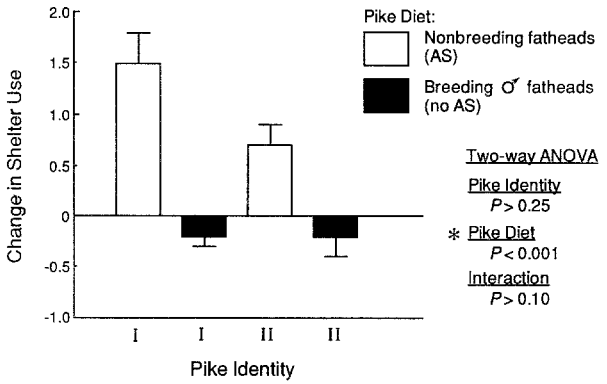

FIG. 1. Mean (±1 SE) change in the index of shelter use by groups of fathead minnows exposed to chemical stimuli from pike that had eaten nonbreeding fathead minnows (with alarm substance cells, AS) versus pike that had eaten breeding male fathead minnows (without alarm substance cells, no AS). Positive numbers indicate an increase in the use of shelter and negative numbers indicate a decrease in the use of shelter.

TABLE 1. NUMBER OF TRIALS IN WHICH FEEDING RESPONSES (NIPPING AT SURFACE OF WATER) WERE EXHIBITED BY FATHEAD MINNOWS EXPOSED TO STIMULUS WATER FROM PIKE FED A DIET OF NONBREEDING FATHEAD MINNOWS (WITH ALARM SUBSTANCE CELLS, AS) OR BREEDING MALE FATHEAD MINNOWS (WITHOUT ALARM SUBSTANCE CELLS, NO AS)

Diet	Feeding response	No feeding response	P^a
Nonbreeding fatheads (AS)	3	17	<0.001
Breeding male fatheads (no AS)	16	4	

[a] Chi-squared test.

fathead minnows (with AS) (1166 minnows) than in traps marked with water from chambers containing pike that were fed breeding male fathead minnows (without AS) (1440 minnows) (Mann-Whitney U test, $Z = 1.88$, $P = 0.03$) (Table 2). The number of individuals that were caught in AS versus no AS traps was not significantly different for any of the other species (Table 2).

If experience or developmental factors played a role in modifying the minnows' responses to the diet-related cues, then individuals in the AS traps should be younger, and therefore smaller, than individuals in the "no AS" traps (in the statistical analysis of the size data, individuals, rather than traps, were treated as independent samples). This prediction was supported by the data for fathead minnows (Mann-Whitney U test, $Z = 3.75$, $P = 0.0001$; Figure 2). This difference in sizes is apparently due to the tendency of larger individuals to be more successful at avoiding the AS traps: fathead minnows that were smaller than 5.0 cm tended to be more frequently found in the AS traps, while those larger than 5.0 cm tended to be more frequent in the no AS traps [change-point test (Siegel and Castellan, 1988), $D = 0.84$, $P < 0.001$; Figure 2].

There were no significant differences in sizes between treatments for individuals of the other three species (one-tailed Mann-Whitney U tests; finescale dace: $Z = 0.44$, $P > 0.30$; pearl dace: $Z = 0.09$, $P > 0.50$; brook stickleback: $Z = 1.03$, $P > 0.10$).

DISCUSSION

Our results support the hypothesis that residual alarm pheromone from ingested fathead minnows is given off as a by-product of digestion by northern pike, resulting in fright responses by pike-naive minnow receivers. This study provides the first evidence of the incorporation of a prey alarm pheromone into the chemical signature of a vertebrate predator. Howe and Harris (1978) described

TABLE 2. MEAN NUMBER OF FISHES (1 SE) CAUGHT IN TRAPS MARKED WITH SPONGES SATURATED WITH WATER FROM TANKS CONTAINING PIKE THAT HAD EATEN NONBREEDING FATHEAD MINNOWS (WITH ALARM SUBSTANCE CELLS, AS) OR BREEDING MALE FATHEAD MINNOWS (WITHOUT ALARM SUBSTANCE CELLS, NO AS).

Species	AS	No AS	Z	P^a
Fathead minnows (*Pimephales promelas*)	53.0 (28.1)	65.5 (29.5)	1.88	0.03
Finescale dace (*Phoxinus neogaeus*)	2.6 (1.1)	4.1 (1.5)	1.16	0.12
Pearl dace (*Margariscus margarita*)	0.6 (0.3)	0.4 (0.3)	0.14	0.56
Brook Stickleback (*Culaea inconstans*)	1.6 (0.4)	2.6 (0.5)	1.48	0.07

[a] Mann-Whitney U test, one-tailed.

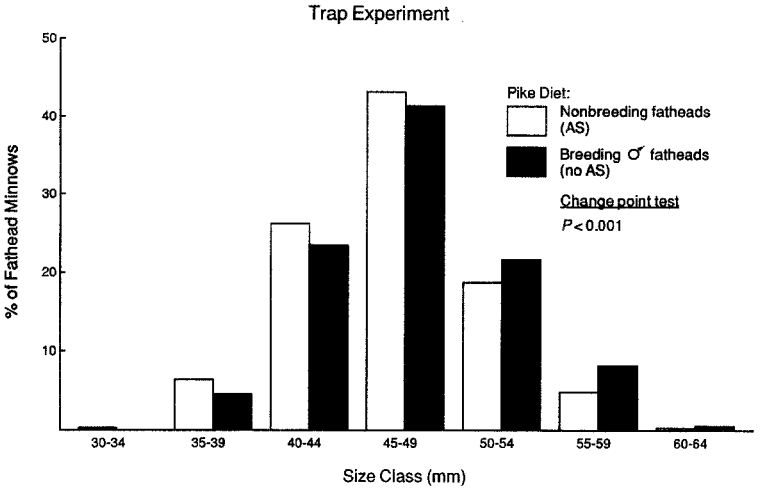

FIG. 2. Percent of fathead minnows in each size class for each treatment. Minnows were caught in traps marked with sponges saturated with water from tanks containing either pike that had eaten nonbreeding fathead minnows (with alarm substance cells, AS) or pike that had eaten breeding male fathead minnows (without alarm substance cells, no AS).

a similar effect for an aquatic invertebrate. In their study, sea anemones (*Anthopleura elegantissima*) exhibited a fright reaction to water containing a predatory nudibranch (*Aeolidia papillosa*) that had eaten conspecific anemones but did not respond to water containing a nudibranch that had been starved for several days. Howe and Harris (1978) used biochemical tests to demonstrate that anthopleurine, the sea anemone alarm pheromone, was incorporated into the tissue of the nudibranch following ingestion of sea anemones. Chemical alarm signals are widespread (e.g., insects: review in Blum, 1985, Purrington et al., 1991; oligochaetes: Ressler et al., 1968; echinoderms: Lawrence, 1991; crustaceans: Rittschof et al., 1992; amphibians: Hews, 1988; fishes: reviews in Pfeiffer, 1977, Smith, 1992), and the incorporation of prey alarm pheromones into the chemical signatures of predators has the potential to be important for predator recognition in a wide range of taxa.

In our study, the prey fishes were of the same species but differed with regard to the presence of alarm substance cells, providing a powerful test of the hypothesis that the minnow alarm pheromone can "label" predators. This experimental design was possible because of the loss of alarm substance cells by male fathead minnows in breeding condition (Smith, 1973). Although breeding and nonbreeding minnows undoubtedly differ in characteristics other than the presence or absence of alarm substance cells (e.g., different levels of hormones),

we feel that it is unlikely that other differences could account for the results of this experiment. Our results correspond with those of a previous study that showed that extracts prepared from the skin of nonbreeding male minnows elicited a fright response from conspecifics while skin extracts from breeding males did not (Smith, 1973); skin extracts exclude gonads, pituitary glands, and other organs that would be expected to exhibit differences between breeding and nonbreeding individuals. Seasonal loss of alarm substance cells is not unique to fathead minnows but has been described for a number of other ostariophysan species (e.g., Smith, 1976; Smith and Smith, 1983). Therefore, intraspecific seasonal or hormonally induced variation in the presence of alarm substance cells may be useful in the design of a wide range of future experiments.

The fright response of ostariophysans to conspecific alarm pheromone is innate and typically develops early (e.g., Pfeiffer, 1963; Waldman, 1982). Therefore, a response to alarm pheromone "labels" would be an efficient means by which inexperienced individuals could recognize predators. The association of the alarm pheromone with other chemical stimuli from the predator may also allow predator-naive individuals to learn to recognize the predator as a potential danger regardless of the predator's recent feeding regime (Mathis and Smith, 1993a).

The ability of naive minnows to avoid predators that have been labeled with the alarm pheromone may be modified by experience. Although all of the minnows in our study were inexperienced with pike, they may have had experience with other predators (invertebrates, birds, mammals); older minnows should, on average, be more experienced with predators than younger minnows. In our field experiment, larger fathead minnows were more successful than smaller minnows at avoiding traps marked with water from tanks containing pike that had eaten nonbreeding minnows (with alarm substance cells). These data are consistent with those of Mathis and Smith (1992), who reported that larger fathead minnows were also marginally more successful than smaller individuals at avoiding chemical stimuli from injured (nonbreeding) conspecifics. Experience with predation also appears to play an important role in the ability of brook trout (nonostariophysans) to discriminate between unfamiliar predators with piscivorous and nonpiscivorous diets (Keefe et al., 1992). In that species, laboratory-reared individuals are apparently unable to recognize predators based on diet-related cues.

The avoidance response that was observed for the fathead minnows in our field experiment was relatively weak in comparison to the results of a previous study in which over 95% of the fathead minnows successfully avoided traps marked with (undigested) skin homogenates from conspecifics (Mathis and Smith, 1992). However, the data from the two studies are not directly comparable; we estimate that the alarm substance stimulus used by Mathis and Smith (1992) was roughly 20 times stronger than that used for traps in this study. The

intensity of the fright response by fathead minnows is positively correlated with the strength of the alarm substance stimulus solution (Lawrence and Smith, 1989). It is impossible to determine whether the comparative weakness of the response to the "digested" alarm pheromone is due to a lower concentration of the alarm pheromone in the pike-stimulus water or whether the alarm pheromone is modified during the digestive process.

We predicted that finescale dace, pearl dace (both ostariophysan species), and brook stickleback would also avoid traps marked with water from tanks containing pike that had eaten nonbreeding fathead minnows. We predicted avoidance by these species because brook stickleback respond to skin extracts from nonbreeding fathead minnows (Mathis and Smith, 1993b) and because the alarm pheromone/fright reaction system is thought to be evolutionarily homologous among ostariophysans (Pfeiffer, 1974). Our analyses of the data for these three species did not detect any statistically significant effects. However, because of the relatively small number of dace and stickleback that were captured during the experiment (Table 1), conclusions concerning the effect of diet-related cues on predator recognition by these species are premature.

Diet-related chemical labeling of predators may have far-ranging consequences for both predators and prey. Prey fish may have evolved specific adaptations to take advantage of such labels. For example, minnows have been shown to gain information concerning predator identity and, possibly, the predator's motivational state during "predator inspection visits" (Magurran and Girling, 1986; Magurran and Pitcher, 1987). Chemical stimuli, including information concerning the predator's recent diet, may provide additional information to minnows during inspection visits. Similarly, predators may reduce labeling effects by adaptations such as the adoption of a more generalist diet, defecation in areas away from feeding sites, or chemical degradation of alarm pheromones.

Our results extend the known benefits that accrue to receivers of alarm pheromones in fishes. By detecting residual alarm pheromone that is excreted by the predator, receivers are able to recognize unfamiliar "labeled" predators as dangerous several days after a predation event has occurred. Furthermore, the consumed minnows may potentially experience benefits in terms of inclusive fitness by warning related individuals of danger.

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SELECTIVELY FLUORINATED ANALOGS REVEAL DIFFERENTIAL OLFACTORY RECEPTION AND INACTIVATION OF GREEN LEAF VOLATILES IN INSECTS

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Abstract—The role of the alkyl terminus of green leaf volatile (GLV) molecules in olfactory reception and inactivation was examined in three diverse insect species: the beet armyworm, *Spodoptera exigua* (Lepidoptera); the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera); and the desert locust, *Schistocerca gregaria* (Orthoptera), using selectively fluorinated analogs of GLVs and electroantennograms (EAGs). When only the magnitude of the depolarization of the EAG is considered (a measure of reception), the order of effectiveness was 1-hexanol (6:OH) = (*Z*)-3-6:OH > 5,5,6,6,6-pentafluoro-(*Z*)-3-6:OH = 5,5-difluoro-(*Z*)-3-6:OH ≫ 5,5,6,6,6-pentafluoro-6:OH. Percent recovery of the EAG (a measure of inactivation) was greater for the pentafluoro-(*Z*)-3-6:OH analog than for the difluoro-(*Z*)-3-6:OH analog. Our results show that the alkyl end of GLV molecules plays an important role not only in reception, but also inactivation processes in insect olfaction. Furthermore, specificities of these two processes may differ.

Key Words—Green leaf volatile, olfaction, reception, inactivation, electroantennogram, fluorinated analogs, *Spodoptera exigua*, *Leptinotarsa decemlineata*, *Schistocerca gregaria*, insect, Lepidoptera, Noctuidae, Coleoptera, Chrysomelidae, Orthoptera, Acrididae.

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INTRODUCTION

Green leaf volatiles (GLVs) are six-carbon alcohols, aldehydes, and their derivatives, e.g., acetates, that are produced by green plants as a product of oxidation of surface lipids (Visser et al., 1979). The most commonly reported GLVs are 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, and their derivatives. These compounds have several roles in insect behavior including: (1) host-plant location (Visser and Ave, 1978); (2) enhancement of insect pheromones (Dickens, 1989; Dickens et al., 1990; 1993a); and (3) host finding by parasitoids of lepidopterous larvae (Whitman and Eller, 1990). Insects detect GLVs through olfactory receptor neurons that are usually housed within porous basiconic (Kaib, 1974; Sass, 1976; Ma and Visser, 1978; Dickens, 1989) or coeloconic (Boeckh, 1967; Kafka, 1971) sensilla on their antennae. These neurons are classified as olfactory generalists or specialized generalists since they may respond to several odors but with differing intensities (Kaissling, 1987). Recently GLVs were shown to activate neurons generally regarded as pheromone specialists in Lepidoptera (Hansson et al., 1989; Dickens et al., 1993b; Mayer, personal communication).

Studies on the mechanisms of detection of GLVs by insects have centered on responses of receptor neurons to functional group analogs (Kafka, 1971; Visser, 1979). Results of these studies indicate chain length, functional group, and the nature of the double bond, if present, as key factors in the stimulatory effectiveness of these compounds. Scant attention has been given to the hydrocarbon end of these molecules in receptor interactions, while even less attention has been afforded the inactivation of these compounds by insect antennae.

In our current study, we show the importance of the hydrocarbon end of GLVs in their reception and inactivation by the beet armyworm, *Spodoptera exigua*. We also demonstrate the general importance of this end of the molecule in comparative studies with the Colorado potato beetle, *Leptinotarsa decemlineata*, and the desert locust, *Schistocerca gregaria*.

METHODS AND MATERIALS

Insects. Spodoptera exigua were obtained as pupae from a laboratory colony maintained for several generations at the Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands. Pupae were sexed and kept in an incubator at 25°C and a photoperiod of 16 hr of light and 8 hr of darkness. Upon emergence, adults were fed a 10% honey–water solution, and maintained under identical conditions as pupae until use one to two days after emergence. *Leptinotarsa decemlineata* and *Schistocerca gregaria* adults were obtained from laboratory colonies maintained at the Department of Entomology, The Agricultural University, Wageningen.

Chemicals. Experimental chemicals used in our studies are represented in

Figure 1A. 1-Hexanol and (Z)-3-hexen-1-ol were obtained from Fluka Chemie AG, Buchs, Switzerland, and Carl Roth GmbH, Karlsruhe, Germany, respectively. 5,5,6,6,6-Pentafluoro-1-hexanol was obtained from Japan Halon. 5,5,6,6,6-Pentafluoro-(Z)-3-hexen-1-ol and 5,5-difluoro-(Z)-3-hexen-1-ol were synthesized as described below. All compounds were >98% pure.

Stimulus Delivery. Chemicals were diluted in pentane. Odorous stimuli were prepared by applying 5-μl aliquots of a serial dilution onto a piece of filter paper (60 × 8 mm, S&S 589[2]) which was placed in a Pasteur pipet. Air that had been purified (Chrompack moisture filter 7971 and charcoal filter 7972) and humidified passed continually over the antennal preparation at a rate of 40 cm/sec (60 ml/sec). The Pasteur pipet containing the stimulus was connected to one of the outlets of a three-way solenoid valve (Lee LFAA1200618H). By operation of the valve, this outlet was connected for 2 sec to a purified air flow of 1 ml/sec, and, in this way, odorous molecules evaporating from the filter paper were injected into the main air flow over the preparation. Intervals between odorous

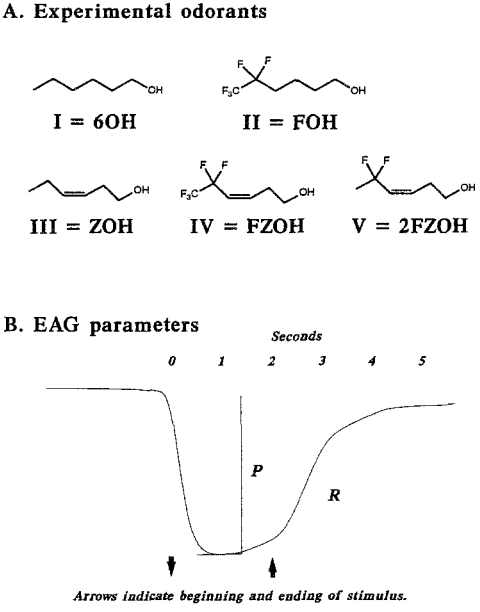


FIG. 1. (A) Odorants used in electrophysiological experiments: 1-hexanol (**I** = 6OH) and (Z)-3-hexen-1-ol (**III** = ZOH) are naturally occurring green leaf volatiles, and 5,5,6,6,6-pentafluoro-1-hexanol (**II** = FOH), 5,5,6,6,6-pentafluoro-(Z)-3-hexen-1-ol (**IV** = FZOH), and 5,5-difluoro-(Z)-3-hexen-1-ol (**V** = 2FZOH) are selectively fluorinated analogs of **I** and **III**. (B) Parameters of the electroantennogram used in our current studies. P = peak; R = recovery. Arrows indicate duration of stimulus. See text for details.

stimuli were at least 3 min. Air-flow rates were set by a flow control unit (Brooks 5878 with two mass flow controllers 5850TR).

Antennal Preparation. For all species, the antenna was amputated at the base. For *S. exigua*, one or two segments at the tip of the antenna were cut off. For *L. decemlineata* and *S. gregaria*, only the tip of the distal antennal segment was amputated. The excised antenna was fixed between two glass electrodes filled with 0.1 M KCl: the indifferent electrode was slipped over the base and the recording electrode contacted with the cut tip. Ag–AgCl wires in the glass electrodes connected the preparation with the recording instruments: an input probe (Grass HIP16A), a preamplifier (Grass P16D, DC rise time = 30 msec), a storage oscilloscope (Philips PM3302), a transient recorder (Krenz TRC4010), and a personal computer (Estate AT386).

Data Collection. At the same time the three-way injection valve opened, a trigger pulse started the storage of the recording. Data were digitized and stored by the transient recorder (200 Hz AD conversion, 12 bits ADC input) and transferred to the AT386 personal computer (Krenz TRS software 4.03a). Each recording of an electroantennogram (EAG) consisted of three parts: (1) a 4-sec prestimulation period (pretrigger), (2) a 2-sec stimulation, and (3) a 14.5-sec poststimulation period, in total of 4096 values.

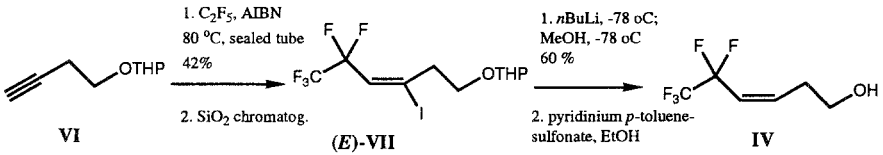
Experimental Protocol. In the first series of experiments, both sexes of *S. exigua* were stimulated with serial stimulus loads of each of the experimental odorants. From these responses, dose–response curves were constructed from stimulus loads of 0.5–500 μg for 1-hexanol (6:OH), (Z)-3-6:OH, and 5,5,6,6,6-pentafluoro-6:OH; and 0.5–50 μg for 5,5-difluoro-(Z)-3-6:OH and 5,5,6,6,6-pentafluoro-(Z)-3-6:OH. Dose–response curves ranged only to 50 μg for the latter two compounds due to their limited availability. This experiment determined the overall sensitivity and responsiveness of antennal receptors for GLVs in *S. exigua* and was used to determine an appropriate stimulus load for use in our second series of experiments. In the second series of experiments, comparative studies were done involving responses of *L. decemlineata* and *S. gregaria* females to 50-μg stimulus loads of the same experimental odorants.

Data Analyses. Electroantennograms (EAGs) were analyzed using a 386 microcomputer and software programs for waveform analyses previously described (Dickens et al., 1993b; Visser, unpublished). Mean normalized peaks (peak = most negative value encountered during 2-sec stimulation period) for each stimulus were calculated as a percent response to the standard (1-hexanol, 50 μg stimulus load). Decay or recovery (a measure of inactivation) was the percent decrease in the EAG 1 sec following the end of the stimulation period relative to the last value in the stimulation period (Figure 1B). EAG parameters were compared using analysis of variance procedures (Ostle, 1963) and Duncan's multiple-range test (Duncan, 1955).

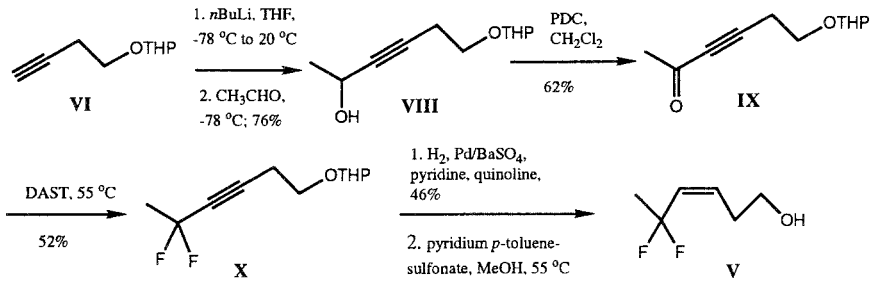
RESULTS AND DISCUSSION

Synthesis. Synthesis of the two fluorinated analogs of (Z)-3-hexen-1-ol [i.e., 5,5,6,6,6-pentafluoro-(Z)-3-hexen-1-ol and 5,5-difluoro-(Z)-3-hexen-1-ol] was accomplished (Ng, 1991) by modifications of earlier methods for fluorinated pheromone synthesis (Sun and Prestwich, 1990; Sun et al., 1992). The pentafluoroethyl analog, 5,5,6,6,6-pentafluoro-(Z)-3-hexen-1-ol **(IV)** was prepared in three steps as shown in Scheme 1. Thus, the THP ether of 3-butyn-1-ol **(VI)** was coupled under radical conditions with pentafluoroethyl iodide in 42% yield, giving the alkenyl iodides **VII** in an *E*/*Z* ratio of 6.6:1. Geometric isomers were separated by silica chromatography, the desired *E* isomer was deiodinated in 60% isolated yield by lithium–halogen exchange (*n*-butyllithium), followed by protonation, both at −78°C. Deprotection with acid catalysis afforded the desired pentafluorinated hexenol adduct **IV.**

The 5,5-difluoro-(Z)-3-hexen-1-ol **(V)** was prepared as shown in Scheme 2. The acetylide anion of alkynyl ether **VI** was condensed with freshly distilled acetaldehyde to give, after aqueous workup and silica chromatography, the propargylic alcohol **VIII** in 76% isolated yield. Oxidation with pyridinium dichromate (PDC) in methylene chloride gave the propargylic ketone **IX** in 62% yield,



SCHEME 1.



SCHEME 2.

and fluorination with neat diethylaminosulfur trifluoride (DAST) at 55°C afforded the alkynyl difluoride **X** in 53% yield. Semihydrogenation of **X** was accomplished in pyridine with 5% $Pd/BaSO_4$ and one drop of quinoline under 1.1 atm of H_2 in 46% yield, and acidic alcoholysis of THP ether gave, after distillation, the desired difluoro analog **V**.

Reception of Green Leaf Volatiles and Analogs. Dose–response curves in response to serial stimulus loads of GLVs and their fluorinated analogs were nearly identical for both sexes of *Spodoptera exigua* (Figure 2A and B). Responses to GLVs 1-hexanol and (*Z*)-3-hexen-1-ol were greater than responses to their fluorinated analogs. The fluorinated analogs of (*Z*)-3-hexen-1-ol were both more active than the fluorinated analog of 1-hexanol.

Substitution of fluorine for hydrogen on the alkyl side chain decreases both the flexibility of the side chain and its hydrophobicity (Filler and Kabayashi, 1982). The greater activity of the fluorinated analogs of (*Z*)-3-hexen-1-ol relative to the fluorinated analog of 1-hexanol may indicate the *cis* conformation as preferred for receptor site interactions. The lack of the *cis* double bond and the increased rigidity of the molecule imposed by the fluorine atoms in the 1-hexanol analog impede such a conformation. The fact that all fluorinated analogs had reduced activities as compared to their nonfluorinated parent compounds indicates the importance of the hydrophobic nature of the alkyl end of the GLV molecules in receptor interactions. The nearly identical activities of the difluoro- and pentafluoro-(*Z*)-3-hexen-1-ol analogs demonstrate that both the terminal and penultimate carbons are important in molecule–receptor interactions. The reduced activity of fluorinated analogs of the GLVs observed in our current study is similar to the reduced activity observed for fluorinated pheromone analogs in other lepidopterous species (McLean et al., 1989; Prestwich et al., 1990; Wu et al., 1992; Bengtsson et al., 1990; Linn et al., 1992).

We realize that what is being compared in these studies is the stimulatory effectiveness of differing stimulus loads of the various compounds. The number of molecules that actually reach the surface of the antenna for transport to the receptor neurons must vary according to the volatility of individual compounds, their release rates from the filter paper, and their adherence to the stimulatory cartridge. However, the reduced activity of the fluorinated analogs relative to their parent molecules cannot be explained based on differences in volatility, since introduction of fluorine atoms actually increases volatility of the resulting molecules (Prestwich et al., 1990; Dickens et al., 1991).

Inactivation of Green Leaf Volatiles and Analogs. Inactivation, as reflected by the time course of recovery of the EAG, was nearly identical for both GLVs in both sexes (Figure 2C). Percent recovery after 1 sec for 1-hexanol and its pentafluoro analog were not significantly different. Percent recovery of the EAG for the pentafluoro-(*Z*)-3-hexen-1-ol was greater than its parent compound in both sexes, but only significantly greater for females ($P < 0.05$).

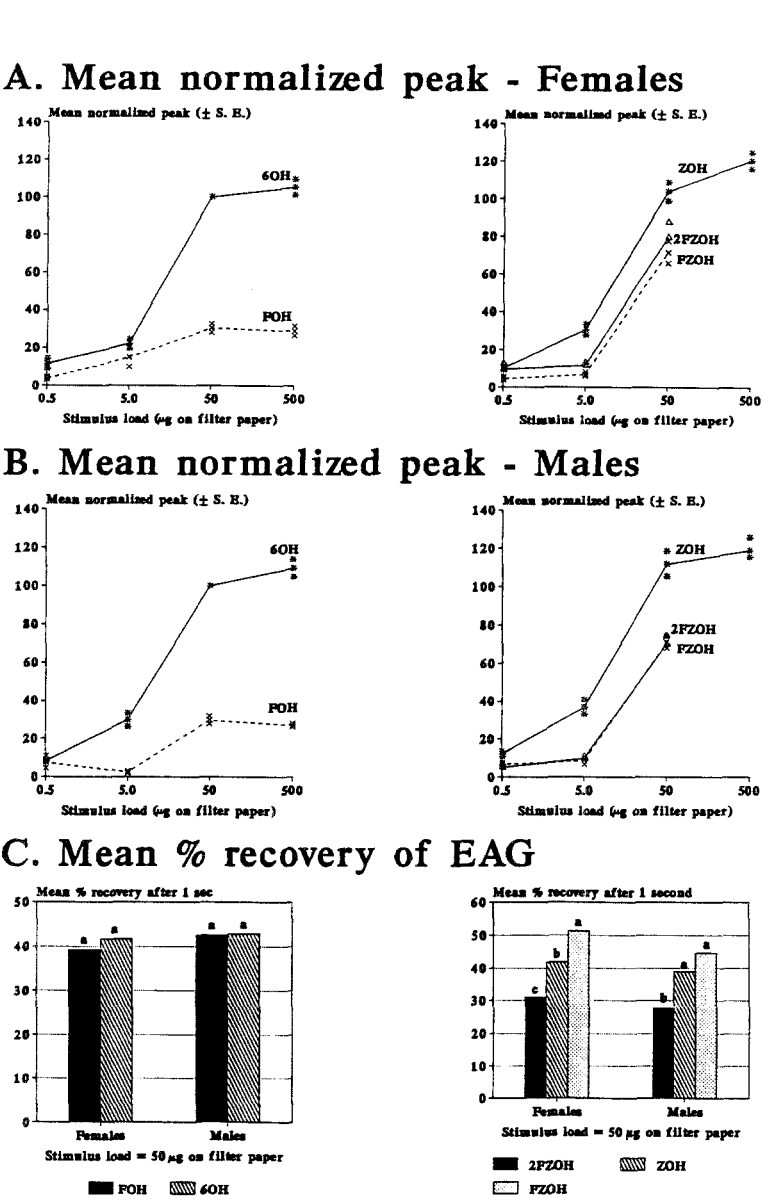


FIG. 2. (A and B) Dose–response curves of mean normalized peaks of female and male *Spodoptera exigua* ($N = 6$ for both sexes) to serial stimulus loads of the green leaf volatiles, 1-hexanol (6OH) and (Z)-3-hexen-1-ol (ZOH), and their fluorinated analogs, 5,5,6,6,6-pentafluoro-1-hexanol (FOH), 5,5,6,6,6-pentafluoro-(Z)-3-hexen-1-ol (FZOH), and 5,5-difluoro-(Z)-3-hexen-1-ol (2FZOH). (C) Mean percentage recovery of EAG of male and female *S. exigua* in 1 sec following stimulation period for green leaf volatiles and fluorinated analogs for 50-μg stimulus loads. Standard errors are represented above and/or below each point. Points and bars marked with different letters are significantly different ($P < 0.05$, Duncan's new multiple-range test).

The relatively faster recovery for the pentafluoro-(Z)-3-hexen-1-ol is similar to the decreased lag observed in EAGs of the western spruce budworm, *Choristoneura occidentalis* Freeman, to a terminally fluorinated pheromone analog relative to its parent pheromone (McLean et al., 1989). We earlier found that pheromone components with perfluorinated alkyl terminals in several Lepidoptera elicited not only reduced neural activity from targeted pheromone receptors, but also diminished poststimulus neural discharge relative to the pheromone at equipotent stimulus levels (Prestwich et al., 1990; Prestwich, 1993; Wu et al., 1992). It was hypothesized that the fluorinated analogs had poor affinity for the hydrophobic region of the pheromone receptor sites and were thus "nonstick" pheromone analogs (Prestwich, 1993). In our current study with GLVs, the more rapid recovery for pentafluoro-(Z)-3-hexen-1-ol relative to its parent GLV may be indicative of a nonstick plant odor analog.

Differing values for percent recovery for 5,5-difluoro-(Z)-3-hexen-1-ol and the pentafluoro analog indicate differing specificities for reception and inactivation mechanisms, since measures of reception based on dose–response curves were nearly identical (Figure 2). This denotes considerable specificity of inactivation involving the alkyl terminus, suggesting the involvement of a specific protein or enzyme, as postulated for inactivation of pheromone molecules (Vogt and Riddiford, 1981). Alternatively, the observed inactivation as measured by percent recovery of the EAG may be explained by compartmentalization of GLV molecules and their fluorinated analogs in the aqueous sensillum lymph based on increasing hydrophilicity of the alkyl terminus, i.e., (Z)-3-hexen-1-ol > 5,5-difluoro-(Z)-3-hexen-1-ol > 5,5,6,6,6-pentafluoro-(Z)-3-hexen-1-ol.

Comparative Studies. Two additional insect species, namely the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera) and the desert locust, *Schistocerca gregaria* (Orthoptera), showed receptor responses to the fluorinated analogs relative to their parent GLVs similar to responses observed for *S. exigua* (Lepidoptera) (Figure 3A). Responses to each fluorinated analog were reduced compared to responses elicited by the parent GLV. While the pentafluorinated analog of 1-hexanol was a less effective stimulus than either fluorinated analog of (Z)-3-hexen-1-ol, the effectiveness of the difluoro- and pentafluoro-(Z)-3-hexen-1-ol analogs were not significantly different ($P < 0.05$). These results are indicative of similar requirements for the hydrophobic terminus of GLVs for interaction with responsive receptors for all three species. Since fluorine atoms not only reduce the hydrophobicity of the alkyl side chain, but also increase rigidity of the molecule (Filler and Kobayashi, 1982), the greater activity of the fluorinated analogs of (Z)-3-hexen-1-ol relative to the fluorinated analog of 1-hexanol indicates that the importance of the *cis* conformation (or a conformation involving flexing of the molecule) for GLV receptor interactions may be widespread.

As was the case for *S. exigua*, mean percent recovery of the EAG differed

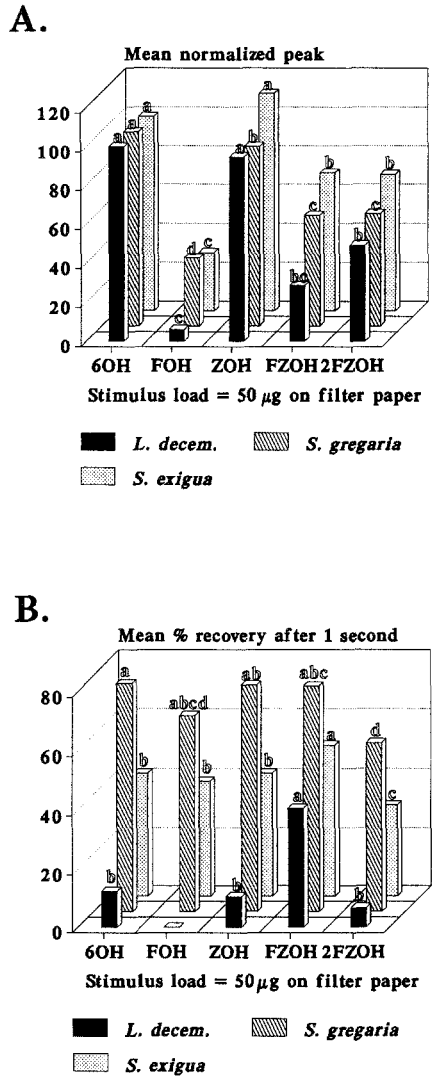


FIG. 3. Mean normalized peaks (A) and mean percentage recovery of EAGs (B) in female *Spodoptera exigua* ($N = 6$), *Schistocerca gregaria* ($N = 3$), and *Leptinotarsa decemlineata* ($N = 3$) to 50-μg stimulus loads of the green leaf volatiles, 1-hexanol (6OH) and (*Z*)-3-hexen-1-ol (ZOH), and their fluorinated analogs, 5,5,6,6,6-pentafluoro-1-hexanol (FOH), 5,5,6,6,6-pentafluoro-(*Z*)-3-hexen-1-ol (FZOH), and 5,5-difluoro-(*Z*)-3-hexen-1-ol (2FZOH). Mean percentage recovery for FOH in *L. decemlineata* not calculated since normalized peaks for this compound did not exceed response to the solvent. For responses within each species, bars with different letters are significantly different ($P < 0.05$, Duncan's new multiple-range test).

significantly for 5,5-difluoro- and 5,5,6,6,6-pentafluoro-(Z)-3-hexen-1-ol in both *L. decemlineata* and *S. gregaria*, even though mean normalized peaks did not ($P < 0.05$) (Figure 3B). These facts provide support for the hypothesis that occurrence of differential reception and inactivation mechanisms for GLVs among insects is rather general.

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CHEMICAL SECRETIONS OF TWO SYMPATRIC HARVESTER ANTS, *Pogonomyrmex salinus* and *Messor lobognathus*

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Abstract—*Messor lobognathus*, an apparent mimic of *Pogonomyrmex salinus*, shows little chemical resemblance to its exemplar. The mandibular glands of *M. lobognathus* gave no volatile compounds. Those of *P. salinus* contain chiefly 4-methyl-3-heptanone. Both species contain a mixture of straight-chain alkanes, alkenes, and methyl-branched alkanes in their Dufour glands. Tridecane (64%) is the major substance in *M. lobognathus* and dodecane (25%) and pentadecane (24%) are the major compounds of *P. salinus*. No secretion induced trail-following in either species. A mixture of 9-, 11-, and 13-methylheptacosane formed the largest peak in the chromatograms obtained from the postpharyngeal glands of both species, but otherwise the hydrocarbon mixtures in this gland too were distinct.

Key Words—Dufour gland, mandibular gland, postpharyngeal gland, cuticle, trail following, mimic, Formicidae, Myrmicinae.

INTRODUCTION

Andrews (1916) described *Messor lobognathus* from four specimens collected in Colorado. It was not reported again for 18 years when Wheeler and Creighton

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(1934) described it, placing it in *Veromessor* (since returned to *Messor* by Bolton, 1982), and stating that its most outstanding characteristic was an extraordinary resemblance to *Pogonomyrmex occidentalis* in size, color, sculpture, and pilosity. The species was not reported again for a further 36 years when Gregg (1955) found it in Colorado, noting that it resembled *P. occidentalis* so closely he had mistaken it for the latter until examined in the laboratory. This statement prompted Wheeler and Wheeler (1956) to reexamine their collection of 100 *P. occidentalis* nests from North Dakota, in which they found one misidentified nest of *M. lobognathus*. Both species are known to occur in Idaho (Yensen et al., 1977).

Wheeler and Wheeler (1989) subsequently made a detailed study of *Messor* (=*Veromessor*) *lobognathus* and have concluded that it is a morphological mimic of *P. occidentalis*, *P. owyheei*, and *P. salinus* (in part of its range). Shattuck (1987) concluded that *P. owyheei* and *P. salinus* are only the northern and southern forms of the one species *P. salinus* Olsen. *P. occidentalis* and *P. salinus* are very closely similar in morphology, and *M. lobognathus* superficially resembles them both. Both *Pogonomyrmex* and *Messor* are harvester ants, i.e., they collect and store seeds, but *Pogonomyrmex* has a functional and painful sting while *Messor* has only a vestigial sting lance and can only bite (Wheeler and Wheeler, 1965).

Some of the chemistry of the secretions of *P. occidentalis* has already been described (Billen et al., 1987a, b). We have now had the opportunity to examine the exocrine secretions of the rare *M. lobognathus* and to compare them with the morphologically very similar and sympatric species, *P. salinus*. The similarity does not extend to their exocrine secretions.

METHODS AND MATERIALS

A large number of workers from three colonies of *Pogonomyrmex salinus* Olsen (PEB #10346, 10339, and 10350) and three colonies of *Messor lobognathus* Andrews (WHC #8540, 8893, and 9174) were collected in the Idaho National Environmental Research Park in southeastern Idaho and transported to Keele, where the glands were dissected and sealed in glass capillaries for chromatography, as described by Morgan (1990). Voucher specimens are deposited in the collections of the Orma J. Smith Museum of Natural History, Albertson College of Idaho, Caldwell (CIDA).

Analysis of the individual glands sealed in capillaries was made by linked gas chromatography–mass spectrometry. Those of *M. lobognathus* were analyzed on one of two fused silica capillary columns, either column A (15 m × 0.32 mm with 0.25 μm film of SE-54) programmed from 30°C to 250°C at 6°/min or column B (12 m × 0.35 mm with 0.33 μm film of OV-1) programmed

from 30°C to 250°C at 8°/min. Those of *P. salinus* were chromatographed either on column B, using the same program or on column C (12 m × 0.2 mm with 0.33 μm film of HP-1) programmed from 30°C to 250°C at 8°/min. The venom glands and the postpharyngeal glands were analyzed on column B with the same program. The injector temperature was held at 140°C with column A, and for the analyses of mandibular glands on columns B and C, otherwise the injector was held at 200°C. The ion source was at 300°C. Retention times in the figures are therefore not exactly comparable.

Mass spectrometry was carried out on a Hewlett Packard 5970B MSD-linked gas chromatograph–mass spectrometer system. The mass spectrometer was set to scan *m/z* 35–500 with ionization energy of 70 eV and scan time of about 1.5 sec.

Double bonds were located in the alkenes of the Dufour gland of *M. lobognathus* by methylthiolation. Two Dufour glands were ground in a small tissue grinder with hexane (100 μl) and transferred to a Keele microreactor (Attygalle and Morgan, 1986), and treated with dimethyl disulfide and iodine (as described by Bagnères et al., 1991a). Normanicone-2(4,6-dimethyl-4-hepten-3-one) was synthesized in poor yield by the method of Faulk and Fry (1970) from isobutyraldehyde and 2-pentanone. Mass spectrum: M^+140 (20%), *m/z* 125 (4), 111 (65), 93 (4), 83 (23), 67 (15), 57 (21), 55 (100), 53 (14), 43 (40), 41 (64), 39 (38).

Tests for trail-following were carried out by the method of Pasteels and Verhaeghe (1974), placing solvent extracts of various body parts in a circle on paper and observing whether or not foraging workers followed the trails.

RESULTS

Neither the mandibular glands nor the venom gland of *Messor lobognathus* yielded any volatile substances; the limit of detection was about 1 ng. The Dufour gland contained a mixture of hydrocarbons (Figure 1) similar to that found in other species of myrmicine harvester ants. The major compound was tridecane, and there were minor amounts of methyl-branched hydrocarbons. No terpenes or oxygenated compounds were detected. The glands were relatively large with about 3.5 μg of secretion on average (Table 1). Two of the colonies, collected three years apart were analyzed. The close correspondence between them is shown in Table 1.

The mandibular glands of *Pogonomyrmex salinus* contained chiefly 4-methyl-3-heptanone (Table 2, Figure 2), a substance found in the mandibular glands of many myrmicine species, and six minor components, including the corresponding alcohol 4-methyl-3-heptanol, and the homologous ketones 4-methyl-3-hexanone and 4-methyl-3-octanone. The three remaining compounds

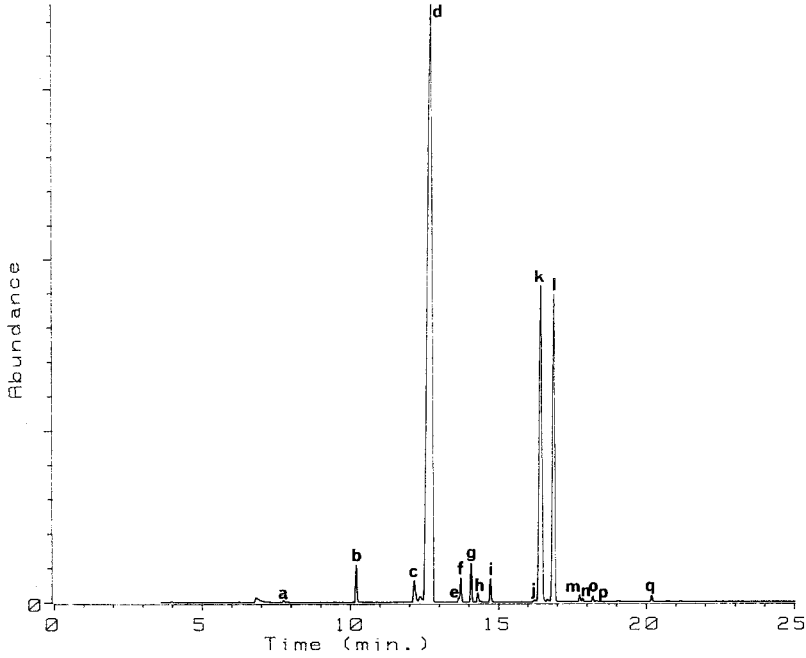


FIG. 1. Total ion chromatogram of a single Dufour gland from a worker of *Messor lobognathus*. The peak identifications correspond to those in Table 1.

are a series of manicone homologs, first found in *Manica rubida* (Bestmann et al., 1988). The mass spectra of manicone and the homomanicone in *P. salinus* are the same as those found in *Manica*, but the spectra of the two normanicones are different. Normanicone-2, the substance in *P. salinus*, was shown to be 4,6-dimethyl-4-hepten-2-one by comparison of its retention time and mass spectrum with those of a sample prepared in the laboratory.

The Dufour glands of *P. salinus*, like those of *M. lobognathus* contain a mixture of hydrocarbons (Figure 3), but the major hydrocarbons, here dodecane and pentadecane, are different in the two species. The glands of *P. salinus* contain many more minor components, and there is a small amount of the terpenoid aldehyde tetramorene-2 (see Ali et al., 1987; Jackson, 1991) present in *P. salinus* (Table 3).

The postpharyngeal glands of *M. lobognathus* and *P. salinus* contain similar mixtures of higher hydrocarbons, both linear and methyl-branched, from C_{20} to C_{30} (Table 4, Figure 4). The same partially resolved mixture of 9-, 11-, and 13-methylheptacosane was the major peak in the gas chromatograms of both species, but otherwise there were no strong similarities between them. Both

TABLE 1. CONTENTS OF DUFOUR GLAND OF TWO SAMPLES OF *Messor lobognathus* (N = 10 and 8)

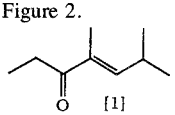
		Mean proportion			
		Nest 1		Nest 2	
Peak[a]	Compound	%	SD	%	SD
a	Undecane	t		0.1	0.04
b	Dodecane	1.2	0.6	1.4	0.2
c	Tridecene	0.6	0.4	1.5	0.1
d	Tridecane	62.7	12.2	63.5	5.6
e	7-Methyltridecane	t		0.2	0.05
f	5-Methyltridecane	1.3	0.8	0.6	0.2
g	3-Methyltridecane	1.5	1.0	1.0	0.3
h	Tetradecene	0.4	0.3	0.3	0.1
i	Tetradecane	0.9	0.7	0.7	0.1
j	Pentadecadiene	t		0.1	0.03
k	6- and 7-Pentadecene	19.6	7.3	17.3	3.7
l	Pentadecane	11.3	2.9	12.6	2.5
m	7-Methylpentadecane	0.2	0.1	0.2	0.07
n	5-Methylpentadecane	0.1	0.1	0.1	0.02
o	3-Methylpentadecane	0.2	0.3	0.1	0.03
p	Hexadecene	t		0.1	0.05
q	Heptadecene	t		0.2	0.05
	Total amount (μg)	4.7	2.2	2.5	2.2

[a]Letters refer to Figure 1.

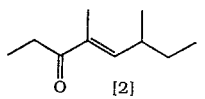
TABLE 2. CONTENTS OF MANDIBULAR GLAND OF *Pogonomyrmex salinus* (N = 8)

		Mean proportion		Mean amount	
Peak[a]	Compound	%	SD	ng/ant	SD
a	4-Methyl-3-hexanone	1.3	0.5	52	37
b	4-Methyl-3-heptanone	90.0	3.2	2817	822
c	4-Methyl-3-heptanol	0.3	0.3	11	11
d	4-Methyl-3-octanone	0.9	0.9	39	37
e	Normanicone [1][b]	2.0	1.0	81	62
f	Manicone-2 [2]	2.4	1.1	94	63
g	Homomanicone [3]	3.1	1.2	119	70
	Total			3210	1043

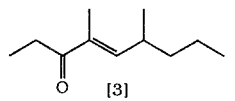
[a]Letters refer to Figure 2.
[b]Structures are:



[1]



[2]



[3]

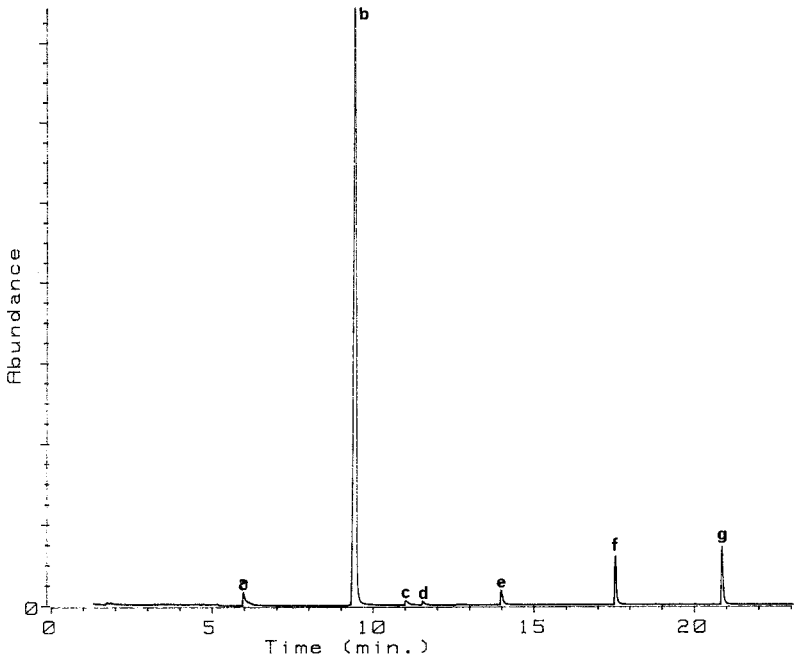


FIG. 2. Total ion chromatogram of the mandibular glands from a single worker of *Messor lobognathus*. For peak identification, see Table 2.

species contained fatty acid amides and *P. salinus* glands also contained fatty acid methyl esters.

No volatile compounds were detected in the poison reservoirs of either species. No activity was observed in the circular trail-following tests with the poison gland, Dufour gland, or whole gaster extracts in hexane with either species. In addition, all extracts of *M. lobognathus* were tested on *P. salinus* and vice versa without observing any activity.

DISCUSSION

Pogonomyrmex and *Messor* are both harvester ant genera, that is, they collect and store seeds for food. *Pogonomyrmex* workers, however, have active stings and a powerful burning venom, while *Messor* have vestigal sting lances and can only bite. The resemblance between *P. occidentalis* or *P. salinus* and *M. lobognathus* is largely external. We wished to see if there was any further evidence of mimicry by a rarely collected ant of its more common exemplars.

The only previous report of exocrine secretions from *Messor* (=*Veromes-*

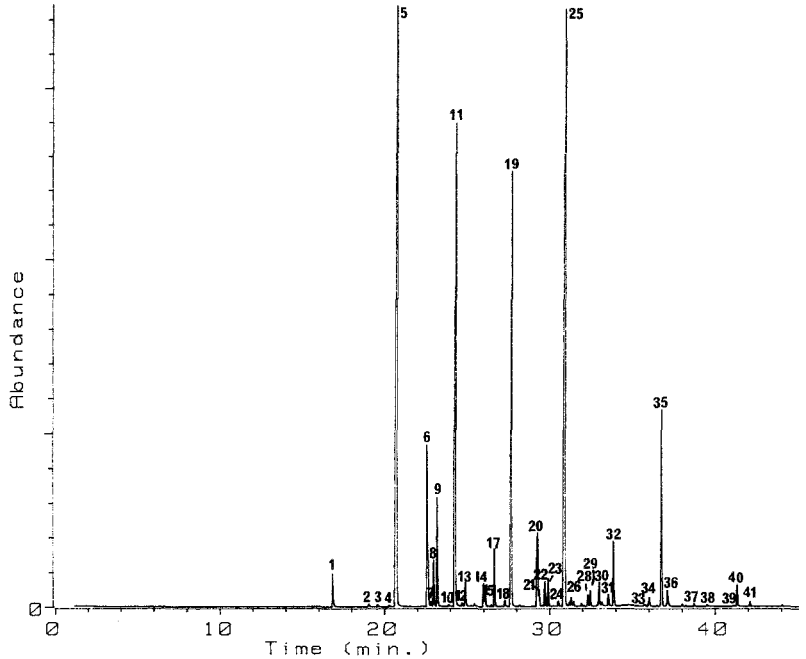


FIG. 3. Total ion chromatogram of the Dufour gland from a worker of *Pogonomyrmex salinus*. Peak numbers correspond to those in Table 3. The numbers of some minor peaks have been omitted for lack of space.

sor) species is that of benzaldehyde in *M.* (=*V.*) *pergandei* mandibular glands (Blum et al., 1969). We have examined mandibular glands of five species of Euro-African *Messor* (Ali et al., 1989; Jackson, 1991) and have found no volatile compounds in any of them. By contrast, the mandibular gland of five species of *Pogonomyrmex* (*P. barbatus*, *P. californicus*, *P. desertorum*, *P. occidentalis*, and *P. rugosus*) all contain 4-methyl-3-heptanone (McGurk et al., 1966). Based on this small body of information, and including the two species that we have now examined (which follow the same pattern), the mandibular gland contents represent a fundamental difference between *Messor* and *Pogonomyrmex*.

The Dufour glands of many *Messor* species are unusual in containing large proportions of unsaturated compounds, e.g., 75% of the total in *M. bouvieri* (Jackson et al., 1991); in *M. lobognathus* the proportion is a more modest 20%. *M. lobognathus* is like *M. minor* in having methyl-branched alkanes, and both have tridecane and pentadecane as major components (see Ali et al., 1989). Whereas the major hydrocarbons in most Dufour glands are of odd number of carbon atoms, *P. salinus* is like *P. rugosus* and *P. barbatus* in having dodecane

TABLE 3. CONTENTS OF DUFOUR GLAND OF *Pogonomyrmex salinus* ($N = 8$)

Peak[a]	Compound	Mean proportion		Mean amount	
		%	SD	ng/ant	SD
1	Undecane	0.7	0.4	36	28
2	5-Methylundecane	0.1	0.1	4	3
3	3-Methylundecane	0.1	0.1	3	2
4	Dodecene	0.1	0.1	2	2
5	Dodecane	24.7	4.2	1124	824
6	6-Methyldodecane	4.6	0.5	217	165
7	5-Methyldodecane	0.2	0.1	6	4
8	4-Methyldodecane	1.0	0.1	50	22
9	3-Methyldodecane	2.4	0.2	113	76
10	Tridecene	0.1	0.1	3	2
11	Tridecane	13.0	1.5	671	486
12	4,6-Dimethyldodecane	0.1	0.1	4	2
13	3,5-Dimethyldodecane	0.9	0.3	28	22
14	7-Methyltridecane	1.0	0.5	29	24
15	5-Methyltridecane	0.4	0.2	13	11
16	4-Methyltridecane	0.1	0.1	2	2
17	3-Methyltridecane	1.1	0.3	62	49
18	Tetradecene	0.2	0.1	7	5
19	Tetradecane	12.5	0.4	557	443
20	6-Methyltetradecane	3.3	0.9	124	95
21	5-Methyltetradecane	0.4	0.2	16	13
22	4-Methyltetradecane	0.5	0.2	25	18
23	3-Methyltetradecane	0.6	0.3	3.0	19
24	Pentadecene	0.1	0.1	5	3
25	Pentadecane	24.0	1.9	1025	767
26	4,6-Dimethyltetradecane	0.1	0.1	5	5
27	3,5-Dimethyltetradecane	0.2	0.1	9	8
28	7-Methylpentadecane	0.3	0.2	14	10
29	5-Methylpentadecane	0.3	0.1	13	10
30	3-Methylpentadecane	0.5	0.2	26	17
31	Hexadecene	0.5	0.3	19	15
32	Hexadecane	1.5	0.1	75	46
33	Heptadecadiene	0.1	0.1	2	2
34	Heptadecene	0.4	0.2	14	10
35	Heptadecane	5.1	0.8	225	165
36	Tetramorene-2	0.6	0.2	24	18
37	Octadecene	0.3	0.2	13	8
38	Octadecane	0.1	0.1	3	2
39	Nonadecadiene	0.1	0.1	1	1
40	Nonadecene	1.7	1.2	26	21
41	Nonadecane	0.1	0.1	1	1
	Total			4626	3867

[a] Numbers refer to Figure 3.

TABLE 4. COMPOSITION OF SECRETION OF POSTPHARYNGEAL GLANDS OF *Messor lobognathus* AND *Pogomyrmex salinus* EXPRESSED AS PERCENTAGE OF TOTAL WITH SAMPLE STANDARD DEVIATION

		M. lobognathus		*P. salinus*	
Peak[a]	Compound	%	±SD	%	±SD
	Methyl palmitate			0.1	0.4
1	Methyl linoleate			1.1	1.6
2	Methyl oleate			6.3	14.5
3	Methyl stearate			0.7	0.9
4	Linoleamide	0.3	0.6	1.9	4.6
5	Oleamide	0.3	1.0	2.0	3.6
6	Stearamide	0.1	0.3	3.7	10.0
7	Tetracosane	t		0.9	2.0
8	9-, 11-, and 13-Methyltetracosane	t		t	
9	3-Methyltetracosane	t		0.1	0.4
10	Pentacosane	5.5	3.2	5.2	3.4
11	9-, 11- and 13-Methylpentacosane	9.4	3.5	3.8	4.8
	7-Methylpentacosane			1.8	2.3
12	5-Methylpentacosane	2.0	3.8	1.2	1.5
13	3-Methylpentacosane	7.7	3.5	3.9	5.9
14	Hexacosane	3.8	1.8	5.5	4.6
15	9-, 11- and 13-Methylhexacosane	7.5	2.7	2.3	3.6
16	5-Methylhexacosane	1.1	0.9	1.2	2.8
17	3-Methylhexacosane	1.4	1.3	0.8	1.6
18	Heptacosane	9.9	5.2	10.6	5.2
19	9-, 11-, and 13-Methylheptacosane	25.9	6.7	11.8	9.8
20	7-Methylheptacosane			2.1	4.5
21	5-Methylheptacosane			6.8	9.3
22	11,15-Dimethylheptacosane	9.5	1.9	4.6	3.9
23	3-Methylheptacosane	6.1	2.2	6.4	7.0
24	Octacosane	3.1	1.9	5.1	5.2
25	Nonacosane	3.7	2.4	5.5	4.4
26	Triacontane	2.3	1.9	4.5	3.7
	Mean total amount (μg)	5.5	±3.9	2.3	±2.1

[a] Numbers refer to Figure 4.

as its principal component (Regnier et al., 1973). It is also noteworthy that the sesquiterpenoid aldehyde tetramorene-2 ($C_{17}H_{30}O$), first discovered in the Dufour glands of *Tetramorium caespitum* and *T. impurum* (Billen et al., 1986) and later found in *Leptothorax acervorum* and *L. nylanderi* (Ali et al., 1987), *Tetramorium meridionale* (Jackson, 1991), and *Daceton armigerum* (Morgan et al., 1992), is thus far known only from myrmicine species. The exact isomeric structure of tetramorene-2 is unknown, the lowest member of the homologous

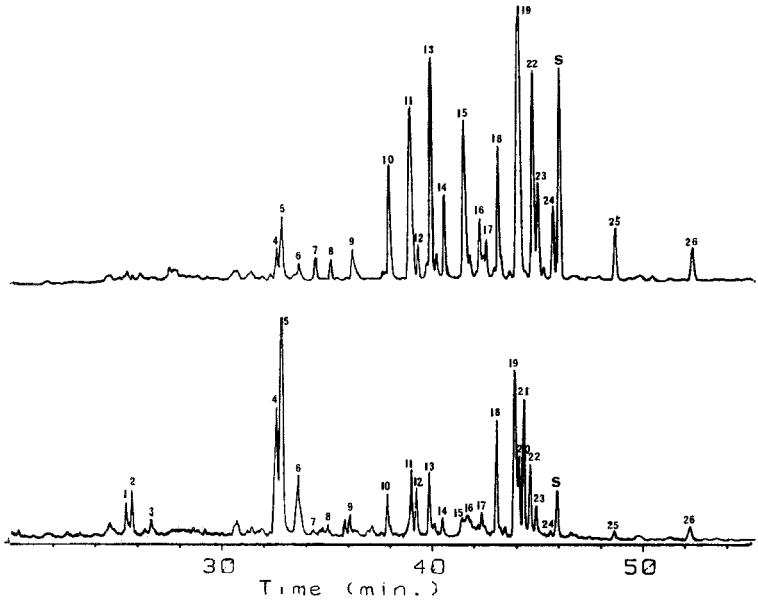


FIG. 4. Total ion chromatograms of the postpharyngeal gland contents of *M. lobognathus* (above) and *P. salinus* (below). The numbers correspond to those in Table 4. The peaks marked S are squalene, a contaminant.

series (tetramorene-0) is 2,3-dihydrofarnesal (Jackson, 1991). *P. occidentalis* contains a bishomofarnesene isomer (Billen et al., 1987a,b).

Blum (1974) reported that workers of *Messor* (= *Veromessor*) *pergandei* follow trails made with their poison glands. We attempted to see if *M. lobognathus* might follow trails of *P. salinus* workers, but we could detect no trail-following response in *M. lobognathus* for their own poison glands nor for any other part of their abdomens nor those of *P. salinus*.

Hölldobler and Wilson (1970) and Hölldobler (1971) have shown that both the poison gland and the Dufour gland of *P. badius* induce recruitment and trail following, but they also say that *P. badius* workers are too excitable and discharge the mandibular gland pheromone too readily to permit reliable assays of the trail pheromone on small groups of workers separated from their colony (Hölldobler and Wilson, 1970).

We cannot say that *M. lobognathus* workers do not use odor trails in their natural surroundings. We have also noticed in some ant species that as the colony nears extinction, surviving workers lose the ability to follow trails. Both species studied here appeared active and healthy, but they had suffered a long journey and a change of climate and altitude.

Cuticular hydrocarbons have been shown to be important as species markers and in interindividual recognition in insects (for a review see Lockey, 1988), and it has been shown that these hydrocarbons may be very similar in cases of mimicry (Bagnères et al., 1991b). We therefore examined the postpharyngeal glands and the cuticle of both species. As we found in other species, there was close correspondence between the pattern in the postpharyngeal gland and the cuticle of that species. There were some similarities between the two species, but there were also large differences (Figure 4). The mean composition of the hydrocarbons of the postpharyngeal glands are given in Table 4. The presence of small quantities of methyl esters and amides of the fatty acids in secretions is noteworthy. They have not previously been recorded as cuticle components (c.f. Lockey, 1988; Blomquist et al., 1987), but we have also found small amounts of methyl esters of fatty acids in the postpharyngeal gland and cuticular wax of the ant *Harpegnathos saltator* (do Nascimento et al., 1993). Their cuticle had somewhere come in contact with petroleum hydrocarbons, which were easily recognized but made quantification of the true cuticular hydrocarbons difficult.

Any mimicry between *P. salinus* and *M. lobognathus* at this stage must be hypothetical and does not include their exocrine secretions. We have no information to suggest *M. lobognathus* is trying to deceive *P. salinus*. The common invertebrate predators of *Pogonomyrmex* (e.g., spiders) may not rely heavily on visual discrimination and vertebrates (such as *Phrynosoma*) do not appear deterred by the sting of *Pogonomyrmex*. Possibly the mimetic relationship developed and existed in a time or place where predation pressure on *Messor* was significant and *Pogonomyrmex* was better able to deter predators.

Acknowledgments—We thank K. Denny for synthesizing 4,6-dimethyl-4-hepten-3-one (normanicone-2). R.R. do Nascimento thanks the Brazilian Government for a research studentship, B.D.J. thanks the Science and Engineering Research Council (G.B.) for a studentship, and E.D.M. thanks S.E.R.C. for a Research grant for the purchase of equipment. We thank O.D. Markham, T.D. Reynolds, and J.B. Johnson for their assistance. Partial support was received from the Idaho National Engineering Laboratory, Radioecology and Ecology Programs sponsored by the Office of Health and Environmental Research, U.S. Department of Energy.

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RUMINAL METABOLISM OF LEAFY SPURGE IN SHEEP AND GOATS: A POTENTIAL EXPLANATION FOR DIFFERENTIAL FORAGING ON SPURGE BY SHEEP, GOATS, AND CATTLE[1]

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Abstract—Leafy spurge (*Euphorbia esula*) is an introduced forb that is invading western rangelands. Goats (*Capra hircus*) readily graze the plant, but cattle (*Bos tarus*) generally and sheep (*Ovis aries*) locally appear to develop conditioned flavor aversions to leafy spurge. They either avoid the plant entirely or graze it reluctantly. We hypothesized that: (1) a diterpene diester that can occur in leafy spurge was an aversive agent, and (2) diet selection differences among ruminant species may be partly a function of differential ruminal metabolism of aversive phytochemicals, and further that cattle and sheep may be reluctant to graze leafy spurge because their ruminal microbes do not metabolize certain leafy spurge chemicals as do ruminal microbes in goats. Sheep did not develop an aversion to a novel food when its consumption was followed by an intravenous injection of ingenol 3,20-dibenzoate ($P = 0.34$). Sheep did develop an aversion to a novel food when its intake was followed by a dose of leafy spurge fermented with sheep ruminal digesta, but not when followed by a dose of leafy spurge fermented with goat ruminal digesta ($P = 0.03$). This suggests that goat ruminal microbes may modify leafy spurge such that it does not elicit an aversion in sheep.

Key Words—Conditioned flavor aversion, diet selection, ruminants, phytotoxins, ingenol, weeds, leafy spurge, *Euphorbia esula*, ruminal metabolism, sheep, *Ovis aries*, goat, *Capra hircus*, cattle, *Bos tarus*.

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INTRODUCTION

In contrast to goats, cattle and sheep can develop feeding aversions to the introduced rangeland and pasture weed leafy spurge (*Euphorbia esula*) (Kronberg et al., 1993a). This finding is consistent with field observations that cattle generally do not graze the plant (Lacey et al., 1985; Lym and Kirby, 1987) and mixed reports that sheep will or will not readily graze it at least in some areas of its infestation (Landgraf et al., 1984; Lacey et al., 1984; Bartz et al., 1985; Kronberg et al., 1992). The finding is also consistent with our field observations in southeastern Idaho that goats graze leafy spurge more willingly than do sheep (Walker and Kronberg, 1992). Our findings suggest that leafy spurge contains one or more chemicals that can elicit an aversive response in cattle and sheep (Kronberg et al., 1993a,b).

The identity of the aversive chemical(s) in leafy spurge is unknown. However, various diterpenoid ingenols have been found in the plant (Kupchan et al., 1976; Upadhyay et al., 1978; Seip and Hecker, 1982; Sorg and Hecker, 1982). Kupchan et al. (1976) found that the diterpene ingenol 3,20-dibenzoate from leafy spurge had inhibitory activity against lymphocytic leukemia in mice, and Sorg and Hecker (1982) found that it had high inflammatory activity on mouse ears. Upadhyay et al. (1978) found two ingenols in leafy spurge that had skin irritant and inflammatory properties, and Seip and Hecker (1982) found a fraction of leafy spurge that exhibited irritant and weak tumor-promoting properties. We suspect that the aversion-eliciting characteristic of leafy spurge may be due to its diterpenoid compounds for two reasons. First, chemicals with cytotoxic activity are typically aversive (Seynaeve et al., 1991; Mattes et al., 1992), and secondly, both lithium and phorbol 12-myristate 13-acetate (PMA) inhibit the gene expression for phosphoenolpyruvate carboxykinase, a key enzyme in gluconeogenesis (Bosch et al., 1992; Chu and Granner, 1986). Lithium is an aversive agent widely used in the study of taste aversion learning (Riley and Tuck, 1985), and PMA and ingenols are related diterpenes (Evans, 1986).

We suspect that ingenol is absorbed into the blood from the gastrointestinal tract. Once in the blood, it reaches the area postrema in the medulla oblongata of the brain, where a chain of events leads to the development of a conditioned flavor aversion (Borison, 1986; Kosten and Contreras, 1989) for leafy spurge.

Diet selection differences among ruminant species may be partly a function of differential ruminal metabolism of phytochemicals. Ruminal microbes can detoxify or produce toxins from phytochemicals (James et al., 1975; Allison et al., 1981, 1990; Carlson and Breeze, 1984; Craig et al., 1992). Rumen microbial populations in different ruminant species can have critically different capacities for degrading plant toxins (Wachenheim et al., 1992); therefore, we rationalized that cattle and sheep may be reluctant to graze leafy spurge because

their ruminal microbes do not metabolize aversive leafy spurge phytochemicals as do ruminal microbes in goats.

The objectives of our study were to learn if one of the ingenols isolated from leafy spurge could elicit an aversive response in sheep and to determine if processes in the rumen might account for interspecific differences in preference for leafy spurge among cattle, sheep, and goats. Specifically, we tested two hypotheses: (1) sheep will develop aversions to a novel food when intake of the food is followed by an intravenous dose of ingenol 3,20-dibenzoate, and (2) sheep will develop aversions to a novel food when intake of the food is followed by leafy spurge that is fermented with sheep ruminal microbes, but not when intake of the novel food is followed by a dose of spurge that is fermented with goat ruminal microbes.

METHODS AND MATERIALS

Experiment 1. This experiment studied the aversiveness of ingenol 3,20-dibenzoate to sheep. Ten weaned white-face lambs (mean wt, 31 $\pm$ 3 kg) were randomly divided into two treatment groups (N = 5/treatment) for an aversion trial. Treatment lambs received the ingenol in ethanol and control lambs received only ethanol. The lambs were about 5 months old.

During the seven-day experiment, except for the brief training or trial periods, lambs were held together in an outdoor pen and had ad libitum access to alfalfa (*Medicago sativa*) hay and salt from 0800 to 1700 hr and had continuous access to water. During a five-day pretrial period, sheep were offered alfalfa pellets from 0800 to 0830 hr in individual outdoor pens to accustom them to the trial procedure.

On the day of the trial, each sheep was offered 300 g of a novel food (milo) in the individual pens from 0800 to 0830 hr. At 0830 hr, after they consumed the novel feed, they were released to their outdoor pen.

Sheep were given jugular injections of ingenol in ethanol (1 mg ingenol 3,20-dibenzoate/ml ethanol) or ethanol alone from 0915 to 0945 hr and from 1015 to 1045 hr. The dosage of ingenol was determined from the concentration of ingenol 3,20-dibenzoate in air-dried leafy spurge (0.0002%) (Kupchan et al., 1976) and a dosage of air-dried leafy spurge that has caused marked elevations of blood cortisol in sheep (0.3% of body weight) indicating physiological stress (Kronberg et al., 1993c) (twice the dosage of air-dried material necessary to cause an aversive response from leafy spurge; Kronberg et al., 1993a). Ingenol 3,20-dibenzoate was purchased from LC Services Corp., Woburn, Massachusetts (Lot BG-101). Each lamb received a dose of 0.6% of body weight (BW) of the appropriate solution at each dosing. On the second day of the trial, each lamb was offered 300 g of novel feed from 0800 to 0830 hr. The amount of novel feed consumed by each animal was recorded daily.

Experiment 2. This experiment studied the effect of fermenting leafy spurge with ruminal digesta from sheep or goats on the subsequent aversiveness of leafy spurge to sheep. Ten ruminally fistulated white-faced lambs (mean wt, 49 ± 11 kg) were randomly divided into two treatment groups (N = 5/treatment) for an aversion trial. The lambs were about 11 months old and were different animals from those used in the first experiment.

Sheep were held in an outdoor pen and had ad libitum access to alfalfa hay and salt from 0700 to 1700 hr and had continuous access to water. During a four-day pretrial period, the sheep were offered alfalfa pellets from 0700 to 0730 hr in individual indoor pens to accustom them to the trial procedure.

On each day of the five-day trial, each sheep was offered 300 g of a novel food (rolled oats) in the individual pens from 0700 to 0730 hr. On the third and fourth days of the trial, after the 30-min period of novel food consumption, sheep were dosed, via their ruminal fistula, with leafy spurge that had been fermented with either sheep or goat digesta for 12.5 hr. The amount of novel food consumed by each animal was recorded daily.

Leafy spurge/digesta fermentations were conducted with the following procedure. For each day that fermented mixtures were dosed, freeze-dried, and ground (1-mm screen) leafy spurge equivalent to 0.10% of body weight (ca. 49 g/animal) was placed into 1-liter Erlenmeyer flasks. Buffer solution was added to each flask and mixed with the leafy spurge, then flasks were placed in an incubator (39°C) to warm. Ruminal digesta from two to four donors of each species was collected into prewarmed insulated containers by species and mixed. Donor animals were maintained on alfalfa. Digesta was added to each flask within a half hour of its collection. Flasks were incubated for 12.5 hr and agitated 3.5 hr after the start of the fermentations. Fermentations were stopped by placing the flasks in ice water. The buffer recipe and proportions of leafy spurge, buffer, and ruminal digesta followed the Barnes modification of the Tilley and Terry in vitro technique (Harris, 1970) with the exception that whole ruminal digesta was used instead of ruminal liquor because the majority of ruminal bacteria are associated with the particulate phase (Forsberg and Lam, 1977; Craig et al., 1987).

Data Analysis. For both experiments, novel food intake after dosing was the dependent variable. Data for experiment 1 were analyzed by analysis of variance, and data for experiment 2 were analyzed by repeated-measures analysis of covariance (SAS, 1986). Novel food intake on the day leafy spurge was first dosed was the covariate, treatment group was the between-animal effect, and day postdose was the within-animal effect.

RESULTS

Experiment 1. The treatment and control groups had similar intakes of novel food on the first day (P = 0.21; 222 and 273 g, respectively) and on the second day (P = 0.34; 300 and 277 g, respectively). Neither group displayed an aversive response to the novel food on day 2 of the trial.

Experiment 2. Sheep dosed with leafy spurge fermented with sheep digesta consumed less novel feed than sheep that received the goat digesta treatment ($P = 0.03$; 122 compared to 203 g, respectively). The aversive effect of spurge fermented with sheep digesta tended to increase after the second dosing with this mixture (treatment × day interaction, $P = 0.16$; Figure 1). The intake of NF on day 3 before dosing (the covariate) accounted for a significant amount of variation ($P = 0.005$) in postdose NF intake.

DISCUSSION

Results from the first experiment suggest that either ingenol 3,20-dibenzoate is not an aversive chemical in leafy spurge or it does not stimulate the development of a conditioned flavor aversion by reaching the area postrema in the blood. Conditioned flavor aversions can also be instigated by stimulation of visceral afferent nerves (Willems and Lefebvre, 1986). Therefore, this ingenol may stimulate flavor aversion development via another route, and this possibility should be investigated. Alternatively, one of the other ingenols found in leafy spurge may be an aversive compound.

Results from experiment 2 suggest that fermenting leafy spurge with goat ruminal digesta may limit its capacity to elicit a conditioned flavor aversion in

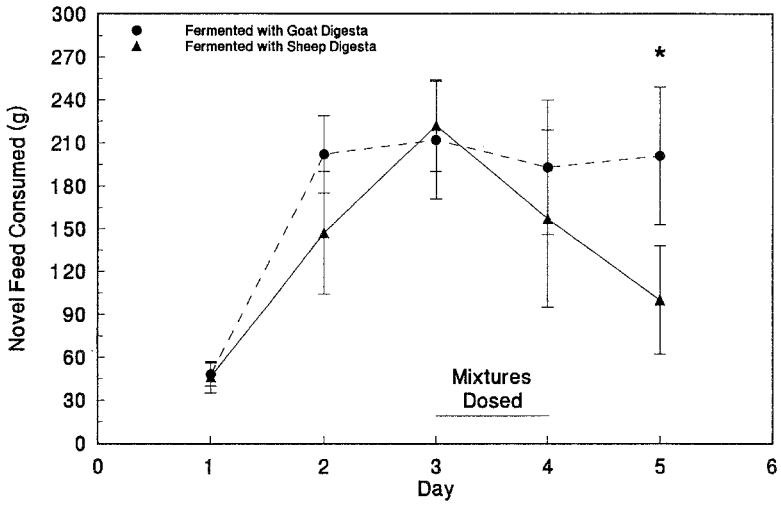

FIG. 1. Consumption of novel feed (g) during a 0.5-hr period by two treatment groups of sheep on each day of a five-day trial. Group 1 and 2 sheep received leafy spurge fermented with ruminal digesta from sheep and goats, respectively. The spurge/digesta mixtures were dosed on days 3 and 4; *indicates that treatment groups differed ($P < 0.05$) in intake of novel feed on that day. Bars represent ± SE.

sheep. In contrast, fermenting leafy spurge with sheep ruminal digesta either (1) does not nullify its capacity to elicit an aversive response from sheep, or (2) creates a by-product during leafy spurge fermentation that is aversive. However, if the second alternative occurs, the spurge/goat-digesta mixture might still have elicited an aversion in sheep.

It is well established that ruminal microorganisms can detoxify various phytotoxins (James et al., 1975; Allison et al., 1990; Craig et al., 1992; Duncan and Milne, 1992), and ruminal microbes can adapt and increase their capacity for detoxifying phytochemicals by altering their number or metabolic capacity or by altering the composition of the microbial community (Lindroth, 1988). However, ruminal microbes in ruminant individuals and species apparently vary in their capacity to degrade particular toxins. Wachenheim et al. (1992) have shown that the ruminal bacteria that metabolize pyrrolizidine alkaloids exist in cattle, sheep, and goats, but only sheep and goats have populations of these bacteria that are large enough to degrade the toxic alkaloids in a timely manner.

Hungate (1966) noted that various ruminal microbes such as *Veillonella alcalescens*, Quin's oval and large selemonads are much more abundant in sheep than in cattle. Several variables may account for differences in composition of rumen microbial communities and fermentation patterns among ruminant species and among individuals of the same species (Hungate, 1966). These include: ruminal pH, osmolarity and redox potential, nutrient availability and recycling to the rumen (via saliva and blood), dietary intake rate and composition, saliva production, and digesta particle size and passage rate (therefore, degree of rumination is indirectly influential) (Hungate, 1966; Owens and Goetsch, 1988; Yokoyama and Johnson, 1988).

Ruminal microbes can be either highly specialized, intermediate, or general in the type of nutrients that they metabolize; consequently, variations in dietary composition among ruminant species is reflected in the variation in rumen microbial compositions (Yokoyama and Johnson, 1988). Ruminal bacteria vary widely in the substrates they ferment (Russell, 1984). For example, *Bacteroides succinogenes*, *Ruminococcus flavefaciens*, and *R. albus* require ammonia, whereas *Selenomonas ruminantium* and *Peptostreptoccus elsdenii* apparently do not (Hungate, 1966). Additionally, the vitamins biotin, folic acid, thiamine, pyridoxine, and pantothenic acid are required by certain species, but not others (Hungate, 1966). Rumen pH, one of the most variable environmental factors, can greatly affect the microbial population (Hungate, 1966; Yokoyama and Johnson, 1988). Reduced feed particle size increases direct contact between bacteria and substrate; consequently, the rate of bacterial adhesion to feed particles and, therefore, fermentation rate can be influenced by the degree of mastication (Cheng, 1987).

There is limited information on variability of factors that could account for variation in rumen microbial communities among ruminant species. Hoppe et

al. (1977) collected blood and ruminal samples from freshly killed Maasai sheep and goats, Thomson's and Grant's gazelles (*Gazella thomsoni* and *G. granti*, respectively), and impalas (*Aepycerus melampus*) during a dry season. Ruminal pH for these species was: 6.12, 6.10, 6.04, 6.01, and 6.30, respectively, and ruminal ammonia nitrogen concentrations were: 13.8, 16.6, 18.5, 14.1, and 21.1 mg/100 ml, respectively. Thornton (1970) fed cattle and sheep the same amount (metabolic BW basis) of the same diet and observed that cattle had slightly lower plasma urea nitrogen (PUN) and ruminal fluid ammonia nitrogen concentrations (1.49 and 0.70 mg/100 ml, respectively) than sheep (1.92 and 0.97 mg/100 ml, respectively). When cattle (crossbreds of *Bos tarus* and *B. indicus*) and sheep were fed the tropical forage spear grass (*Heteropogon contortus*) ad libitum, cattle and sheep had daily nitrogen intakes of 0.09 and 0.08 g/kg of BW/day, respectively, and PUN levels of 5.6 and 14.5 mg/100 ml, respectively (Siebert and Kennedy, 1972). When they were fed a diet of 80% spear grass and 20% alfalfa, cattle and sheep had daily nitrogen intakes of 0.27 and 0.24 g/kg of BW/day, respectively, yet cattle still had lower PUN levels than sheep (12.1 and 19.5 mg/100 ml, respectively) (Seibert and Kennedy, 1972).

Plasma urea nitrogen was linearly related to salivary urea concentration in cattle (Bailey and Balch, 1961) and sheep (Somers, 1961) up to a certain concentration of PUN, and the diffusion of urea from the blood across the rumen epithelium (urea is hydrolyzed to ammonia as it diffuses through the epithelium) was linearly related to blood urea concentration (Houpt and Houpt, 1968). Therefore, it is logical to assume that lower levels of urea were entering the rumens of cattle than of sheep in Thornton's (1970) and Seibert and Kennedy's (1972) studies, and this difference probably influenced the composition of their rumen microbial communities.

Huston et al. (1986) fed forage sorghum (*Sorghum* spp.) hay to sheep, goats, and white-tailed deer (*Odocoileus virginianus*). The digestibility of sorghum hay was different among sheep, goats, and deer (56.4, 61.5, and 51.8%, respectively) as was mean retention time of sorghum particles in the gastrointestinal tract (35.5, 59.6, and 33.0 hr, respectively). Reid et al. (1990) compared the utilization of warm- and cool-season forage hays by cattle, sheep, and goats and observed that: (1) cattle generally had higher intakes than sheep and goats, and (2) sheep and goats had faster particle passage rates than cattle. Playne (1978) fed cattle and sheep similar amounts (BW basis) of low-quality tropical grass hay and observed that the digestibility of this hay was much higher in cattle than in sheep (49.6 vs 34.6%, respectively) and that cattle had higher serum SO_4-S concentrations and had a better sulfur balance than sheep did. Playne (1978) concluded that this difference was not due to the diet selected by the two species, but was a result of greater neutral detergent fiber (NDF) digestion by the cattle, and suggested that differential NDF digestion may result from

differences between cattle and sheep in their ability to recycle nutrients to their rumen (e.g., sulfur) and, consequently, differences in rumen microbial activity. These reports support our contention that there is variation in ruminal environments and probably differences in rumen microbial communities among ruminant species. This variability may account for some of the diet selection differences among these species.

Results from our second experiment may explain why goats generally graze leafy spurge more readily than do sheep. If the level of aversive postingestive feedback derived from a food is low and/or delayed and its nutritional value (positive feedback) is high and/or promptly experienced, ruminants appear to have more attraction than aversion to it and increase their intake of the food (Launchbaugh and Provenza, 1992; Provenza and Cincotta, 1992). Leafy spurge has high crude protein and digestible energy levels similar to alfalfa (Fox et al., 1991). We speculate that leafy spurge offers more positive than negative stimuli for goats because of its treatment in the goat's rumen; therefore, they continue to consume it. In contrast, leafy spurge causes more negative than positive stimuli for sheep because of their ruminal treatment of spurge; therefore, they do not continue to consume it. Provenza et al. (1992) offered a similar explanation for observations of cattle either grazing or avoiding tall larkspur (*Delphinium barbeyi*).

We interpret our findings to suggest that differences among livestock species in selection for leafy spurge may be caused by processes occurring in the rumen. We believe that this report offers the first evidence that differential activity in the rumen of two ruminant species may account for differences in their diet selection. However, it is also possible that degradation of spurge chemicals in the goat occurs within its own tissues (e.g., its liver). The goal of our research is to discover the mechanisms that allow goats to consume leafy spurge with apparent impunity and to use this knowledge to bestow similar abilities to other species of livestock.

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NONLETHAL RODENT REPELLENTS: DIFFERENCES IN CHEMICAL STRUCTURE AND EFFICACY FROM NONLETHAL BIRD REPELLENT

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Abstract—At least some anthranilates (e.g., methyl anthranilate), and acetophenones (e.g., orthoaminoacetophenone) are aversive to mice as well as to birds. Here we systematically examined nine acetophenone isomers (ortho, meta, para) and moieties (amino, hydroxy, methoxy) previously tested as drinking and feeding repellents for European starlings (*Sturnus vulgaris*). All nine substances reduced intake by mice in single-bottle tests. When molecular characteristics were examined, amino group reactivity and, to a lesser extent, isomeric position (i.e., resonance), were related to the strength of the avoidance response. Unlike effective avian repellents, the presence of intramolecular hydrogen bonds did not appear to affect avoidance responding.

Key Words—Acetophenone, anthranilate, chemosensory, mouse, *Mus musculus*, repellent.

INTRODUCTION

Few nonlethal chemicals are available for rodent control, although they would be desirable in situations where lethal control poses health or aesthetic risks. Those substances that are available are either indiscriminately offensive, such

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as capsaicin (Meehan, 1988), or unpredictable in performance, such as denatonium benzoate, which varies inter- and intraspecifically (Beauchamp and Mason, 1991).

A promising strategy for the development of new rodent repellents may be molecular modeling where chemical structure is related to biological activity. Using this approach, we recently tested 36 derivatives of benzoic acids as bird repellents (Mason et al., 1991; Clark and Shah, 1991; Clark et al., 1991). Acetophenones are structurally similar to anthranilates, and both groups contain effective avian repellents. Three molecular features contribute to repellency: (1) the basicity of a substituted phenyl ring, (2) the presence of an electron-donating group in resonance with an electron withdrawing group on a phenyl ring, and (3) a heterocyclic ring in the same pi cloud as the phenyl ring, with the ring comprised of an intramolecular hydrogen bond or covalently bonded heteroatoms (Clark and Shah, 1991).

At least one anthranilate (methyl anthranilate), and one acetophenone (orthoaminoacetophenone) are aversive to mice (Nolte et al., 1993) as well as to birds (Mason et al., 1991). Here, we examined the repellency of additional acetophenone isomers (ortho, meta, para) and moieties (amino, hydroxy, methoxy) to clarify the effects of isometric changes on repellency (viz., Clark and Shah, 1991). We studied amino, methoxy and hydroxy substitutions because these configurations allowed a systematic test for the effects of basicity, position (isomerization), and hydrogen bonding on repellency.

METHODS AND MATERIALS

Subjects. Two hundred sixteen experimentally naive 30- to 35-day-old male mice (*Mus musculus*) were individually caged (27 × 21 × 14 cm) under a 12:12 light–dark cycle (light onset at 0700 hr) at 23°C and given free access to 8604-00 Wayne Rodent Blox. Prior to testing, the animals were allowed free access to tap water presented in 10-ml syringes fitted with sipper tubes. These same tubes were used during the experimental procedures described below.

Chemicals. Orthoaminoacetophenone (CAS #551-93-9), metaaminoacetophenone (CAS #99-03-6), paraaminoacetophenone (CAS #99-92-3), orthomethoxyacetophenone (CAS #579-74-8), metamethoxyacetophenone (CAS #586-37-8), paramethoxyacetophenone (CAS #100-06-1), orthohydroxyacetophenone (CAS #118-93-4), metahydroxyacetophenone (CAS #121-71-1), and parahydroxyacetophenone (CAS #99-93-4) were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Each moiety had a phenyl ring with an electron-donating primary amino, hydroxy, or methoxy group and an electron-withdrawing carbonyl group. The strength of donation was ranked as: amino > methoxy > hydroxy. Isomers of each moiety differed only in their substitution patterns on the phenyl ring (Figure 1).

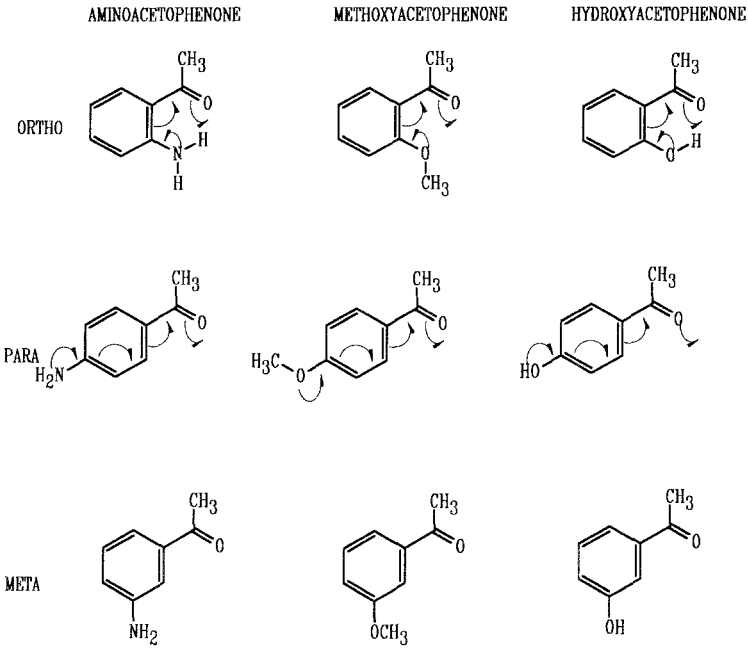

FIG. 1. Molecular structure of the isomers of amino-, methoxy- and hydroxyacetophenones. The arrows indicate donation paths of lone pairs of electrons.

Because acetophenones are generally insoluble in water, we mixed each compound in water under low heat to yield saturated emulsions with concentrations at 1.0% (w/w). Lower concentrations of 0.5% and 0.25% were prepared by serial dilution.

Procedure. Mice were randomly assigned to 27 treatment groups (N = 8/group), and adapted to an 18-hr (1500–0900 hr) water-deprivation schedule. Adaptation was followed immediately by four days of pretreatment. On each pretreatment day, tap water was presented at 0900 hr, and water intake between 0900 and 1200 hr was determined to the nearest 0.1 ml. Between 1200 and 1500 hr, animals were provided ad libitum access to tap water.

A four-day treatment period immediately followed pretreatment. Treatment procedures were identical to those described for pretreatment, except that each group was given a different chemical and concentration during the 3-hr measurement period.

Analysis. Prior to treatment, we tested whether water intake among the 27 groups was equal using a one-way repeated-measures analysis of variance (ANOVA). Equality of water intake among groups was a precondition for further

testing, allowing an unbiased estimate of concentration, chemical, and pre- vs. treatment differences in subsequent analyses (Games, 1979).

We tested for differences among experimental factors using a four-way repeated-measures ANOVA. Fluid intake was the dependent variable. Between-subjects factors were chemical (nine levels) and concentration–group (three levels). It is important to bear in mind that mice received water only during the pretreatment period and chemicals only during the treatment period, thus concentration effects were confounded with group effects. However, since the qualifying one-way analysis of variance described above indicated no pretreatment differences for intake among groups, any observed effects were reasoned to be due to chemical concentration effects. Within-subjects (i.e., repeated) factors were period (two levels, pre- vs. treatment period) and day (four levels).

Because four-factor analyses of an experiment are often difficult to interpret, we used Duncan's multiple-range tests to identify post-hoc differences among means for specific structured comparisons of interest (e.g., chemical effects during the treatment period) (von Eye, 1990).

RESULTS

Pretest Fluid Intake Criterion. There was no interaction between days and groups, indicating that all groups of mice had the same fluid intake pattern across days ($P = 0.301$). Furthermore, there were no day effects ($P = 0.352$). More importantly, the lack of a group effect ($P = 0.805$) indicated that assignment of groups to chemical and concentration treatment could proceed without biasing further analyses.

Overall Four-Factor Effects. All but three of the main and interaction effects of the four-way repeated measures ANOVA were significant (Table 1). Because there were no group effects during the pretest period (above), all period $\times$ day $\times$ within-subjects and period $\times$ within-subjects effects were assumed to be related to aversion of chemically treated water. This aversion is illustrated by inspection of the period $\times$ concentration $\times$ chemical-group profiles ($P < 0.001$). Furthermore, aversions were more pronounced at higher concentrations ($P < 0.001$).

Factors involving day effects were generally significant. Fluid intake during the treatment period tended to decrease as a function of time ($P < 0.001$; Table 1). While day interactions with chemical or concentration group were also significant, there was no systematic pattern for fluid intake that could be attributed to biological or chemical effects.

There were differences among chemicals ($P < 0.001$). Post-hoc range tests indicated that the aminoacetophenones, the most basic of the compounds considered, were also the most repellent (Figure 2). The remainder of chemicals

TABLE 1. *F* AND *P* VALUES FOR THE FOUR-WAY REPEATED MEASURES ANOVA

	df	MS	*F*	*P*
Between-subjects effects				
Constant	1	21371	5144.98	<0.001
Chemical	8	49.63	11.96	<0.001
Concentration group (CG)	2	139.12	33.99	<0.001
Chemical × CG	16	4.87	1.17	= 0.293
Error	188	4.15		
Period × within-subjects effects				
Period	1	2686.46	1809.31	<0.001
Chemical × period	8	50.13	33.76	<0.001
CG × period	2	134.48	90.57	<0.001
Chemical × CG × period	16	3.85	2.60	<0.001
Error	188	1.48		
Day × within-subjects effects				
Day	3	1.46	5.25	<0.001
Chemical × day	24	.78	2.80	<0.001
CG × day	6	.53	1.92	= 0.076
Chemical × CG × day	48	.43	1.54	= 0.013
Error	564	.28		
Period × day × within subjects effects				
Period × day	3	3.11	9.68	<0.001
Chemical × period × day	24	0.83	2.53	<0.001
CG × period × day	6	0.24	0.76	= 0.603
Chemical × CG × period × day	48	0.53	1.65	<0.005
Error	564	0.32		

yielded a graded aversion response. Ortho isomers of both methoxy and hydroxy (less basic) functions tended to be more repellent than the meta isomers. Para isomers were intermediately effective repellents with respect to ortho- and meta isomers of the acidic functions.

The four-way repeated measures ANOVA indicated a significant chemical × period × concentration × day effect ($P < 0.001$). Post-hoc tests were undertaken for all concentration groups for each chemical to elucidate the nature of the period and day effect.

Aminoacetophenones. Post-hoc tests indicated that fluid intake during the treatment period was reduced relative to the pretreatment period for all aminoacetophenone groups (Figure 3A–C). Fluid intake was similar across the four-day treatment period for all concentration groups of orthoaminoacetophenone (Figure 3A). Fluid intake decreased during the treatment period for all concentration groups of paraaminoacetophenone (Figure 3B) and all concentration groups of metaaminoacetophenone (Figure 3C). There was no indication that

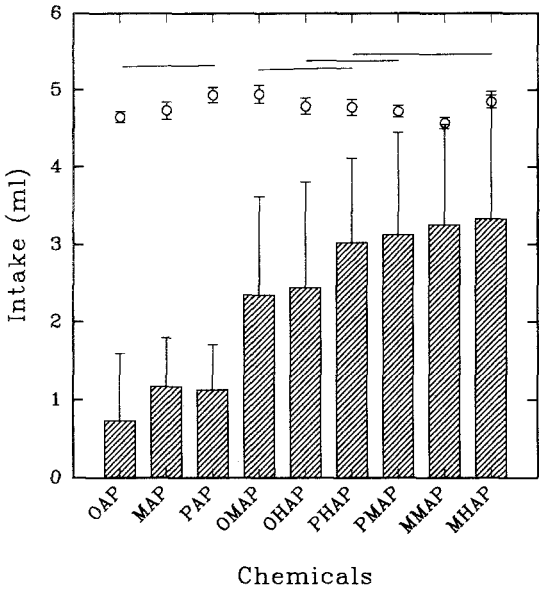

FIG. 2. The mean fluid intake by mice during the treatment period (hatched bars) for each of the test chemicals. Values were averaged over all concentration groups. The horizontal lines depict homogeneous subsets as determined by Duncan's multiple-range tests. The open circles depict the mean pretreatment water ingestion by mice given each of the chemicals. Vertical capped bars depict 1 SE.

habituation or sensitization towards any of these repellents occurred, even after exposure for four days.

Methoxyacetophenones. Fluid ingestion decreased during the treatment period relative to the pretreatment period for the 1.0% and 0.5% concentration groups of orthomethoxyacetophenone (Figure 3D). There was no evidence of habituation or sensitization towards the repellent after four days. The repellent effect was weak for the 0.25% concentration group (Figure 3D).

Fluid intake was similar across all pretreatment and treatment days for the lowest concentration-group (0.25%) of paramethoxyacetophenone (Figure 3E). There were clear reductions, however, during the treatment period for the two higher concentration groups (Figure 3E). Again, there was no evidence of habituation or sensitization during the four-day treatment period.

There was no repellent effect for metamethoxyacetophenone for the 0.25% concentration group (Figure 3F). Intake was less during the treatment period than during the pretreatment period for the 0.5% concentration group (Figure 3F). Ingestion across days within each of the periods was similar. A similar trend was seen for the 1.0% concentration group (Figure 3F). As before, there

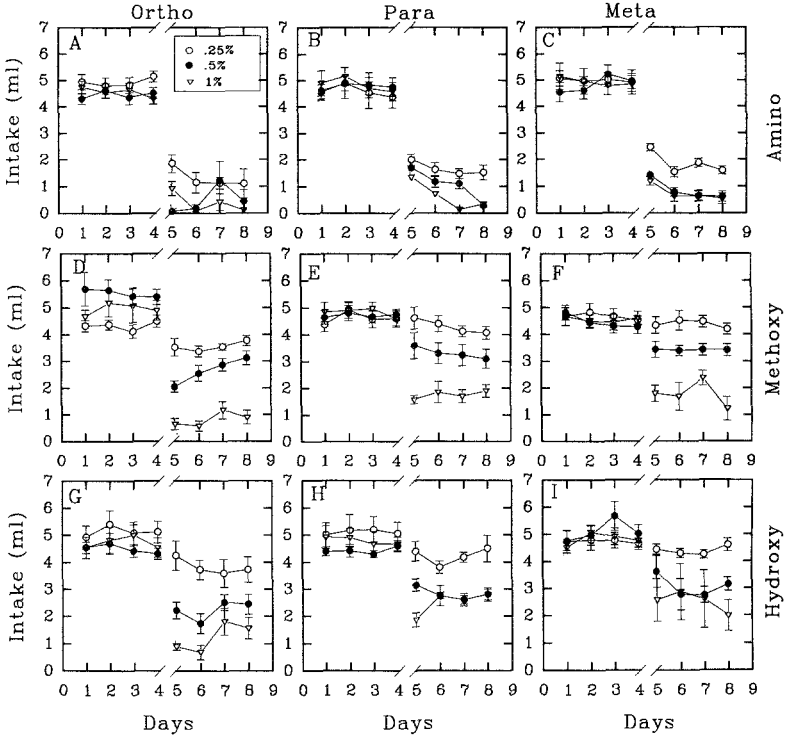

FIG. 3. Profiles of fluid ingestion by mice for the four-way interaction of chemical × day × period × concentration group. The pretreatment period consisted of days 1–4. The treatment period consisted of days 5–8. The break in the *x* axis represents the beginning of the treatment period. The vertical capped bars depict 1 SE.

was no evidence of an enhanced avoidance response across days as a function of time.

Hydroxyacetophenones. Fluid ingestion for orthohydroxyacetophenone decreased during the treatment period for the 1.0% and 0.5% concentration groups (Figure 3G). There was no evidence of habituation or sensitization towards these repellents after four days. Post-hoc tests showed that there was no significant difference in ingestion over time for the 0.25% concentration group.

Fluid ingestion for parahydroxyacetophenone decreased during the treatment period relative to the pretreatment period for the 1.0% and 0.5% concentration groups (Figure 3H). There was no indication of reduced repellency after four days of exposure to the repellent. At the lower concentration of 0.25%, there was no indication of a repellent effect (Figure 3H).

Fluid intake for metamethoxyacetophenone tended to be lower for the 1.0% and 0.5% concentration groups during the treatment period, although the level of ingestion on some treatment days was similar to pretreatment days (Figure 3I). There was no such ambiguity, however, for the 0.25% concentration group. At this concentration, there was no repellent effect.

DISCUSSION

In previous experiments with avian repellents (Clark and Shah, 1991; Clark et al., 1991; Mason et al., 1991), the strength of repellency decreased as a function of the positional isomer; ortho isomers were better repellents than para isomers, and these, in turn, were better than meta isomers, suggesting that electron donation by resonance was an important feature of repellency. Basicity (and/or electron-donating ability of the substituent group) was also important. Thus, amino substituents were both the most basic and the strongest repellent, whereas hydroxy substituents were the least basic and the weakest repellents. Finally, intramolecular hydrogen-bonding capacities were associated with repellency, although such bonding was not necessary for strong repellency to occur.

In this study we found that amino substituents were more repellent to mice than either methoxy or hydroxy substituents. Unlike with birds, however, we found no strong differences in repellency between methoxy and hydroxy substituents. For these less basic substituents, resonance appeared to contribute to repellency. The relative ranking of fluid intake by mice was consistent with this notion (i.e., ortho < para < meta). This is in contrast to birds, where the relative contribution of basicity to repellency was more important that resonance.

It is clear that mice differ from the previously tested bird species in their sensitivity towards acetophenones. A comparison of the concentration–response functions for birds and mammals suggests that birds have a lower threshold for aversion to the compounds tested (Clark and Shah, 1991). In mammals, except for the amino substituent, repellent effects disappeared at concentrations of 0.25%. Thus, it may be possible that for the nonamino substituents, mammals are responding to a concentration effect rather than structural, positional, or electronic features of molecules, which might manifest themselves at lower concentrations. This interpretation may be favored because, even though intake of methoxy and hydroxy substituents was a function of resonance, the degree of overlap among the chemicals suggests that the contribution of resonance to repellency may have been weak. Thus, we conclude that amino reactivity rather than basicity per se is the primary feature relating to repellency in mammals.

Management Implications. While we are cautious about extrapolating from the laboratory to the field, the present results have clear practical implications. For example, granular agricultural chemicals are a hazard to rodents and birds

that unwittingly ingest them. An additive with both rodent and bird repellent capabilities would be useful, particularly since no such repellent is available at present (Beauchamp and Mason, 1991). Cowbirds (*Molothrus ater*) reduce their intake of pellets containing pesticides when methyl anthranilate is present (Mason et al., 1993).

The repellents tested here might also have application as additives to packaging, plastics, and fabrics where any rodent damage is undesirable. Avoidance of all the chemicals in the present experiment was strong on the first day of treatment, at least for the higher ($\geq 0.5\%$) concentration. This suggests that at least some of these chemicals may be useful in inhibiting damage caused by sampling or explorative gnawing of rodents.

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PHEROMONAL BASIS OF AGGREGATION IN EUROPEAN EARWIG, *Forficula auricularia* L. (DERMAPTERA: FORFICULIDAE)

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Abstract—The aggregation behavior of the European earwig *Forficula auricularia* was investigated. Bioassays of frass extracts, cuticular washings, and the defensive exudate have been conducted to locate the source of an aggregation pheromone, and aggregation behavior has been demonstrated with extracts of frass and the washings of male cuticular lipids. Chemical investigations revealed the presence of a unique pattern of typical normal, monomethyl-, and dimethylalkanes, along with a series of fatty acids and the well-known defensive quinones from these insects. It has been concluded from the bioassays of a number of authentic compounds and the lack of a chemically discernible difference between male and female extracts that the aggregation pheromone of *F. auricularia* is quite probably a minor component of the male cuticular lipids.

Key Words—Aggregation pheromone, cuticular hydrocarbons, fatty acids, quinones, Dermaptera, Forficulidae, *Forficula auricularia*, European earwig, social behavior.

INTRODUCTION

The European earwig, *Forficula auricularia*, is a suburban nuisance pest in Virginia whose behavior is characterized by aggregations numbering from a

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dozen to several hundred insects. This behavior appears to be associated with contact between conspecific individuals and frass. Observations of *F. auricularia* invariably yield a clumped distribution of individuals (Lamb, 1975) and large amounts of frass in and around the refuge and in immediate contact with the insects.

The chemical defenses of *F. auricularia* are some of the earliest elucidated in insects, consisting of 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone (Schildknecht and Weis, 1960). These compounds are produced and stored in a pair of abdominal glands and are readily released when the animals are handled. They are well known as components of the defensive secretions of many other arthropods (Blum, 1981).

Forficula auricularia is dorsoventrally flattened, and has a smooth, slippery cuticle. In addition, tibial glands have been described (Brousse-Gaury, 1983), whose function is unknown.

In order to assess the aggregation behavior of *F. auricularia*, we have conducted detailed analyses and comparative bioassays of extracts from the frass, cuticle, and legs of this insect.

METHODS AND MATERIALS

Insects. Insects were collected in Blacksburg and Christiansburg, Virginia between May and October 1990, 1991, and 1992 using groove-board traps (Crumb et al., 1941) placed near areas of natural refuge or using a battery-operated car vacuum cleaner fitted with a Plexiglas nozzle. The insects were maintained in 1-liter Mason jars fitted with screen tops, supplied with food (bee pollen, cat chow, and vegetables) and water ad libitum and kept in a room illuminated by natural light. Temperature was maintained ca. 20–25°C.

Chemical Analyses. Frass and cuticular and leg washings were analyzed by gas chromatography and mass spectroscopy. GC analysis was conducted using a Shimadzu GC-9A gas chromatograph equipped with a 30-m × 0.5-mm-ID open DB-17 column with a 1.0-μm film thickness. The temperature was programmed from 60 to 215°C, set to run at 10°C/min, and the carrier gas flow rate was 15.0 ml/min. On any given day, retention temperatures were reproducible to 1°C. Mass spectra were obtained in the EI mode at 70 eV using either a LKB-2091 GC-MS equipped with a 30-m × 0.25-mm-ID 5% Db-5 column or a Finnigan model 4500 GC-MS equipped with a 30-m × 0.32-mm-ID column with a 5% DB-1 phase. Normal hydrocarbons, fatty acids, and quinones were identified by comparison of their mass spectra with published data (NIST/EPA/MSDC, 1990). The methyl alkanes were identified from their parent ions and diagnostic fragment ions (Lockey, 1988; Bagnères and Morgan, 1990).

Extracts. The defensive exudate from 7–10 individuals of each sex was obtained by pinching or pulling its forceps. The secretion was taken up in 0.5-μl microcapillaries, which were quickly crushed in 1.0 ml of CH_2Cl_2. GC-MS analysis indicated the presence of 2-methyl- and 2-ethyl-1,4-benzoquinone. GC analysis consistently showed that ethylquinone predominated in the defensive chemistry, with about 0.05 μg/μl per sample or 0.005 μg/μl per animal, and about 0.025 μg/μl methylquinone per sample or 0.0025 μg/μl per animal. There was no discernible sexual dimorphism in quantity of quinones produced.

Total cuticular extracts were obtained by immersing 10 insects in 1.0 ml of either CH_2Cl_2 or pentane. Three samples were prepared from each sex. A mixture of normal, monomethyl-, and dimethylalkanes was identified by GC-MS (Table 1). The hydrocarbons identified comprise >88% of the detectable volatiles. The many unidentified minor components (>30 at less than 1% each) that were detected were present in both male and female washings. Similar samples were used for bioassays.

Frass produced by a mixture of nymphs and adult males and females was collected from field traps, natural refuges, and laboratory colonies maintained with controlled diets. In each case, approximately 2.0 g of frass was extracted with 2.0–5.0 ml of solvent (distilled water, acetone, CH_2Cl_2, and CH_2Cl_2–distilled water together) and concentrated for bioassays. Frass from animals

TABLE 1. SATURATED CUTICULAR HYDROCARBONS OF *Forficula auricularia*

Peak	Percent	Compound	Diagnostic ions	Mol. mass
1	1.0	Tricosane		324
2	5.8	Pentacosane		352
3	1.3	13-MeC_{25}	196/197	366
7	5.6	Heptacosane		380
8	14.7	9-, 11-, 13-MeC_{27}	140/141, 280/281; 168/169, 252/253; 196/197, 224/225	394
13	0.5	Nonacosane		408
14	19.1	9-, 11-, 13-, 15-MeC_{29}	140/141, 308/309; 168/169, 280/281; 196/197, 252/253; 224/225	422
15	5.5	*x*, *y*-$DiMeC_{29}$		436
16	5.5	9, 21-$DiMeC_{29}$	140/141, 323	436
17	16.6	9-, 11-, 13-, 15-MeC_{31}	140/141, 336/337; 168/169, 308/309; 196/197, 280/281; 224/225, 252/253	450
18	5.5	*x*, *y*-$DiMeC_{31}$		464
19	5.5	9, 23-$DiMeC_{31}$	140/141, 351	464
20	1.2	11-MeC_{33}	168/169, 336/337	478
Total	87.8% of detectable components			

raised on exclusive diets of either carrots, cat chow, or bee pollen was extracted in pentane in a similar manner and analyzed by GC-MS. Insect frass equivalents (mean milligrams of frass per day per insect) were determined by sequestering five replicates of 10 males and 10 females, separated by sex, in individual weighed vials. After 24 hr, the insects were removed, and the vials reweighed. Mean frass produced per day per male was 1.238 mg (SD = 0.14 mg), and mean frass produced per day per female was 1.336 mg (SD = 0.38 mg); frass production did not differ significantly by sex. Analysis revealed the presence of a set of common fatty acids (Table 2) along with the same pattern of cuticular hydrocarbons shown in Table 1.

The legs of 25 nymphs were amputated at the femoral–trochanteral joint with a pair of forceps and were placed in 2.0 ml of CH_2Cl_2. A similar sample was also prepared from 50 adults that had been previously frozen. Both samples were subjected to GC-MS analysis and used for bioassays. Analysis of these extracts revealed the same fatty acids found in the frass except that one, with a mass spectrum very similar to that of linoleic acid, predominated in the mixture. The usual cuticular hydrocarbons (Table 1) were also detected , along with cholesterol. In addition, two minor (<5% each) components were detected having mass spectra (m/z = 348 (M+), 96, 95, 82, 81, 67 (100), 57, and 55; 376 (M+), 110, 96, 95, 82, 81, 67 (100), 57, and 55 indicating $C_{25:2}$ and $C_{27:2}$ alkadienes. These components eluted before the normal alkanes in this mixture.

Bioassays. Bioassays of attractancy of compounds were performed by allowing test insects a choice between control and experimental shelters. Experimental compounds (100–250 μl of natural extracts or 200–400 μl of synthetic compounds) were applied to 2.5- × 6.25-cm strips of Fisher Scientific chromatographic paper and allowed to dry. Control papers were identical except that only the solvent used in the extraction was applied to the paper. Papers were folded into a W shape (Ross and Tignor, 1986) and placed on end opposite each other in the bioassay arena to form artificial shelters. All tests were conducted

TABLE 2. FATTY ACID CONTENT IN *F. auricularia* FRASS

Peak	Percent	Acid	Mol. wt.	IE/day (mg)[a]
2	1.8	Myristic	228	0.008
4	17.9	Palmitic	256	0.083
5	1.0	Heptadecanoic	270	0.005
6	15.2	Linoleic	280	0.071
7	44.6	Oleic	282	0.207
8	18.8	Stearic	284	0.087

[a] IE = insect equivalent.

during daylight hours in a room illuminated by natural light and maintained ca. 20–25°C. Bioassays were performed in arenas of two different designs, one in which a large glass cylinder was inverted over a 645-cm^2 piece of glass, and the other in which three 11.4-cm-diameter plastic Petri dishes were cut and glued together to form three overlapping chambers with a section of the walls removed to allow movement from one chamber to another (Nagel and Cade, 1983). In the latter design, the control and experimental papers were placed in the far chambers, and the insects introduced into the central chamber. Test insects were removed from laboratory colonies without regard to sex, developmental stage, or body size approximately 1 hr before testing. Insects were then sequestered in groups of 20 in large glass vials. At the start of each experiment, vials were inverted over the central arena. Position of all insects were recorded 1 hr later as on or touching the control paper, on or touching the experimental paper, or neither. Arenas were thoroughly washed and dried after each test. No significant difference between insect response to the two bioassay designs was found.

Statistics. Attractivity of compounds was evaluated by the Student's t test (Sokal and Rohlf, 1981). Level of attractivity was determined by using the aggregation index (Roth and Cohen, 1973) in which a value of 0 represents random choice of papers, negative values suggest a tendency toward dispersal, and positive values a tendency toward attraction/aggregation.

RESULTS

Chemistry

Three classes of compounds have been detected in the extracts of the defensive exudate, frass, and cuticle of *Forficula auricularia*. The simple quinones, methyl- and ethylquinone, were identified in the defensive exudate as previously described by Schildknecht and Weis (1960). A series of fatty acids were identified in the extracts of the frass and in the extracts of the legs of nymphs and adults (Table 2). Finally, a series of normal and methyl-branched alkanes were identified in the cuticular washings of male and female *F. auricularia* (Table 1). This same pattern of compounds was also detected in the frass and leg extracts that were examined. In addition, cholesterol and small amounts ($<5\%$) of C_{27} and C_{29} alkadienes were detected in the washings from the legs.

Behavior

Natural Extracts. Behavioral bioassays of frass extracts showed that frass is clearly attractive to earwigs. Significant aggregative activity was obtained with all solvents (Table 3). Response to frass produced exclusively by nymphs, males, or females was not specifically evaluated; however, it appears that frass,

TABLE 3. AGGREGATION BIOASSAYS OF NATURAL EXTRACTS OF *F. auricularia*

Extract	Replicates	Response (*P*)	AI[a]
Frass			
Distilled H_2O	8	0.0210	0.3750
Acetone	8	0.0030	0.5670
CH_2Cl_2	8	0.0001	0.7320
Distilled H_2O + CH_2Cl_2	8	0.0001	0.8250
Cuticle washes			
Male	15	0.034	0.2020
Female	15	NS	−0.1340
Leg washes			
Nymphs	8	NS	0.0336
Adults	10	−0.004	−0.2351
Leg trails	8	NS	−0.1330

[a]AI = Aggregation index.

regardless of origin, aggregates nymphs and adults of both sexes equally. Extracts of female cuticle were not attractive, but male extracts were (Table 3). Bioassays of nymph leg extract were not significantly attractive. On the contrary, bioassays of adult leg extract were significantly repellent. These results suggested an assay of potential leg "trails," residual compounds that might be left on the substrate by the tibial glands. For each test, 20 insects were confined to 1-liter jars lined with Whatman filter paper for 24 hr, after which papers were removed and extracted in 1.0 ml CH_2Cl_2 except for areas obviously spotted with excreta; these areas were not extracted. However, these extracts were not significantly attractive and were, in fact, slightly, but not significantly, repellent (Table 3).

Synthetic Fatty Acids. Linoleic and oleic acids were not attractive at any concentration tested, and stearic and palmitic acids were significantly attractive only at higher concentrations, at about 50 insect equivalents/day. Oleic and linoleic acids, assayed alone and together, did yield slight repellency, although this was not significant in any case.

Synthetic Hydrocarbons. None of the hydrocarbons tested produced a significant response. Tricosane was weakly, but not significantly, repellent. Bioassays of pentacosane and heptacosane, applied singly and together, were not significant.

Synthetic Quinone. 2-Methyl-1,4-benzoquinone was significantly repellent at concentrations of 0.01 M and 0.10 M, except for 400 μl at 0.01 M. Repellency also increased with increasing dosage at the 0.10 M concentration.

DISCUSSION

Male and female *Forficula auricularia* produce a nearly identical mixture of cuticular hydrocarbons, which may serve as a taxonomic character (Grunshaw et al., 1990) in future research. It is noteworthy that mono- and dimethylalkanes predominate in this mixture and that the branching is at odd-numbered carbons and at position C-9 or higher. A review of the literature reveals that this methyl branching pattern is not unusual (Brown et al., 1990). On the other hand, *F. auricularia* are typically found in dark, humid areas, so that cuticular hydrocarbons that form efficient waterproofing layers would not be as essential as in other insects (Lockey, 1976; Howard and Blomquist, 1982). *F. auricularia* are notoriously difficult to grasp, and it may be that their cuticular hydrocarbons serve a defensive function, imparting the characteristic slipperiness to the insect. In addition, these hydrocarbons may enhance the dissolution of the quinones on the surface of the insect's cuticle, increasing the efficacy of the insect's defense, in a manner similar to the tenebrionid beetle *Argoporis alutacea*, which improves its defense by spreading a quinone exudate over its body and that of its attacker (Tschinkel, 1972). Since quinones also appear to act as an intraspecific repellent in *F. auricularia*, they may serve the dual roles of defensive secretion and alarm pheromone, as commonly observed in the membracids, aphids, and cimicids (Nault and Phelan, 1984).

Gregarious behavior may have several biological benefits including enhancement of juvenile growth and development (Fuchs et al., 1985), facilitation of mate selection (Antony et al., 1985), and predator defense (Hamilton, 1971). Its significance in *F. auricularia* is currently unknown, but may include some or all of these factors.

Sauphanor (1992) recently suggested that compounds contained within the tibial glands (Brousse-Gaury, 1983) of *F. auricularia* are responsible for its gregarious behavior on the basis that extracts of amputated legs and of filter paper "trails" caused aggregation. The results of the present study, however, showed that neither leg extracts nor leg trails aggregated *F. auricularia*—in fact, extracts of adult legs, shown to contain relatively large quantities of unsaturated fatty acids, were repellent, and indeed, oleic acid has been shown to be a generalized repellent (Howard et al., 1982). A probable reason for the discrepancy in the results of the test of leg trails was that in our study, we removed areas of paper spotted with excreta before extracting filter papers; since frass is attractive, papers with spots of excreta might bias the results of any test for aggregating ability of possible compounds deposited by the legs. Sauphanor (1992) tested responses of nymphs, whereas we evaluated adult response, but it seems unlikely that only nymphs would be sensitive to the pheromone given that gregarious behavior is so pronounced in earwigs both in the field and in

laboratory colonies, regardless of maturity. Also in contrast to our results, frass extracts were not attractive in the Sauphanor (1992) study. A factor potentially leading to this difference is freshness of the frass: we observed that frass must be fresh in order to attract.

This study presents evidence for an aggregation pheromone for *F. auricularia* that appears to be located on the male cuticle. The location of the male cuticle for pheromones is not unusual (Schaner and Jackson, 1992). Since earwigs are in close contact with each other and because they regularly consume carcasses and shed exuviae, it is not surprising that frass also promotes gregariousness. The same hydrocarbon pattern observed in the cuticle was also seen in the frass. Frass mediates aggregation in several insects, including *Blaberus discoidalis*, *Acheta domestica*, *Blattella germanica* (Ishii, 1970; McFarlane and Alli, 1985), and *Phylctinus callosus* (Barnes and Capatos, 1989). Thus, it is possible that the pheromone does originate in the frass. On the other hand, this would imply that the cuticular hydrocarbon mixture acquired activity by contact with the frass, and so the female cuticular washings should also show aggregation activity, which they did not. The hydrocarbons identified in *F. auricularia* do not seem to play a role in aggregation, since only the cuticular washings of males are attractive. The aggregation pheromone appears to be a minor component of the hydrocarbon profile, since male and female cuticular profiles are identical to within a few percent (and we have identified nearly 88% of the total hydrocarbons). Some other insects produce a very small quantity of pheromone compared to total volatiles emitted, such as *Ips pini*, whose synergistic aggregation compound lanierone constitutes only 0.2% of the amount of ipsdienol emitted (Teale et al., 1991). Fatty acids have been shown to aggregate other insects (McFarlane et al., 1983; Fuchs et al., 1985), but do not appear to be responsible for gregarious behavior in earwigs since attractive concentrations were greater than those found in frass and, therefore, probably indicate attraction to a food source rather than presence of a pheromone.

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SEMIOCHEMICALS FROM ANAL EXUDATE OF LARVAE OF TSETSE FLIES *Glossina morsitans morsitans* WESTWOOD AND *G. morsitans centralis* MACHADO ATTRACT GRAVID FEMALES

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Abstract—Tsetse flies (Diptera: Glossinidae: *Glossina*) mature their offspring in utero, giving birth to mature larvae that burrow into soil and pupariate. During the hot dry seasons, puparia of some species of tsetse are aggregated in areas of deep shade in dense thickets. We have confirmed the presence of a semiochemical from the prepupariation excretions of larvae of *Glossina morsitans morsitans* Westwood and report a similar semiochemical in *Glossina morsitans centralis* Machado. These semiochemicals are attractive to gravid females and result in the aggregation of puparia. Behavioral studies with *G. m. centralis* showed that a higher percentage of females larviposited over moist sand conditioned by the anal exudate of larvae. Electroantennogram analyses of extracts of sand conditioned by *G. m. centralis* and *G. m. morsitans* confirmed the presence of olfactory receptors on the antennae for the semiochemicals. Both subspecies responded to extracts of the semiochemicals of the other, with *G. m. morsitans* more responsive to lower concentrations of extract of *G. m. centralis* than the converse.

Key Words—*Glossina morsitans morsitans*, *Glossina morsitans centralis*, tsetse flies, Diptera, Glossinidae, semiochemicals, larviposition, breeding sites.

INTRODUCTION

Tsetse flies (Diptera: Glossinidae: *Glossina*) infest about 40% of tropical Africa and transmit protozoans, *Trypanosoma* spp., which cause human sleeping sick-

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ness and trypanosomiasis (nagana) in animals (Rodgers and Randolph, 1985). Tsetse and the ectoparasitic Diptera pupipara are the sole insect groups with adenotrophic viviparity (Tobe and Langley, 1978). Larvae mature singly, in utero, and are provided a rich source of proteins and lipids produced by modified accessory glands (Pimley, 1983). At birth (parturition), the fully mature larvae burrow into the substrate and pupariate within 2 hr (Rowcliffe and Finlayson, 1981; Zdarek and Denlinger, 1991). Just before pupariation, larvae release an anal exudate that coats the larval cuticle (Rowcliffe and Finlayson, 1981; Zdarek and Denlinger, 1991; Kinghorn, 1912; Lamborn, 1915; Fiske, 1920; Nash et al., 1976). Pupation occurs within the puparium, and adults emerge in about a month (Azevedo and Pinhao, 1964).

Gravid females play the primary role in selecting the sites where parturition occurs (Rowcliffe and Finlayson, 1981). Aggregations of puparia often occur in specific locations during the dry season, in contrast to the more generalized and widespread distribution during the wet season (Rowcliffe and Finlayson, 1981; Nash, 1933, 1937; Buxton, 1955; Adkinson, 1971). Nash (1969) speculated that a pheromone from larvae that attracted gravid females might be one factor contributing to the aggregation of puparia. Nash et al. (1976) showed that a higher percentage of gravid *G. morsitans morsitans* Westwood larviposited in the presence of extracts of the anal exudate of larvae. From these findings they proposed that this exudate contains a pheromone attractive to gravid females. Rowcliffe and Finlayson (1981), however, repeated the work of Nash et al. (1976) and found no evidence for a pheromone. We reexamined the question of a putative semiochemical from larvae of *G. morsitans centralis* Machado and *G. m. morsitans*.

METHODS AND MATERIALS

Rearing. G. m. centralis and *G. m. morsitans* were reared in the International Centre for Insect Physiology and Ecology (ICIPE) rearing facility at 25°C, 50% relative humidity and a 12:12 hr light–dark photoregime. Females were separated at eclosion and mated on day 3. Flies were blood-fed on rabbits three times a week. We used flies in the first cycle of parturition.

Conditioning of Sand. Sand, after being extracted with solvents and heat-sterilized at 100°C for 24 hr, was placed in trays under cages containing gravid *G. m. centralis* or *G. m. morsitans*. Flies were blood-fed in the morning and placed over the trays in midafternoon to reduce possible contamination from feces, since flies normally defecate during or shortly after blood-feeding. The female sex pheromone, from cuticular hydrocarbons, was considered not to be a potential source of contamination, since it does not have an olfactory component (Langley et al., 1987); Wall, 1989). At parturition, larvae burrowed

through the mesh bottoms of the cages, dropped into the sand, and pupariated. Puparia were removed by sifting. Sand, freshly conditioned by anal exudate of larvae of *G. m. centralis*, was used for larviposition choice tests. Alternatively, the exudate was extracted from sand conditioned by both subspecies for electroantennogram (EAG) analyses.

Larviposition Choice Tests. Eighteen-day-old *G. m. centralis* were moved from the rearing facility to a room maintained at 25°C, 50 ± 5% relative humidity, and a 12 : 12 hr light–dark photoregime. The fluorescent lights were shaded with black cloth to approximate the low light levels preferred by gravid females (Abdel Karim and Brady, 1984).

Gravid females were exposed to a choice of 25 cm^3 of sterile sand or an equivalent amount of sand conditioned by an average of 1.8 pupariations of *G. m. centralis*. Sand was placed in heat-sterilized 7-cm glass Petri dishes. Ten gravid females were placed in clean, ovoid, polyvinyl chloride (PVC) cages 19.5 × 7 × 6 cm with mesh tops and bottoms. The cages were supported on two Petri dishes, one containing conditioned and the second, sterilized sand. Flies could not contact the sand, and each dish accounted for about 30% of the basal area of each end of the cage. A shallow pan, in which the Petri dishes and cage were placed, had sterilized sand in the bottom to collect puparia from larvipositions that occurred in the center of the cage between the Petri dishes. Larvae deposited on the mesh above Petri dishes fell into and pupariated in the dishes.

Tests were run for 96 hr. Most larviposition occurred in late afternoon as noted in other studies (e.g., Rowcliffe and Finlayson, 1981). Puparia were removed each morning, and pans were rotated 180°. To reduce effects of additional conditioning, sand surrounding the puparia was also removed and replaced by an equivalent amount of sterilized or conditioned sand.

Two choice tests were conducted, one comparing responses to dry conditioned vs. dry unconditioned sand, and the second comparing moist conditioned vs. moist unconditioned sand. In the test using moistened sand, 10 ml of distilled water was added to each Petri dish on day 1, and 8 ml on days 2 and 3.

Each test consisted of 200 naive gravid females in their first cycle of parturition. There were four replicates of five cages each containing 10 gravid females. Data were analyzed with Student's *t* test.

Electroantennogram Experiments. The active components from ca. 500 cm^3 of sand conditioned by 90 pupariations of either *G. m. centralis* or *G. m. morsitans* were extracted with *n*-hexane (AR grade). The extract was evaporated to 1 ml (neat) from which serial dilutions were made. Electroantennogram (EAG) responses were recorded according to the technique and instrumentation described elsewhere (den Otter and Saini, 1985; Saini and Hassanali, 1993). Antennae were amputated from gravid *G. m. morsitans* and *G. m. centralis* one to two days before parturition.

Ten microliters of each test compound was applied on filter paper in a glass

syringe that served as an odor cartridge. A standard stimulus of 10 μl of *n*-hexane (AR grade) was applied 60 sec before and after each stimulation with the test compound to correct for gradual diminishing of the EAG response during an experiment and to allow for comparisons among different preparations. The amplitude of the response to the test compound was then expressed as a percentage of the mean response to the reference presented before and after stimulation with the test compound.

EAGs were recorded from at least eight gravid females for each stimulus. Each preparation was exposed to its larval semiochemical and to that of the other subspecies.

RESULTS

Choice Tests. Gravid *G. m. centralis* presented moistened conditioned vs. moistened sterilized sand deposited 68% of 101 larvae over the conditioned sand ($P < 0.001$). Flies provided dry conditioned vs. dry unconditioned sand showed no preference, with 40% of the 40 larvae deposited over conditioned sand ($P > 0.05$).

Electroantennogram Studies. We expanded our study to include *G. m. morsitans* in EAG analyses. In our preliminary studies, *G. m. morsitans* and *G. m. centralis* showed positive EAG responses to consubspecific distilled water and *n*-hexane extracts of conditioned sand, larvae covered with anal exudate, newly formed puparia, and sand pellets surrounding puparia cemented by the anal exudate of larvae. We saw no EAG responses to distilled water, aqueous or hexane extracts of unconditioned sand, washes of larvae that had not released anal exudate, or sand that had been conditioned two weeks before being extracted for EAGs.

To compare responses of antennal preparations of *G. m. centralis* and *G. m. morsitans*, we used *n*-hexane extracts of conditioned sand. EAG responses increased with increasing extract concentrations (Figure 1). Preparations of both subspecies showed equivalent EAG responses to neat extract, but *G. m. morsitans* was more responsive to lower concentrations. Antennal preparations of both subspecies responded extracts of sand conditioned by the other subspecies. Antennal preparations of *G. m. morsitans*, however, were more responsive to extracts of *G. m. centralis* than the converse.

DISCUSSION

Tsetse flies are of interest because of their unique reproductive biology and their role in transmission of trypanosomiasis. One aspect of their biology, the seasonal differences in selection of breeding sites, has been the source of spec-

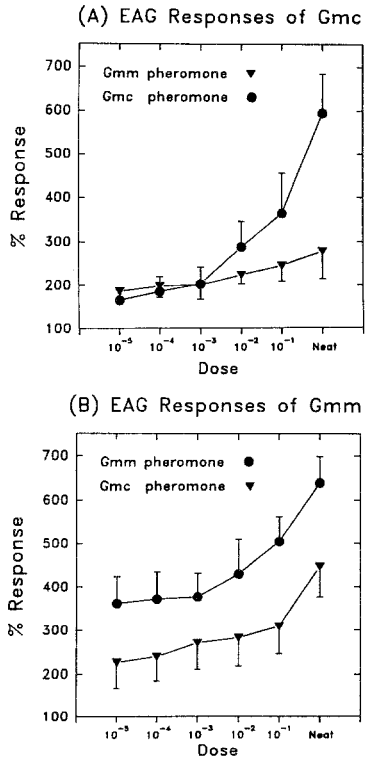

FIG. 1. Electroantennogram (EAG) responses of antennal preparations of *G. morsitans centralis* (Gmc) (A) and *G. morsitans morsitans* (Gmm) (B) exposed to hexane extracts of semiochemicals from conditioned sand. Both subspecies show equally strong responses to their own extracts at the highest concentration (neat). At lower concentrations, the responses of *G. m. morsitans* to its own and to *G. m. centralis* extracts were higher. Responses are averages from at least eight antennal preparations and expressed as a percentage response of a reference signal (hexane). Bars indicate one standard error of the mean.

ulation. Although the report of Nash et al. (1976) of a pheromone in the larval exudate in *G. m. morsitans* was questioned by Rowcliffe and Finlayson (1981), we confirm that gravid *G. m. centralis* respond to semiochemicals from larval exudate. Our choice tests showing 68% of larvipositions over moistened conditioned sand closely approximate data of Nash et al. (1976). Those authors found 64% of larvipositions of *G. m. morsitans* occurred in pots baited with water-soluble extracts of excretions from two larvae and 65% of larvipositions in pots baited with ether-soluble extracts of excretions from 2.5 larvae.

The lack of difference in number of larvipositions of gravid *G. m. centralis*

over dry conditioned sand vs. dry sterile sand and the marked preference shown when females had a choice of wet conditioned vs. wet sterile sand is of interest. This suggests that gravid females are most responsive to the semiochemicals when the differential between soil moisture and ambient humidity is large. Such conditions would occur during the dry season when ambient humidity levels are lower. Under such conditions, moist sites with semiochemicals would be attractive to gravid females seeking a locality in which to larviposit and could explain the aggregation of puparia reported in the literature. This apparent relationship with soil moisture might also clarify the disparity in results of previous studies. No difference in attraction was found in tests conducted at high relative humidity (80 $\pm$ 5%) (Rowcliffe and Finlayson, 1981). This contrasts with positive and equivalent responses to extracts of anal exudates of larvae in tests conducted at relative humidities of 60 $\pm$ 5% (Nash et al., 1976) and 50 $\pm$ 5% (this study).

Viviparity results in an extremely low reproductive capacity in tsetse flies and a large maternal investment in maturing larvae singly, in utero (Rogers and Randolph, 1985; Tobe and Langley, 1978). The first larva is matured and deposited in about 20 days with subsequent larvipositions at 9- to 10-day intervals thereafter (Tobe and Langley, 1978). Twenty offspring are recorded from a laboratory-reared *G. morsitans morsitans* (Azevedo and Pinhao, 1964), but in nature it is likely that fewer progeny are produced (Rowcliffe and Finlayson, 1981). Use of a semiochemical to identify sites where conditions of moisture are optimal would result in a higher survival rate of the few offspring produced by each female.

Availability of sufficient levels of moisture have been shown to be important to survival of tsetse. Population levels of tsetse are lower and sites suitable for larviposition are limited in the hot dry season (Nash, 1937). Other than short periods spent foraging for blood meals, flies remain inactive in resting sites, such as dense thickets, to avoid lethal temperatures (Brady and Crump, 1978). In these habitats, moisture is important, particularly for offspring. Females of *G. morsitans orientalis* Vanderplank select areas of high humidity to larviposit, and larvae prefer moist substrate for pupariation (Finlayson, 1967). Arid conditions affect pupal survival and adult eclosion in *G. m. morsitans* (Azevedo and Pinhao, 1964; Willemse, 1991), although excess moisture deters pupariation (Zdarek and Denlinger, 1991).

The antennal responses of both subspecies to the semiochemical(s) of the other (Figure 1) attest to the close affinity of the two subspecies, as shown by Curtis (1972) in crossing experiments with *G. m. morsitans* and *G. m. centralis*.

The possible occurrence of such semiochemicals in other species of tsetse and the identification of the compounds comprising the anal exudates of *G. m. morsitans* and *G. m. centralis* await further research. The semiochemicals of tsetse larvae may be analogous to pheromones on eggs of some species of mosquitoes and sandflies that aid gravid females in locating suitable breeding

sites (Pile et al., 1991; Elnaiem and Ward, 1991). Whether the larval semiochemicals in tsetse function solely as a cue to aggregate gravid females or as a stimulus for larviposition remains in question.

Trypanosomiases are among Africa's most devastating diseases, and efforts to control tsetse have been conducted for over 70 years (Dransfield et al., 1991). Current control techniques incorporate visual and host odors as cues to attract flies to traps and targets (Dransfield et al., 1990). It requires at least nine months, however, to reduce tsetse populations to tolerable levels (Dransfield et al., 1990; Vale et al., 1988; Willemse, 1991), and visual or odor cues now used do not attract gravid females (Randolph and Rodgers, 1981; Randolph et al. 1991). The semiochemicals from larval exudate might prove useful as a means of increasing the efficiency of traps and targets in management programs for tsetse and trypanosomiases.

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EFFECTS OF α-TERTHIENYL ON THE MIDGUT DETOXIFICATION ENZYMES OF THE EUROPEAN CORN BORER, *Ostrinia nubilalis*

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Abstract—The biochemical basis for the tolerance of the European corn borer, *Ostrinia nubilalis*, to the phototoxin α-terthienyl was investigated by measuring the midgut polysubstrate monooxygenases and glutathione *S*-transferase activities. α-Terthienyl administered in the diet to the corn borers increased the level of cytochrome b_5, NADH-cytochrome *c* reductase, *O*-demethylase, and glutathione *S*-transferase activities. The induced detoxification enzyme activities should enable the corn borer to metabolize α-terthienyl more efficiently and therefore render the corn borer highly tolerant to α-terthienyl.

Key Words—*Ostrinia nubilalis*, European corn borer, Pyralideae, Lepidoptera, cytochrome P-450, cytochrome b_5, cytochrome *c* reductase, glutathione *S*-transferase, α-terthienyl, phototoxin.

INTRODUCTION

α-Terthienyl (α-T) is a natural phototoxin occurring in plants of the Asteraceae family (Arnason et al., 1981). It is highly toxic to the larvae of mosquitoes, black flies, and several species of Lepidoptera and has shown potential for use in insect control (Hasspieler et al., 1991; Philogène et al., 1985; Iyengar et al.,

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1987). The toxicity of α-T increases in the presence of near-UV light, but it is also toxic under nonphotosensitizing conditions (Arnason et al., 1981). A comparative study showed that the toxicity of α-T varied considerably among the larvae of three species of Lepidoptera, the European corn borer, *Ostrinia nubilalis*; the tobacco budworm, *Heliothis virescens*; and the tobacco hornworm, *Manduca sexta* (Iyengar et al., 1987). *O. nubilalis* larvae are nearly 70 times more tolerant to α-T compared to *M. sexta*, and the tolerance was related to the fast rate of excretion of ingested α-T in the feces of *O. nubilalis* larvae (Iyengar et al., 1987). In vitro metabolism of α-T by the midgut microsomal fraction of *O. nubilalis* was 30 times faster as compared to *M. sexta* (Iyengar et al., 1990). In vivo topical application of piperonyl butoxide to corn borer larvae that had ingested α-T led to a decrease in metabolites in the feces (Iyengar et al., 1990), suggesting that polysubstrate monooxygenases (PSMOs) are involved in the metabolism of α-T in *O. nubilalis*.

The detoxification processes of xenobiotics usually occur in two stages. Initially, xenobiotics are metabolized to less active primary products. These products can be excreted directly or can be further metabolized through conjugation to increase their solubility and enhance their exportability (Berenbaum, 1991). The PSMOs are typical primary detoxification enzymes, and secondary detoxification enzymes are exemplified by glutathione *S*-transferase, which conjugate the reduced glutathione with a wide range of lipophilic toxicants bearing electrophilic sites (Habig et al., 1974). α-Terthienyl likely undergoes PSMO-mediated epoxidation at one of the double bonds on the outer thiophene rings, and this epoxide is a suitable substrate for conjugation to reduced glutathione by glutathione *S*-transferase (Figure 1) (J. Atkinson, personal communication). The involvement of glutathione *S*-transferases in the metabolic process of α-T was also proposed in *Culex tarsalis* larvae by Hasspieler et al. (1991).

The following study was conducted to investigate whether α-T can induce the activities of PSMO and glutathione *S*-transferase, which are probably involved in the metabolism of α-T in *O. nubilalis*.

METHODS AND MATERIALS

Experimental Insects. A laboratory colony of *Ostrinia nubilalis*, which originated from animals obtained from both M. Hudon (Agriculture Canada, St. Jean, Québec) and G. McLeod (Agriculture Canada, London, Ontario), was maintained in the laboratory under a 16:8 light–dark photoperiod at 25°C, 85% relative humidity, and reared on a meridic diet according to the method of Guthrie et al. (1985), modified by addition of corn cob grits. Nine milligrams of α-T were dissolved into 0.5 ml of acetone and mixed into 300 g of freshly prepared warm (45°C) meridic diet mixture. Control meridic diet contained only

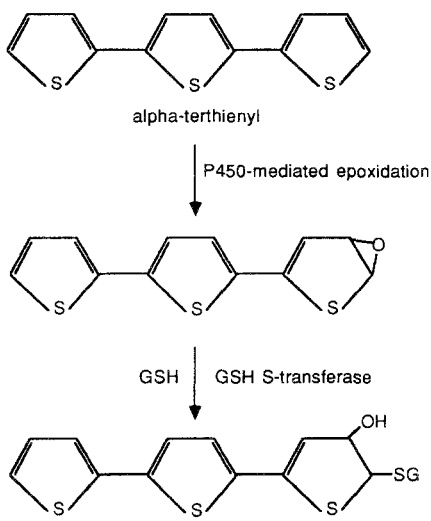

FIG. 1. Possible metabolism of α-terthienyl by P-450 and glutathione (GSH) *S*-transferase in *Ostrinia nubilalis*.

the same amount of the solvent. The final concentration of α-T in the diet was 30 μg/g diet, a level that in an earlier experiment did not cause significant reduction in the growth of the corn borers (Iyengar et al., 1987). Early fifth-instar borers were transferred onto the fresh control diet or diet containing α-T and fed for 48 hr before dissection. Since α-T is a phototoxin, during the α-T diet preparation, lights were dimmed and the bottle containing the diet with α-T and the corn borers was covered with aluminum foil so the European corn borers could freely consume the α-T diet without being adversely affected by the phototoxic effects of α-T.

Enzyme Preparation and Activity Assays. Corn borer larvae were dissected under 0.15 M NaCl solution and the intact midgut was removed and cleared of its contents by splitting it longitudinally. Eighty midguts were homogenized in 2 ml of 0.1 M sodium phosphate buffer, pH 7.5, with 10 passes of a motor driven glass–Teflon homogenizer. The homogenate was diluted with the same buffer to a total volume of 18 ml and centrifuged at 10,000*g*, 4°C for 15 min. The resulting supernatant was recentrifuged at 100,000*g*, 4°C for 1 hr in a Sorvall RC 28S centrifuge. The supernatant was used to determine glutathione *S*-transferase activity. The microsomal pellet was resuspended in 4 ml of 0.1 M sodium phosphate buffer, pH 7.5 (about 1 mg protein/ml). Two milliliters of the microsomal suspension was used for cytochrome P-450 measurement and the remaining 2 ml of microsomal suspension was diluted with 2 ml of the same buffer and used for PSMO assays and protein determination.

Cytochrome P-450 and cytochrome b_5 were determined by the method of Omura and Sato (1964) using a Cary 2000 double-beam spectrophotometer. NADH–cytochrome *c* and NADPH–cytochrome *c* reductases, NADH and NADPH oxidases, *N*- and *O*-demethylases, and glutathione *S*-transferase were assayed by the procedures reported previously (Feng et al., 1992). Protein concentrations were determined by the method of Lowry et al., (1951) using bovine serum albumin (Fraction V, Sigma Chemical, St. Louis, Missouri) as standard.

Statistical Analysis. Each enzyme activity was measured two or three times from each preparation (80 midguts/preparation), and data collected from three independent preparations were subjected to Student's *t* test.

Chemicals. α-Terthienyl (>99%) was synthesized by a Grignard Wurz procedure (Philogène et al., 1985) and provided by P. Morand, University of Ottawa. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, and NADH were purchased from Boehringer Chemical Company (Larval, Québec, Canada). Cytochrome *c*, NADPH, and *p*-chloro-*N*-methylaniline were from Sigma. *p*-Nitroanisole, 3,4-dichloronitrobenzene, and *p*-dimethylamino-benzaldehyde were from Aldrich Chemical Company (Milwaukee, Wisconsin). Other chemicals and solvents were of analytical grade and purchased from commercial suppliers.

RESULTS AND DISCUSSION

The results (Table 1) demonstrate that the cytochrome P-450 content of the European corn borer was not affected by dietary α-T, which is the same as the finding by Iyengar et al. (1990) on the same species treated with α-T at the same rate. Cytochrome P-450 has multiple isozymes and regulation of cytochrome P-450 is very complex, depending upon the particular form of P-450 isozyme. Each isozyme has specific and overlapping substrate specificities (Ronis and Hodgson, 1989). Induction of cytochrome P-450-based activities is not always accompanied by increased levels of total cytochrome P-450 (Ronis and Hodgson, 1989). Cohen et al. (1989) did not find an increase in total P-450 accompanying a fourfold increase in P-450-mediated xanthotoxin-metabolizing activity in the black swallowtail, *Papilio polyxenes*, treated with dietary xanthotoxin. The increased *O*-demethylase activity and decreased *N*-demethylase activity in the corn borer (Table 1) indicate that α-T probably selectively induced one or more P-450 isozymes and decreased one or more simultaneously. Therefore, the overall amount of cytochrome P-450 was not changed in the α-T-treated corn borers compared to that of control. That P-450 inducers can selectively induce one isozyme, inhibit another, and have no effect on the total P-450 level has been reported by other researchers (Rose and Terriere, 1980; Yu, 1984). The absorbance maximum of cytochrome P-450 in the α-T-fed corn

TABLE 1. LEVELS OF MIDGUT POLYSUBSTRATE MONOOXYGENASE AND GLUTATHIONE *S*-TRANSFERASE ACTIVITIES OF FIFTH INSTAR EUROPEAN CORN BORER LARVAE FED ON CONTROL DIET OR DIET WITH 30 μg/g α-TERTHIENYL[a]

	Measurements		
	No α-T	30 μg α-T	% control
nmol/mg protein			
Cytochrome P-450	0.15 ± 0.02	0.15 ± 0.01	100
Cytochrome b_5	0.17 ± 0.01	0.25 ± 0.02[b]	147
nmol/min/mg protein			
NADPH–cytochrome *c* reductase	76.37 ± 4.78	72.61 ± 6.98	95
NADH-cytochrome *c* reductase	427.09 ± 14.66	717.07 ± 69.75[b]	168
NADPH oxidase	6.84 ± 0.30	3.51 ± 0.17[b]	51
NADH oxidase	2.89 ± 0.32	3.60 ± 0.50	125
N-demethylase	7.40 ± 0.41	5.84 ± 0.34[b]	79
O-demethylase	3.23 ± 0.12	3.71 ± 0.07[b]	115
Glutathione *S*-transferase	19.76 ± 0.89	24.71 ± 1.03[b]	126

[a] Measurements are mean ±SE from three preparations, each (80 midguts/preparation) with a minimum of two duplicate determinations

[b] Significantly different from control at $P < 0.05$ determined by Student's *t* test.

borers was at 453 nm, which is different from that of control corn borers with a P-450 absorbance maximum at 449 nm (Figure 2A). This again indicates changes of cytochrome P-450 isozyme composition in the α-T-fed corn borers compared to control corn borers.

Cytochrome b_5 and NADH–cytochrome *c* reductase form the second electron transport chain of the PSMO system (Ronis and Hodgson, 1989). The levels of cytochrome b_5 and NADH–cytochrome *c* reductase activity were significantly increased by dietary α-T compared to those of control corn borers (Table 1). NADH oxidase and NADPH–cytochrome *c* reductase activities were unaffected by dietary α-T. NADPH oxidase activity was significantly decreased by α-T treatment. The increased levels of second electron transport chain enzymes indicate that they might be actively participating in the metabolism of α-T. The absorbance maximum of cytochrome b_5 was at 429 nm (Figure 2B), which is different from that of the corn borers fed on control diet (Figure 2B), indicating that different isozymes of cytochrome b_5 might be induced in the α-T-treated corn borers. Cytochrome b_5 does not directly combine with substrates, and the function of different possible isozymes is not clear. Perhaps different isozymes have different efficiencies in transducing electrons to the terminal oxidase, cytochrome P-450.

Glutathione *S*-transferase activity was significantly increased in the α-T-

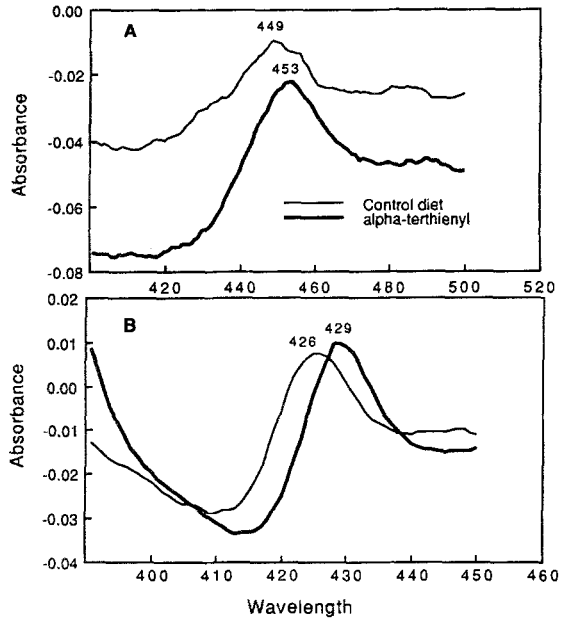

FIG. 2. Difference spectra of CO dithionite-reduced cytochrome P-450 (A) and cytochrome b_5 (B) from the midgut of fifth-instar European corn borer larvae fed on control diet or on diet with 30 μg/g α-terthienyl.

treated borers compared to that of untreated controls (Table 1), suggesting that α-T is an inducer for this phase II detoxification enzyme. These data also suggest that glutathione *S*-transferase, as well as the PSMO system, is involved in the metabolism of α-T in the corn borer (Iyengar et al., 1990). The involvement of glutathione *S*-transferase in the metabolism of α-T was proposed by Hasspieler et al. (1991) based on the fact that α-T is extremely lipophilic and a significant amount of water-soluble metabolites were excreted by Malpighian tubules in *C. tarsalis* larvae. Moreover, many plant allelochemicals induce enzyme activities responsible for their own detoxification (Wadleigh and Yu, 1987; Nitao, 1988; Cohen et al., 1989). Xanthotoxin, a linear furanocoumarin phototoxin present in some plants of the Apiacieae and Rutaceae families, was metabolized in *P. polyxenes*, by PSMOs (Bull et al., 1986), which are inducible by xanthotoxin itself (Cohen et al., 1989). The corn borer larvae are nearly 70 times more tolerant to α-T than *M. sexta*, but only 30 times faster in metabolizing α-T by microsomal fraction compared to *M. sexta* (Iyengar et al., 1990). The increased glutathione *S*-transferase activity demonstrated in this study may explain, in part, the difference.

α-Terthienyl is a phototoxin whose toxicity is attributable primarily to the production of singlet oxygen, which is deleterious to a variety of biomolecules including lipids, proteins, and nucleic acids (Spike and Straight, 1987). The antioxidant enzymes, superoxide dismutase, catalase, and glutathione reductase, usually provide an important line of defense against reactive species of oxygen in living organisms (Halliwell and Gutteridge, 1989). However, a recent study showed that these antioxidant enzyme activities were generally lower in *O. nubilalis* than in *M. sexta* (Aucoin et al., 1991). These findings indirectly support the notion that both PSMO and glutathione *S*-transferase may participate in the detoxification of α-T to bring about the high tolerance in the corn borer towards α-T toxicity. α-Terthienyl, with three aromatic rings (Figure 1), is probably not amenable to direct attack by glutathione *S*-transferase. However, after epoxidation by the PSMO system and presumably subsequent epoxide ring opening, α-T is a very likely candidate substrate for glutathione conjugation enzymes.

These studies have shown that detoxification enzyme activities are induced by α-T in the corn borer, and these inductions may be contributory to the corn borer's high tolerance to this phototoxin. Studies on the effects of α-T on the detoxification enzymes of α-T-sensitive lepidopterans, coupled with definitive α-T metabolism studies in these insects, would provide a clearer picture of the differences in response to α-T toxicity in different species of Lepidoptera.

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CHEMICAL AND ULTRASTRUCTURAL ANALYSIS OF CORN CUTICULAR LIPIDS AND THEIR EFFECT ON FEEDING BY FALL ARMYWORM LARVAE

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Abstract—The cuticular lipid composition of lower and upper leaves of five genotypes of field-grown corn, *Zea mays* L., was determined by combined gas chromatography–mass spectrometry. Surface lipids of the upper leaves had a higher proportion of *n*-alkanes (45–52%) than the lower leaves, while the lower leaves had higher percentages of fatty alcohols (12–18%) than the upper leaves. Scanning electron microscopy showed that the upper leaves of two corn genotypes, MpSWCB-4 and Cacahuacintle X's, had a smooth amorphous appearance, while the lower leaves had a dense array of wax crystals. *Spodoptera frugiperda* (J.E. Smith) larvae weighed more and developed more rapidly when they were reared on diet containing corn foliage from which the cuticular lipids had been removed than when they were fed untreated foliage. However, growth was not inhibited when larvae were fed diet containing the cuticular lipid extracts or individual cuticular lipid components.

Key Words—*Spodoptera frugiperda*, Lepidoptera, Noctuidae, *Zea mays*, scanning electron microscopy, cuticular lipids, host-plant resistance.

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INTRODUCTION

Plant resistance to insect pests has played an important role in integrated pest management programs (Painter, 1968; Williams et al., 1983). Several insect-resistant crop lines have been bred and made available to growers (Wiseman, 1987). A more complete understanding of the various mechanisms by which plants are able to resist insect pests would allow for a more effective breeding program (Smith and Fisher, 1983; Stockel et al., 1987).

The plant surface, with which an insect pest first comes in contact, plays an important role in plant–insect interactions (Woodhead and Chapman, 1986). Many herbivorous insects seem to select their host plants based upon the chemical characteristics of the plant surface (Chapman and Bernays, 1989). Variations in the chemical and ultrastructural characteristics of the plant surface may affect many aspects of insect behavior such as oviposition, orientation and feeding, and thereby result in differential host-plant resistance (Eigenbrode et al., 1991; Espelie et al., 1991; Bergman et al., 1991). The cuticular lipids of all plants have a characteristic chemical composition that can vary depending upon the age of the plant, the part of the plant examined, and the environmental conditions under which the plant was grown (Baker, 1982; Blaker and Greyson, 1988; Blaker et al., 1989). A better understanding of the role that plant surface chemistry plays in host-plant resistance to insect pests would allow for more efficient incorporation of these characteristics into breeding programs.

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a major insect pest of corn in the southeastern United States (Sparks, 1986). Although several corn genotypes have been reported to be resistant to the fall armyworm (Wiseman and Davis, 1979; Wiseman et al., 1981; Williams et al., 1983), the mechanisms of resistance are not well understood. In the present paper, we characterize the cuticular lipids of resistant and susceptible genotypes of corn grown under different environmental conditions, and we examine the role that these cuticular lipids play in the development of fall armyworm larvae.

METHODS AND MATERIALS

Plant Material. Four fall armyworm-resistant corn genotypes, MpSWCB-4, Pioneer X304C, Mp496, and Antigua 2D-118, and three susceptible genotypes, Cacahuacintle X's, Pioneer 3192, and Pioneer 3369A (Scott and Davis, 1981a,b; Gross et al., 1982), were planted during the spring and summer of 1991 in Tifton, Georgia, using agronomic practices common to the area. No soil or foliar insecticides were utilized. Six corn genotypes, MpSWCB-4, Pioneer X304C, Mp496, Antigua 2D-118, Cacahuacintle X's, and Pioneer 3369A, were planted in pots in a greenhouse in the spring of 1989 in Athens, Georgia, using field soil from Tifton, Georgia. The plants were fertilized and watered as nec-

essary. Four corn genotypes, MpSWCB-4, Pioneer X304C, Cacahuacintle X's, and Pioneer 3192, were planted in the greenhouse in the fall of 1991 in Athens, Georgia, using Hyponex Potting Soil and these plants were fertilized and watered as necessary with a 12 : 12 light–dark regime.

Extractions. Greenhouse-grown plants from six genotypes in 1989 were excised at the stem base at the late-whorl stage and air-dried for two weeks. The dried leaves were collected as the first, second, third, and up to 12th leaves, for each genotype. The fourth and eighth fresh leaves of greenhouse-grown (four genotypes) corn plants were collected at the mid-whorl stage in 1991. Each leaf sample (10 g for dried, and 20 g for fresh foliage) was cut into pieces (10 cm in length) and dipped in redistilled chloroform (300 ml) for 1 min at room temperature. A brief extraction period was used, so that only surface lipids would be recovered (Espelie et al., 1980; Misra and Ghosh, 1991). Solvent extracts were reduced to a volume of 5 ml on a rotary evaporator at 40°C, and stored at −20°C prior to chemical analysis.

Chemical Analysis. Aliquots of the leaf extracts (from 1 g air-dried foliage or 5 g fresh foliage) were dried under a stream of N_2 and then derivatized with *N,O*-bis(trimethylsilyl)acetamide at 110°C for 10 min. Excess derivatizing reagent was removed under N_2, and the derivatized extract was resuspended in 1 ml of hexane. Aliquots (0.1%) were analyzed by combined gas chromatography–mass spectrometry (Hewlett-Packard 5890A/5970). The capillary column (25 m cross-linked methyl silicone, 0.2 mm internal diameter, 0.33 μm film thickness) with helium as the carrier gas was held at 55°C for 3 min after sample injection (splitless), and then the oven temperature was raised to 305°C at a rate of 15°C/min and held at this temperature for 20 min. The column was connected to a mass spectrometer, and mass spectra were recorded at 70 eV at 0.8-sec intervals. Individual components were identified by their mass spectra, which were compared to those of standards, and were matched by computer search with the National Bureau of Standards Mass Spectral Library. Quantitation was based upon total ion chromatogram integrations, which were corrected for response factors by utilizing a standard for each class of cuticular lipid component (Yang et al., 1992). Standards were *n*-nonacosane, 1-triacontanol, tetradecanal, hexadecanoic acid, β-sitosterol, and β-amyrin.

Scanning Electron Microscopy. Lower (fourth) and upper (eighth) fresh leaves of two genotypes, MpSWCB-4 and Cacahuacintle X's, were harvested from greenhouse-and field-grown corn plants at the mid-whorl stage in 1991. Leaf samples were cut into small pieces (0.6 × 0.6 cm), fixed overnight in 2% glutaraldehyde in 0.2 M cacodylate, washed in 0.1 M cacodylate buffer with 5% sucrose for 2–3 min, postfixed in 1% osmium tetroxide for 1 hr, washed again in 0.1 M cacodylate buffer with 5% sucrose, dehydrated sequentially in 30%, 50%, 70%, 80%, 95%, and 100% ethanol (each for 10 min), rinsed twice in 100% ethanol, and critical-point dried. Samples were then mounted on alu-

minum stubs with double sticky tape and silver paint, coated with gold–palladium (40 nm thickness) with a Hummer X Sputter Coater, and examined with a Phillips 505 scanning electron microscope located at the Center for Advanced Ultrastructure Research, University of Georgia, Athens, Georgia. In each case the same region of the leaf was examined: 15 cm from the leaf axil and 1 cm from the midrib. Three samples of adaxial (upper surface) and three samples of abaxial (lower surface) leaf surfaces were examined for both upper and lower leaves of the two genotypes.

Fall Armyworm Growth on Diet Containing Corn Foliage, Extracted Corn Foliage, or Corn Foliage Extracts. The lower (fourth) and upper (eighth) fresh leaves of field-grown corn plants from seven genotypes in 1991 were excised at the leaf axil, cut into pieces (10 cm long), and divided into two equal fractions. One fraction (80 g) was dipped in redistilled chloroform for 1 min at room temperature to remove the cuticular lipids, and the leaves were then air-dried in a hood for 1 hr. The extract was reduced to a volume of 2 ml on a rotary evaporator at 40°C, and the entire extract was mixed with Celufil (1 g) and air-dried. The other foliage fraction was not treated. Foliar fractions (either extracted or unextracted) were blended with 300 ml pinto bean diet plus 100 ml distilled water and distributed into 30 diet cups (30 ml) (Wiseman et al., 1984). Similar diets were prepared by blending the entire extract in Celufil with pinto bean diet and water as above. Each diet cup was infested with a single neonate fall armyworm larva and maintained at 28°C in an environmentally controlled chamber. Fall armyworm larvae were obtained from a colony maintained at the Insect Biology and Population Management Research Laboratory, USDA-ARS, Tifton, Georgia (Burton and Perkins, 1989). Tests were arranged as a three-factor factorial design (seven genotypes, two leaf locations, and three treatments) with 30 replicates plus Celufil and diet checks. The following fall armyworm developmental parameters were recorded: 7- and 10-day larval weights, days to pupation, and days to adult emergence.

Fall Armyworm Growth on Diet Containing Individual Cuticular Lipid Components. Seven commercially available compounds, *n*-nonacosane, 1-triacontanol, dodecanoic acid, hexadecanoic acid, β-sitosterol, α-amyrin, and β-amyrin, were selected as representatives of each class of components of corn foliar cuticular lipids. Chemicals (1, 10, or 100 mg) were dissolved in chloroform (2 ml), mixed with 1 g Celufil, air-dried, blended with pinto bean diet (300 ml) and distilled water (50 ml), and distributed into 30-ml diet cups. Each diet cup was infested with a fall armyworm neonate and kept at 28°C. Tests were arranged as a two-factor factorial design (seven compounds and three concentrations) with 30 replicates plus a Celufil check. Fall armyworm developmental parameters were monitored as described above.

All data were analyzed by ANOVA and means were separated by Fisher's protected least significant difference (LSD) at $P < 0.05$ level (Ott, 1988).

RESULTS

Chemical Composition of Foliar Cuticular Lipids of Greenhouse- and Field-Grown Corn Plants. The cuticular lipids of corn leaves were comprised primarily of *n*-alkanes (with chain lengths of C_{23}–C_{35}), aldehydes (C_{24}–C_{32}), free fatty alcohols (C_{18}–C_{32}), and fatty acids (C_{12}–C_{24}) (Table 1). Although there were minor differences between the cuticular lipid compositions of the five genotypes, the most striking variations were between the surface lipids found on upper leaves compared to those on the lower leaves. The major cuticular lipid component on the upper leaves of each genotype was *n*-nonacosane, which ranged from 14% to 23% of the total surface lipids (Table 1). This alkane comprised lower proportions on the surface of the lower leaves of each genotype (3–17%). The fatty alcohol, 1-dotriacontanol, was present in much larger amounts on the surface of the lower leaves (3–30%) than it was on the upper leaves (<0.1–6%). The cuticular lipids of the lower leaves of MpSWCB-4 grown in the greenhouse had a large amount (17%) of dotriacontanal. This aldehyde was present in small amounts (0.1–1%) on the surface of the lower leaves of field-grown plants, and it was not detected on the upper leaves of these plants (Table 1).

The total amount of alkanes extracted from the upper leaves was greater than that recovered from the lower leaves for each genotype (Table 2). This difference, however, was relatively small for Cacahuacintle X's, Pioneer 3192, and Pioneer 3369A. Conversely, there was a higher proportion of total fatty alcohols on the lower leaves than there was on the upper leaves for each genotype (Table 2). The fatty alcohols on the lower leaves had a longer chain length than those on the upper leaves where 1-tetracosanol and 1-hexacosanol were the major components in this class of cuticular lipids (Table 1). Triterpenols (α-and β-amyrin) comprised a higher percentage of the surface lipids of the upper leaves (4–7%) than they did of the lower leaves for each genotype (Table 2).

The differences observed between the cuticular lipid compositions of lower and upper leaves was examined in more detail with foliage from six genotypes grown in the greenhouse. The surface lipids of MpSWCB-4 leaves varied most dramatically from the lower to the upper portion of the plant in the proportions of fatty alcohols and *n*-alkanes (Figure 1A). Leaves from Pioneer X304C and Cacahuacintle X's also had increasing percentages of *n*-alkanes (from lower to upper leaves) and decreasing proportions of fatty alcohols (Figures 1B and 1C). The leaves of Pioneer 3369A also had decreasing percentages of fatty alcohols with increasing leaf number, but there were relatively minor changes in the proportions of *n*-alkanes (Figure 2A). The major class of components for all leaves of Pioneer 3369A was free fatty acids. The cuticular lipid compositions of the different leaves of Mp496 (Figure 2B) were very similar to those of the

TABLE 1. CUTICULAR LIPID COMPOSITION OF LOWER (4TH) AND UPPER (8TH) FRESH LEAVES FROM GREENHOUSE- AND FIELD-GROWN PLANTS OF SELECTED CORN GENOTYPES

		Cuticular lipid composition (%)											
		Greenhouse, MpSWCB-4		Field									
				MpSWCB-4		P. X304C		Cacahuacintle		P. 3192		P. 3369A	
R.T.[a]	Component	4th	8th	4th	8th	4th	8th	4th	8th	4th	8th	4th	8th
14.76	Dodecanoic acid	0.3	0.6	0.6	0.2	0.8	0.4	0.5	0.3	0.4	0.3	0.7	0.2
15.55	Tridecanoic acid	0.3	D[b]			0.6	0.4		0.4	0.4	0.3		
16.31	Tetradecanoic acid	2.9	3.6	3.3	3.5	5.0	3.1	3.2	3.5	4.9	3.2	4.9	2.5
17.04	Pentadecanoic acid	1.7	2.1	2.1	1.4	2.6	2.0	2.0	0.9	1.4	1.7	2.2	1.5
17.58	Hexadecenoic acid	3.0	4.4	3.1	3.4	3.1	2.2	2.5	3.5	1.9	2.4	2.6	2.2
17.73	Hexadecanoic acid	6.9	9.2	9.3	12.1	12.1	11.7	8.4	11.3	9.7	9.0	9.9	10.6
18.38	Heptadecanoic acid	0.8	1.4	1.7	1.0	0.8	0.5	0.9	0.8	0.3	0.6	0.6	1.0
18.51	1-Octadecanol	1.2	1.9	0.6	1.2	0.8	0.5	0.6	1.0	0.5	0.7	0.8	1.0
18.83	Octadecadienoic acid[c]	D	D	3.9	2.3	2.0	2.2	3.0	2.8	1.8	1.5	1.5	2.0
18.89	Octadecenoic acid[c]	4.6	5.2	7.0	6.1	5.5	5.5	6.4	5.6	4.0	3.4	3.9	4.8
19.01	Octadecanoic acid	3.7	3.8	5.0	4.5	4.3	1.8	4.0	2.9	2.6	1.5	3.7	2.0
19.41	*n*-Tricosane	2.1	1.6	1.1	6.1	1.0	1.3	1.0	1.3	0.7	0.8	1.6	0.9
19.71	1-Eicosanol	0.6	0.2		0.9	1.2	0.5	1.0	1.7	1.0	0.5	1.5	0.6
20.01	*n*-Tetracosane	1.9	1.8	0.8		0.8	D	0.7	D	0.5	D	1.5	
20.21	Eicosanoic acid	1.9	D	1.6	1.2	D	D	0.7		0.3	D		D
20.65	*n*-Pentacosane	1.1	1.3	2.0	3.0	2.0	1.9	3.3	1.9	2.3	1.3	4.4	1.9
20.95	1-Docosanol	0.8	0.7	0.6	0.8	0.5	0.5	0.8	0.8	0.8	1.0	0.9	0.9

21.30	*n*-Hexacosane	0.5	0.7	0.9		1.0		1.5	D	1.5		2.7	D
21.46	Tetracosanal						D				0.8	0.8	0.8
21.53	Docosanoic acid	0.3		1.2	D	D		0.6		D			
22.08	*n*-Heptacosane	2.2	4.8	5.9	9.1	7.9	10.9	7.9	8.6	9.8	9.4	10.4	8.0
22.41	1-Tetracosanol	1.5	3.7	1.8	1.6	2.3	3.2	2.5	3.0	3.6	4.0	2.0	2.8
22.87	*n*-Octacosane	0.5	1.6	1.9	D	1.7	1.0	2.0	0.6	2.3	1.0	2.6	1.1
23.05	Squalene	3.1	3.6		6.7		4.2		4.3		4.2		3.3
23.11	Hexacosanal			1.8	0.3	2.3		2.2	1.0	2.5		2.6	0.9
23.19	Tetracosanoic acid	D		0.6				0.5					
23.63	Nonacosene				2.2		1.5		1.1		0.8		
23.89	*n*-Nonacosane	3.2	13.8	11.8	17.3	14.4	20.9	11.7	18.3	16.6	22.9	13.3	19.6
24.29	1-Hexacosanol	0.7	2.8	1.5	1.7	2.3	3.8	2.5	4.5	4.3	5.7	2.2	4.1
24.93	*n*-Triacontane	0.5	1.7	1.3	D	1.1	0.5	1.2	D	1.4	1.0	1.1	1.1
25.26	Octacosanal		D	0.7		D	0.7	1.0	1.4	0.9	1.1	1.0	1.5
26.24	*n*-Hentriacontane	4.9	11.1	11.8	8.8	10.9	11.3	9.6	10.5	9.8	11.0	9.3	12.1
26.82	1-Octacosanol	D	D	0.5	D	D	0.7	0.8	0.9	1.2	1.4	0.8	1.2
27.72	*n*-Dotriacontane		1.0	D				D	D	D		D	
28.28	Triacontanal		0.9	0.3				D	D		0.6	D	0.8
29.64	*n*-Tritriacontane	D	3.4	2.4	2.5	2.0	3.8	2.2	3.6	2.0	3.9	1.8	4.3
30.60	1-Triacontanol	1.2	0.6	0.5	D	0.4	0.4	0.9	0.5	0.7	0.8	0.5	0.5
32.00	β-Amyrin	D	D	1.1	D	2.1	0.8	1.5	0.9	2.5	1.6	2.9	3.0
32.66	Dotriacontanal	16.5	3.8	1.1		0.5		1.0		0.6		D	
32.78	α-Amyrin			0.5	D	0.5	D	0.7	D	0.6	0.3	0.8	1.0
34.50	*n*-Pentatriacontane		0.7	D	D		D	D	D	D	D	D	D
35.80	1-Dotriacontanol	29.7	5.9	7.5	D	5.3	D	8.7	D	4.8	D	2.9	D

[a] Retention time (min). Components are listed only if they were identified by their mass spectra.
[b] Detectable, but less than 0.1%.
[c] Estimated by selected ion chromatography.

TABLE 2. COMPOSITION BY CLASS OF CUTICULAR LIPIDS OF LOWER (4TH) AND UPPER (8TH) LEAVES FROM GREENHOUSE- AND FIELD-GROWN PLANTS OF SELECTED CORN GENOTYPES

	Cuticular lipid composition (%)											
	Greenhouse, MpSWCB-4		Field									
			MpSWCB-4		P. X304C		Cacahuacintle		P. 3192		P. 3369A	
Component (Chain length)	4th	8th	4th	8th	4th	8th	4th	8th	4th	8th	4th	8th
Alkanes (C_{23}–C_{35})	16.9	43.5	39.9	46.8	42.8	51.6	41.1	44.8	46.9	51.3	48.7	49.0
Alkenes ($C_{29:1}$)				2.2		1.5		1.1		0.8		
Alcohols (C_{18}–C_{32})	35.7	15.8	13.0	6.2	12.8	9.6	17.8	12.4	16.9	14.1	11.6	11.1
Aldehydes (C_{24}–C_{32})	16.5	4.7	3.9	0.3	2.8	0.7	4.2	2.4	4.0	2.5	4.4	4.0
Fatty Acids (C_{12}–C_{24})	26.4	30.3	39.4	35.7	36.8	29.8	32.7	32.0	27.7	23.9	30.0	26.8
Triterpenols	3.1	3.6	1.6	6.7	2.6	5.0	2.2	5.2	3.1	6.1	3.7	7.3

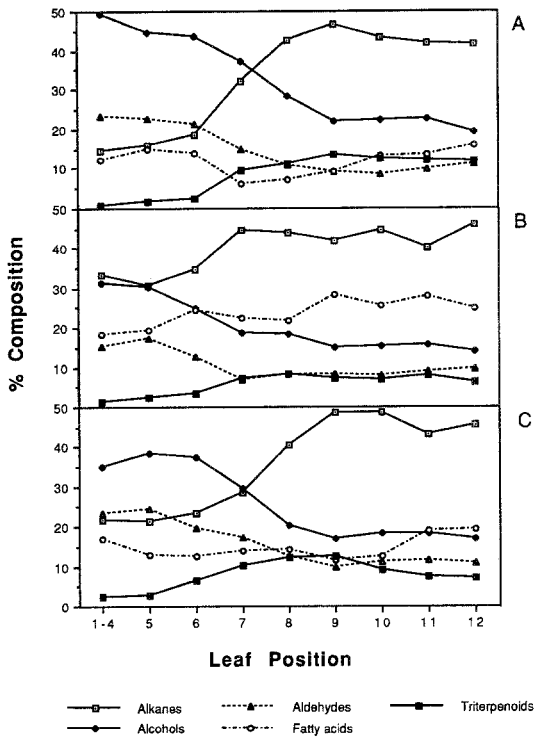

FIG. 1. Percent composition of major classes of cuticular lipids from different leaf locations of three genotypes of greenhouse-grown corn: (A) MpSWCB-4; (B) Pioneer X304C; (C) Cacahuacintle X's.

leaves of Antigua 2D-118 (Figure 2C). For both of these genotypes, *n*-alkanes were the major class of lipid components for all leaf positions.

Corn Leaf Ultrastructure. The ultrastructural appearance of the foliar surfaces of the insect-resistant corn genotype MpSWCB-4 and the susceptible genotype Cacahuacintle X's was very similar. However, there were differences between the adaxial (Figure 3A) and abaxial (Figure 3B) surface of upper leaves of both greenhouse- and field-grown leaves of both genotypes. There were large double or single trichomes and small spines on the adaxial surface of the upper leaves in both genotypes (Figure 3A and 3C), which were not found on the abaxial surface of the upper leaves or on either side of the lower leaves. At higher magnification, the adaxial and abaxial surfaces of upper leaves in both genotypes of greenhouse- and field-grown plants exhibited an amorphous appearance (Figure 3B).

There was a dense, irregular array of wax crystals on both the adaxial

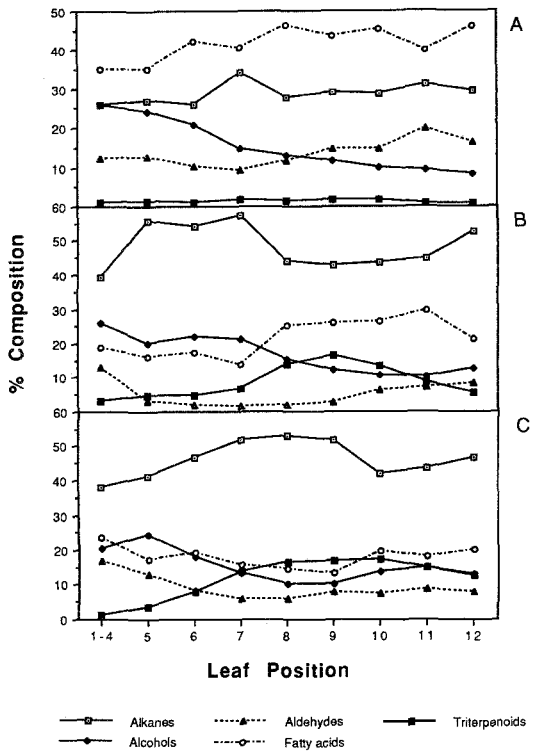

FIG. 2. Percent composition of major classes of cuticular lipids from different leaf locations of three genotypes of greenhouse-grown corn: (A) Pioneer 3369A; (B) Mp-496; (C) Antigua 2D-118.

(Figures 3D–F) and the abaxial (Figures 3G–I) surfaces of the lower corn leaves. This array of crystals was heavier on both the adaxial (Figure 3D) and the abaxial (Figure 3G) surfaces of field-grown plants than it was on the adaxial and abaxial sides of greenhouse-grown plants of MpSWCB-4 (Figures 3E and H) and Cacahuacintle X's (Figure 3F and I).

Fall Armyworm Growth on Diet Containing Corn Foliage, Extracted Foliage, or Foliage Extracts. Fall armyworm larvae were significantly larger at seven days when they were reared on diet containing corn leaves from which the cuticular lipids had been removed than when they were fed diet containing foliage that had not been extracted with chloroform (Table 3). The largest differences in developmental parameters were observed between larvae reared on diets containing extracted and unextracted leaves from the upper part of the plants. For instance, larvae reared on diet containing upper leaves from

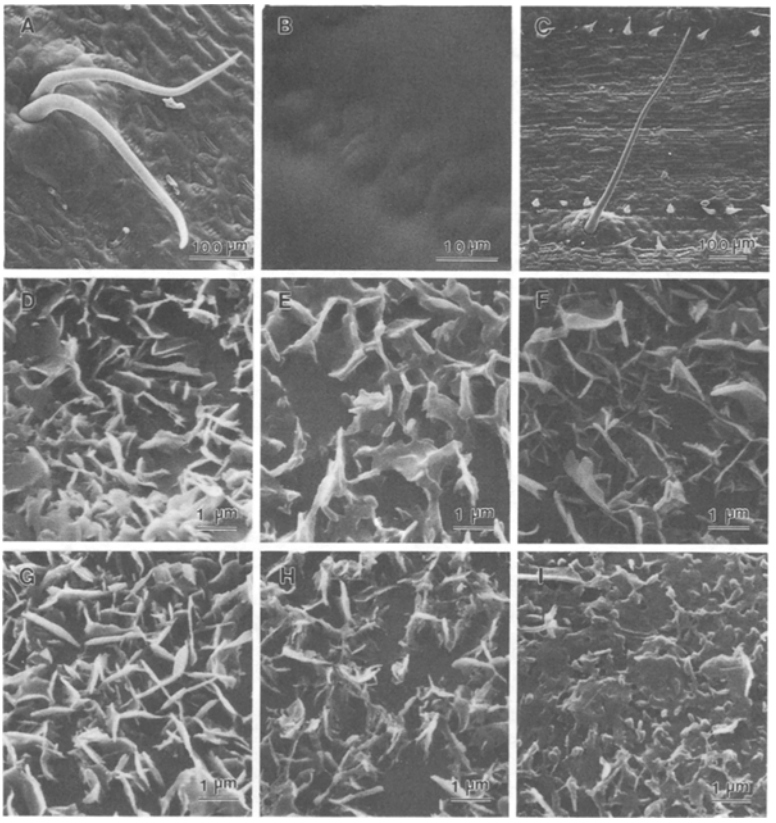

FIG. 3. Scanning electron micrographs (SEMs) of upper (8th) leaves of greenhouse-grown corn: (A) adaxial surface of MpSWCB-4; (B) abaxial surface of MpSWCB-4; (C) adaxial surface of Cacahuacintle X's. SEMs of adaxial surface of lower (fourth) leaves of: (D) field-grown Cacahuacintle X's; (E) greenhouse-grown Cacahuacintle X's; (F) greenhouse-grown MpSWCB-4. SEMs of abaxial surface of lower leaves of: (G) field-grown Cacahuacintle X's; (H) greenhouse-grown Cacahuacintle X's; (I) greenhouse-grown MpSWCB-4.

MpSWCB-4 plants weighed 35.9 mg when the foliage was untreated, but larvae weighed 59.9 mg when they were fed foliage from which the cuticular lipids had been removed. Larvae reared on diet containing extracted upper leaves weighed significantly more at 10 days than larvae fed diet containing unextracted foliage for each of the seven corn genotypes (Table 3). Larvae reared on diet with extracted lower leaves also weighed more than those fed diet containing untreated foliage, but the weight difference was not significant for some of the genotypes. Larvae that were fed cuticular lipid extracts from both upper and

TABLE 3. FALL ARMYWORM DEVELOPMENT ON DIET CONTAINING UPPER (8TH) OR LOWER (4TH) EXTRACTED FOLIAGE, NONEXTRACTED FOLIAGE, OR FOLIAGE EXTRACTS FROM FIELD-GROWN PLANTS OF SELECTED CORN GENOTYPES[a]

Genotype	Upper leaves			Lower leaves		
	Extracted foliage	Foliage	Extract	Extracted foliage	Foliage	Extract
			Larval 7-day weight (mg)			
MpSWCB-4	59.9 ± 16.5ax	35.9 ± 16.4cdz	51.3 ± 19.0ay	57.8 ± 15.9axy	50.9 ± 10.4aby	53.1 ± 20.9axy
Pioneer X304C	56.3 ± 11.3awx	35.5 ± 14.1cdz	56.4 ± 14.9awx	57.2 ± 22.0aw	48.0 ± 14.2aby	49.2 ± 11.0axy
Cacahuacintle X's	52.3 ± 15.2ay	35.9 ± 14.0cdz	53.9 ± 21.1ay	62.7 ± 17.6ax	41.1 ± 12.5cz	50.6 ± 11.9ay
Pioneer 3192	56.9 ± 13.6ax	31.8 ± 12.5dy	57.8 ± 25.5ax	60.3 ± 17.1ax	23.0 ± 9.2dy	60.0 ± 27.7ax
Pioneer 3369A	56.3 ± 21.1ax	40.6 ± 12.8bcz	51.6 ± 12.0axy	54.2 ± 18.0ax	45.1 ± 12.2bcyz	53.5 ± 18.4axy
Mp496	58.1 ± 13.8ax	44.6 ± 15.0by	51.2 ± 16.6axy	55.9 ± 19.6ax	52.1 ± 16.0axy	56.7 ± 23.8ax
Antigua 2D-118	51.6 ± 13.0ay	34.4 ± 13.6cdz	50.8 ± 16.3ay	60.7 ± 20.6ax	41.1 ± 10.9cz	54.2 ± 13.8axy
Diet check	53.4 ± 17.7a	53.4 ± 17.7a	53.4 ± 17.7a	51.2 ± 12.9a	51.2 ± 12.9ab	51.2 ± 12.9a
Celufil check			52.4 ± 21.2a			49.2 ± 21.1a
			Larval 10-day weight (mg)			
MpSWCB-4	400 ± 125ax	190 ± 54dz	363 ± 150axy	413 ± 118ax	304 ± 83bcy	370 ± 137ax
Pioneer X304C	383 ± 105abx	208 ± 52cdz	416 ± 105ax	424 ± 136ax	328 ± 114aby	326 ± 102ay
Cacahuacintle X's	312 ± 95cdx	192 ± 50dz	352 ± 121ax	442 ± 116aw	250 ± 74dy	345 ± 98ax
Pioneer 3192	360 ± 112abcx	185 ± 46dy	405 ± 147ax	413 ± 95ax	173 ± 45ey	412 ± 148ax
Pioneer 3369A	373 ± 104abx	240 ± 60bcy	355 ± 97ax	366 ± 142ax	265 ± 62cdy	331 ± 112ax
Mp496	393 ± 103abx	260 ± 58bz	350 ± 121axy	401 ± 142ax	320 ± 118aby	376 ± 138axy
Antigua 2D-118	275 ± 102dy	192 ± 49dz	344 ± 140ax	426 ± 132aw	237 ± 74dyz	386 ± 114awx
Diet check	341 ± 113bc	341 ± 113a	341 ± 113a	363 ± 112a	363 ± 112a	363 ± 112a
Celufil check			323 ± 116a			340 ± 123a

			Days to pupation			
MpSWCB-4	13.9 ± 0.8byz	15.4 ± 1.1ax	14.0 ± 0.9ay	13.5 ± 0.6az	14.1 ± 0.6cdy	13.9 ± 0.7ayz
Pioneer X304C	13.8 ± 0.7by	15.1 ± 0.9abx	13.7 ± 0.7ay	13.7 ± 1.0ay	13.9 ± 0.6dy	14.1 ± 0.6ay
Cacahuacintle X's	14.1 ± 0.8abz	15.2 ± 0.9abx	14.0 ± 1.0az	13.8 ± 0.7az	14.6 ± 0.7by	14.0 ± 0.8az
Pioneer 3192	14.0 ± 0.6abz	15.2 ± 0.8aby	13.9 ± 0.9az	13.7 ± 0.6az	15.8 ± 0.9ax	13.8 ± 1.0az
Pioneer 3369A	13.9 ± 1.0bz	14.8 ± 0.7bcx	14.0 ± 0.6 ayz	13.9 ± 1.2az	14.4 ± 0.5bcxy	14.0 ± 0.7ayz
Mp496	13.8 ± 0.5by	14.6 ± 0.6cx	13.9 ± 0.7ay	14.0 ± 1.0ay	14.1 ± 1.0cdy	13.7 ± 0.8ay
Antigua 2D-118	14.4 ± 1.0axy	15.2 ± 0.9abw	14.0 ± 0.9ayz	13.8 ± 0.8az	14.5 ± 0.5bx	13.7 ± 0.6az
Diet check	13.8 ± 0.7b	13.8 ± 0.7d	13.8 ± 0.7a	13.9 ± 0.7a	13.9 ± 0.7d	13.9 ± 0.7a
Celufil check			13.9 ± 0.8a			14.0 ± 0.7a
			Days to adult emergence			
MpSWCB-4	22.2 ± 1.0cz	24.4 ± 1.3ax	22.9 ± 1.0ay	22.1 ± 0.9az	22.5 ± 1.0cyz	22.5 ± 1.0ayz
Pioneer X304C	22.8 ± 0.9abyz	23.7 ± 1.0bcx	22.5 ± 1.2ayz	22.3 ± 1.1az	22.6 ± 0.9cyz	22.9 ± 1.1ay
Cacahuacintle X's	23.1 ± 1.2axy	24.0 ± 1.0abw	22.2 ± 1.5az	22.6 ± 1.1ayz	23.2 ± 0.9bx	22.4 ± 1.1az
Pioneer 3192	22.7 ± 1.2abcy	24.0 ± 1.1abx	22.2 ± 1.2ayz	22.3 ± 0.8ayz	24.5 ± 0.9ax	22.1 ± 0.9az
Pioneer 3369A	22.3 ± 0.9bcz	23.3 ± 0.9cx	22.5 ± 1.0ayz	22.6 ± 1.6ayz	23.0 ± 1.0bcxy	22.4 ± 1.1az
Mp496	22.5 ± 0.8bcyz	23.2 ± 1.0cx	22.1 ± 1.1az	22.7 ± 1.1axy	22.7 ± 1.0bcxy	22.4 ± 1.2ayz
Antigua 2D-118	23.1 ± 1.3ay	23.9 ± 1.2abx	22.5 ± 1.1az	22.4 ± 1.0az	23.2 ± 0.8by	22.2 ± 0.9az
Diet check	22.3 ± 1.2bc	22.3 ± 1.2d	22.3 ± 1.2a	22.7 ± 0.8a	22.7 ± 0.8bc	22.7 ± 0.8a
Celufil check			22.4 ± 1.3a			22.5 ± 0.9a

[a]Means (±SD, $N = 30$) followed by the same letter in a column (a, b, c, d, or e) or in a row (w, x, y, or z) for each developmental parameter are not significantly different ($P > 0.05$; Fisher's protected least significant difference; Ott, 1988).

lower foliage of all genotypes did not differ significantly from larvae reared on diet checks.

Fall armyworm reared on diet containing extracted upper leaves from each of the seven corn genotypes pupated in a significantly shorter period of time than fall armyworm fed diet containing unextracted corn foliage (Table 3). Insects reared on extracted lower leaves pupated sooner than those fed diet containing leaves from which the surface lipids had not been removed, but the difference in pupation time was not significant for diets containing Pioneer X304C or Mp496. Pupation time for fall armyworm fed diet containing the cuticular lipid extracts, from either upper or lower foliage, did not differ significantly from the controls. Fall armyworm adults emerged more quickly when the larvae were reared on diet containing extracted leaves than when they had been fed diet with untreated foliage (Table 3). The difference in adult emergence time was significant for fall armyworm fed diet containing upper leaves from each of the seven genotypes, but for larvae fed on lower leaves the difference was significant only for those reared on diets containing Cacahuacintle X's, Pioneer 3192, and Antigua 2D-118. None of the cuticular lipid extracts had a significant effect on fall armyworm adult emergence time.

Fall Armyworm Larval Growth on Diet Containing Individual Cuticular Lipids. There were no significant differences in the 7- or 10-day weights of fall armyworm larvae reared on artificial diet containing the lowest concentration of individual cuticular lipid components (Table 4). At the intermediate concentration (10 mg/350 ml diet) larvae fed hexadecanoic acid weighed 134 mg after seven days while larvae on the control diet weighed only 98 mg. Fall armyworm larvae that were reared on diet containing the highest concentration (100 mg) of individual cuticular lipid components weighed more than the control. At 10 days, these differences were significant for each treatment, except for larvae fed diets containing β-sitosterol (Table 4). The only chemical treatments that resulted in larvae weighing less than those insects reared on control diet were the low and intermediate concentrations of β-sitosterol. However, the weight differences for the larvae reared on these treatments were not significant (Table 4).

DISCUSSION

The difference between the chemical compositions of the lower and upper corn leaves found by GC-MS analysis of the cuticular lipid extracts (Table 2) was supported by the results obtained by scanning electron microscopy (Figure 3). The higher proportion of *n*-alkanes found on the upper corn leaves resulted in an amorphous ultrastructural appearance (Figure 3B), while the higher percentage of fatty alcohols on the lower leaves caused these surfaces to have a crystalline appearance (Figures 3D–I). Variations in cuticular lipid chemistry

TABLE 4. FALL ARMYWORM LARVAL GROWTH ON DIET CONTAINING VARIOUS CONCENTRATIONS (1, 10 OR 100 mg) OF INDIVIDUAL CUTICULAR LIPID COMPONENTS[a]

Component	7-Day larval weight (mg)			10-Day larval weight (mg)		
	1 mg	10 mg	100 mg	1 mg	10 mg	100 mg
n-Nonacosane	98 ± 43ay	106 ± 55by	135 ± 45ax	391 ± 123ay	412 ± 116ay	496 ± 102ax
1-Triacontanol	118 ± 57ax	117 ± 53abx	115 ± 34abx	457 ± 177ax	442 ± 147ax	456 ± 95ax
Dodecanoic acid	114 ± 42axy	107 ± 53by	138 ± 47ax	450 ± 110axy	408 ± 149ay	494 ± 97ax
Hexadecanoic acid	115 ± 47ax	134 ± 57ax	116 ± 48abx	447 ± 117ax	477 ± 149ax	464 ± 125ax
β-Sitosterol	91 ± 53axy	88 ± 51by	121 ± 58abx	377 ± 176ax	375 ± 165ax	445 ± 180abx
α-Amyrin	117 ± 35ax	112 ± 46abx	NT[b]	439 ± 114ax	435 ± 133ax	NT
β-Amyrin	117 ± 47a	NT	NT	446 ± 125a	NT	NT
Celufil	98 ± 50a	98 ± 50b	98 ± 50b	387 ± 178a	387 ± 178a	387 ± 178b

[a] Means (±SD, $N = 30$) followed by the same letter in a column (a or b) or in a row (x or y) for each fall armyworm growth parameter are not significantly different ($P > 0.05$; Fisher's protected least significant difference; Ott, 1988).

[b] These concentrations were not tested due to lack of chemicals.

are known to affect the ultrastructural appearance of the leaf cuticle (Baker, 1982; Jeffree and Sandford, 1982; Jeffree, 1986). Our data support previous results that reported differences between the cuticular lipid compositions of young and old corn leaves (Blaker and Greyson, 1988; Blaker et al., 1989). Similar differences in the cuticular lipid compositions of younger and older alfalfa leaves (Bergman et al., 1991) and oak leaves (Gülz and Boor, 1992) also resulted in dramatic differences in the ultrastructural appearance of the leaf surface.

The observed difference in cuticular lipid composition between upper and lower corn leaves may affect the feeding behavior of fall armyworm. The cuticular lipids of cabbage leaves affect the behavior of diamondback moth larvae, and changes in the lipid composition result in increased rates of movement and decreased levels of feeding (Eigenbrode and Shelton, 1990; Eigenbrode et al., 1991). The surface lipids of upper corn leaves have a higher proportion of *n*-alkanes (Table 2), which have been shown to be deterrent to several species of herbivorous insects (Woodhead, 1983; Woodhead and Chapman, 1986), while the lower leaves have a higher percentage of fatty alcohols, which have been shown to be feeding stimulants (Mori, 1982). It is important to note, however, that specific *n*-alkanes have also been shown to stimulate insect feeding (Klingauf et al., 1978). In laboratory studies, neonate fall armyworm larvae traveled farther and moved more rapidly when they were placed on excised upper leaves of corn plants than when they were placed on lower corn leaves (Yang et al., 1993b). Similar results were obtained in field tests where neonate larvae placed on upper leaves of corn plants reached feeding sites (whorl tissue) more quickly than larvae that were placed on lower leaves (Yang et al., 1993c).

There were relatively minor differences between the cuticular lipid compositions of the fall armyworm-resistant genotypes, MpSWCB-4, Mp496, Pioneer X-304C, and Antigua 2D-118, and the susceptible genotypes, Cacahuacintle X's and Pioneer 3369A (Figures 1 and 2). The susceptible genotype, Pioneer 3369A, had the lowest proportion of triterpenols, and the upper leaves of this genotype showed no increase in these compounds (Figure 2A). The triterpenols, α- and β-amyrin, have been shown to inhibit the feeding of *Locusta migratoria* (Chapman, 1977), and the palmitate ester of α-amyrin inhibits growth of several species of lepidopteran larvae (Shankaranarayana et al., 1980). Recently, α- and β-amyrin were found in much higher amounts in the cuticular lipids of a cabbage variety that is resistant to diamondback moth than in the lipids of a susceptible cabbage variety (Eigenbrode et al., 1991), and β-amyrin was shown to be present in higher quantities in the cuticular lipids of raspberry varieties resistant to the aphid *Amphorophora idaei* (Robertson et al., 1991). Both α- and β-amyrin have been found as major components in the frass of the leaf beetle, *Pyrrhalta viburni*, which these insects deposit as a protective layer over their eggs (Hilker, 1992). The structural similarity of the amyrins to sterols may cause them to be toxic to some insects, and the presence of these triterpenols

in plant cuticles may play a role in resistance to herbivory (Eigenbrode et al., 1991).

The higher proportion of alcohols and aldehydes found on the leaf surface of greenhouse-grown corn compared to the cuticular lipids of leaves from field-grown plants, which had more alkanes and fatty acids (Table 2), may have been due to the different environmental conditions. There have been very few analyses comparing the cuticular lipids from plants grown in the greenhouse to those from field-grown plants. Woodhead (1981) found that there were higher levels of phenolic acids in sorghum grown in the field compared to plants grown in growth chambers, and these phenolic acids were shown to deter herbivores (Woodhead and Bernays, 1978). Scanning electron microscopy studies have indicated that the ultrastructural appearance of the leaf cuticle of greenhouse-grown plants is the same as that of field-grown plants (Wetzstein and Sommer, 1983; Sutter, 1985; Sutter et al., 1988). The lower leaves of field-grown Cacahuacintle X's plants (Figures 3D and 3G) were also very similar in appearance to the lower leaves of greenhouse-grown plants (Figures 3E and 3H), although the array of wax crystals was more dense on those plants grown in the field.

The increased growth of larvae reared on diet containing foliage from which the surface lipids had been removed, compared to the growth of larvae fed diet containing unextracted corn leaves (Table 3), supported previous results indicating that cuticular lipids inhibited fall armyworm development (Quisenberry et al., 1988; Yang et al., 1991, 1993a). Since the total lipid extracts did not inhibit fall armyworm growth (Table 3), and since several individual surface lipid components enhanced larval growth (Table 4), the role of the cuticular lipids of corn in fall armyworm feeding behavior remains obscure. It is possible that the cuticular lipids may interact with other components in the corn leaf to inhibit the development of fall armyworm larvae. Alternatively, there may be volatile components on the surface of the corn leaf that were lost during our extraction procedure and that were, therefore, not present in the diets containing the cuticular lipid extracts.

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RESPONSE OF *Meteorus leviventris*, (HYMENOPTERA: BRACONIDAE) TO MUSTARD OILS IN FIELD TRAPPING EXPERIMENTS

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Abstract—Trapping experiments were carried out near Saskatoon, Canada, from May through August 1990 to assess the response of the braconid wasp, *Meteorus leviventris*, to four selected mustard oils or isothiocyanates (IC) at a release rate of 4 mg/day, and for allyl IC only, at 40 mg/day. Only allyl IC at 4 mg/day was significantly attractive when trap captures were compared to the captures in the control traps. The others (*n*-propyl IC, 2-phenylethyl IC, and ethyl IC) were not attractive, nor was allyl IC at the higher dose, although trap captures with the latter bait were the second highest.

Key Words—Mustard oils, isothiocyanates, kairomone, biological control, Hymenoptera Braconidae, *Meteorus leviventris*.

INTRODUCTION

Meteorus leviventris (Wesmael) is a common braconid parasitoid widely distributed in North America, which attacks cutworms (Lepidoptera: Noctuidae) including *Euxoa auxiliaris* (Grotė), *E. messoria* (Harris), and *Agrotis ipsilon* Hufnagel (Soteres et al., 1984; Schoenbohm and Turpin, 1977; Cheng, 1977). In a 1989 field trapping experiment, using mustard oils to investigate flea beetle population dynamics, it was noted that *M. leviventris* was frequently caught in baited traps. As this insect parasitizes important insect pest species, a better knowledge of its response to mustard oils could be useful in the management of cutworms. Such knowledge would also allow the comparison of mustard oil specificity in a species of a third insect order to those already carried out (Pivnick et al., 1991, 1992). Consequently, in 1990, a field trapping survey was under-

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taken using four isothiocyanates (mustard oils), found naturally in plants, to examine their degree of attraction for *M. leviventris*.

METHODS AND MATERIALS

Field trapping adjacent to cultivated fields was carried out from May 7 to August 31, 1990, at the Agriculture Canada experimental farm in Saskatoon. Traps were set out on the ground in a randomized complete block design, with four replicates and a minimum of 10 m between traps within a replicate, and 400 m between replicates. Yellow plastic boll weevil traps with a clear plastic funnel and ball trap top were used (Pest Management Specialists, Starkville, Mississippi). Baits were held upright with adhesive tape in the center of each trap base. The baits were isothiocyanates (IC), all neat liquids, and were released from vials made from glass tubing, sealed at one end, or 3.5-ml serum vials, as in Pivnick et al. (1991). The release rate of any given compound was directly proportional to the internal diameter of the container and inversely proportional to the length of the air column above the meniscus of the compound. The dimensions used were designed (different for each compound to adjust for volatility) to give the approximate desired release rates with little change over two weeks, at which time the baits were changed. It would take a doubling of air column length to halve the release rate and, in practice, the air column length changed little. Actual release rates were variable, as weather conditions could modify the release rates but would do so uniformly across all baits.

The compounds tested (allyl IC, 2-phenylethyl IC, *n*-propyl IC, and ethyl IC) were obtained from Aldrich Chemical Co., and all are known to occur in plants.

Vial dimensions used for release of compounds were as follows: Air column lengths were 50, 85, and 10 mm and internal diameters were 3.7, 3.7, and 6.5 mm for intended release rates of 4 mg/day for allyl IC, ethyl IC, and 2-phenylethyl IC, respectively (based on prior empirical tests). Dimensions for *n*-propyl IC vials were identical to those for allyl IC. For 2-phenylethyl IC only, two vials were used per trap to achieve the desired estimated rate of release. For 40 mg/day (allyl IC only), a half-filled 7-dram serum vial was used, capped with a rubber septum with a pipe cleaner inside as a wick. Blank control traps were not baited. Actual release rates for all compounds were determined by measuring the change in the length of the air column every two weeks when changing the baits. The release rates of allyl IC in the serum vials were not measured here but were previously measured at a mean of 69 mg/day (Pivnick et al., 1991). Traps were emptied every two weeks, and the total number of *M. leviventris* was counted (but not sexed). Total numbers of wasps captured per trap over the season were analyzed by two-way ANOVA followed by protected LSD tests.

RESULTS AND DISCUSSION

M. leviventris adults were caught in significantly greater numbers in traps baited with allyl IC than those baited with other ICs or blank traps (Table 1). The higher dose of allyl IC attracted fewer wasps than the lower dose, although the capture was still higher (but not significantly so) than traps with other baits. There was an early summer peak of trap capture, and a larger late summer peak (Figure 1). Throughout, however, more wasps were caught with allyl IC than other baits.

Volatile chemicals produced by host insects and their food sources are important cues used by parasitoids to select a suitable host (van Alphen and Vet, 1986). *Diaeretiella rapae*, a braconid species that attacks aphids on plants in the family Brassicaceae, is attracted to the odor of collards, *Brassica oleracea*, as well as to allyl IC (Read et al., 1970). On the other hand, *Diadromus pulchellus* Wesmeal, an ichneumonid parasitoid whose primary host feeds on leeks, *Allium porrum*, is inhibited in its movements by the presence of any of three ICs tested, but walks faster and turns more in the presence of characteristic leek volatile components (Lecomte and Thibout, 1984).

Only one dose was used of three of the mustard oils tested. Yet in tests with other insects, the mustard oil that is most attractive at one dose, tends to be most attractive at all doses until an upper limit is reached, at which point mustard oils become repellent (Pivnick et al., 1991, 1992, unpublished data).

The strong response of *M. leviventris* to allyl IC only of the ICs tested may indicate that allyl IC is a primary volatile constituent released by a common host plant of one or more of the parasitoid's host noctuid species. In fact, allyl IC is one of the most widespread in occurrence of the ICs in cruciferous plants

TABLE 1. PARASITE WASPS *Meteorus leviventris* CAPTURED IN YELLOW BOLL WEEVIL TRAPS BAITED WITH SELECTED ISOTHIOCYANATES

Compound	Estimated rate of release (mg/day)	Mean measured rate of release (mg/day) (±SE)	Mean number (±SE; $N = 4$) of wasps caught per trap over the season[a]
Allyl IC[b]	4	3.8 ± .5 ($N = 22$)	49.3 ± 13.6 a
Allyl IC	40		18.5 ± 8.0 b
n-Propyl IC	4	3.1 ± .3 ($N = 23$)	6.8 ± 2.1 b
Ethyl IC	4	4.4 ± .6 ($N = 18$)	3.3 ± 1.5 b
Blank			2.8 ± 1.0 b
2 Phenylethyl IC	4	12.9 ± 2.7 ($N = 36$)	2.3 ± 1.1 b

[a]Mean in columns followed by different letters are significantly different at $P < 0.05$ based on a two-way ANOVA followed by a protected LSD test.
[b]IC = isothiocyanate.

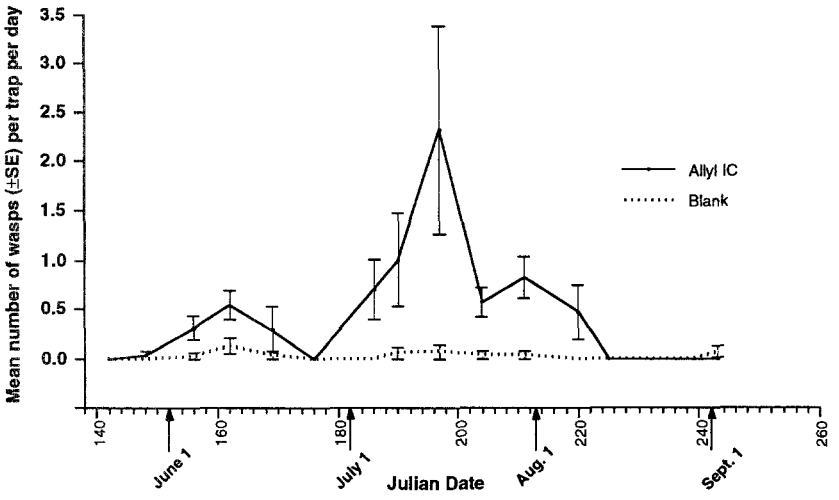

FIG. 1. Mean trap captures (N = 4) of *Meteorus leviventris* in Saskatoon in 1990 in yellow boll weevil traps baited with allyl isothiocyanate (releasing ca. 4 mg/day) and in unbaited traps.

(Kjaer, 1960). These results suggest that the planting along agricultural fields of borders or strips of a plant releasing allyl IC, such as *B. juncea*, may enhance activity of *M. leviventris* and hence, improve biological control of cutworm pests. Initial evidence that such an approach can increase rates of parasitism has been obtained with a parasitic fly, *Cyzenis albicans* (Fall.) (Roland et al., 1989).

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FOLIAR AND FLORAL PYRETHRINS OF *Chrysanthemum cinerariaefolium* ARE NOT INDUCED BY LEAF DAMAGE

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Abstract—Pyrethrins are a class of potent insecticides produced by *Chrysanthemum cinerariaefolium*. Simulated herbivory does not affect concentrations of pyrethrins in damaged and undamaged expanding and fully expanded leaves, or flowers of greenhouse or field-grown plants.

Key Words—Induced defense, *Chrysanthemum cinerariaefolium*, pyrethrins, leaf damage.

INTRODUCTION

In at least some plant species, examples of secondary compounds that increase in response to leaf damage come from every major biosynthetic class of secondary metabolites (Baldwin, 1993; Rosenthal and Berenbaum, 1991; Tallamy and Raupp, 1991). Thus, few generalizations can be made about how inducible a class of metabolites will turn out to be. Many secondary metabolites are synthesized in specialized tissue types that are active only during particular periods of leaf ontogeny and thus their inducibility may be morphologically constrained. For example, many terpenoids are stored in secretory idioblasts, laticifers, and glandular trichomes—cell types active early in leaf development—which may constrain induced responses to the next leaves produced after damage (Gershenzon and Croteau, 1991). However, plant development and organization is known for its plasticity, so it should not be surprising to find biochemical plasticity in differentiated tissues and to find that some tissues are more plastic

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than others. For example, Nitao (1988) found no evidence for induction in total or specific furanocoumarins in the fruits of wild parsnip, but Zangerl (1990) and Zangerl and Berenbaum (1990) found dramatic evidence for furanocoumarin induction in leaf tissues in the same genotypes of the same species.

The leaves and flowers of *Chrysanthemum cinerariaefolium* (Trev.) contain the potent insecticides, pyrethrins. The highest concentration of the six esters that constitute the natural pyrethrins are found in the flower heads, where they are localized in secretory canals and oil glands of the achenes (Brewer, 1973). The concentration of pyrethrins is also highly correlated with the number of oil glands in the leaves (Zito et al., 1983). Here we examine the effects of leaf damage on the pyrethrin concentrations of damaged and undamaged leaves and flowers of greenhouse and field-grown plants.

METHODS AND MATERIALS

Plant Growth and Sampling. C. cinerariaefolium were grown from seed obtained from the Park Seed Co. (Greenwood, South Carolina; lot #2925) in 25-cm pots with Cornell mix A (Boodley and Sheldrake, 1977) supplemented with 5 g of Osmocote 14–14–14 fertilizer in a glasshouse under supplemental lighting from 400-W sodium vapor lamps for 13 hr/day. Greenhouse experiments were conducted with 2-year-old plants. Four vigorously growing vegetative plants of similar dimensions were sampled for pyrethrins in both expanding and fully expanded leaves. After the initial sampling, half of each plant was cut with scissors, removing the top 3 cm of all leaves on the damaged half of the plant. The cutting removed approximately 24% of the plants' leaf mass. Expanding and fully expanded leaves were sampled on both the damaged and undamaged halves of each plant at the times indicated in Figure 1. Fifteen 0.12-cm^2 leaf disks (one disk from the between-vein portion of the lamina of 15 different leaves) were removed with a cork borer, pooled by leaf developmental stage, and extracted immediately in absolute methanol. Each of the sampled leaves was resampled at each sampling time. At the last sampling, additional samples of leaf disks were removed, weighed, dried at 50°C, and reweighed.

Since some damage-induced chemical responses are inhibited in pot-bound plants (Baldwin, 1988; Karban et al., 1989), a second experiment was conducted on plants transplanted to a field plot where root growth would remain unrestricted. Two-year-old greenhouse-grown plants were transplanted into a rototilled field plot in Amherst, New York, on June 5 with a between-plant spacing of 50 cm. On August 17, ten flowering plants growing in the center row of the plot were selected for the experiment. Every other plant was cut with scissors as previously described and five to six fully expanded undamaged leaves were removed from each plant at the times indicated in Figure 2, placed on ice, and

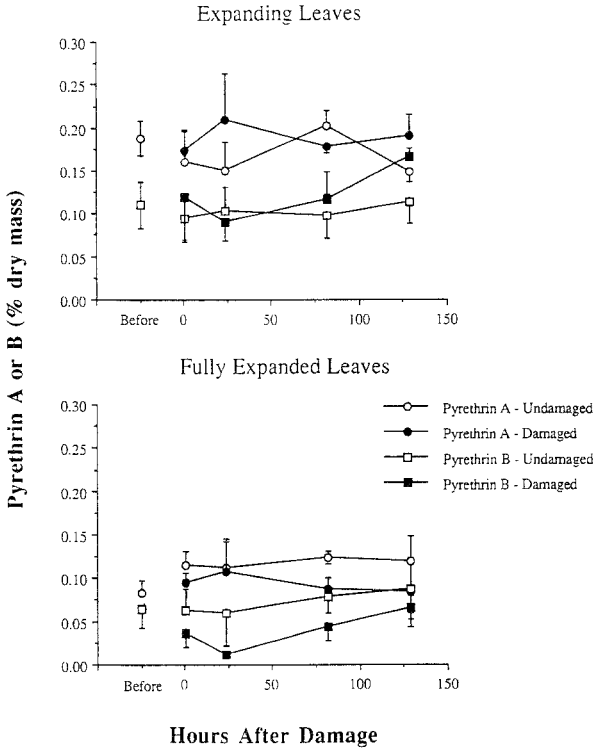

FIG. 1. Mean pyrethrin A and B (±SEM) concentrations for expanding and fully expanded leaves of four greenhouse-grown *C. cinerariaefolium* plants. After initial sampling (designated as before), the top 3 cm of half of each plant was cut with scissors. Expanding and fully expanded leaves were then resampled on both the damaged and undamaged halves of each plant at 0.75, 24, 82, and 129 hr. Pyrethrin A represents total pyrethrin I, jasmolin I, and cinerin I amounts, and pyrethrin B represents total pyrethrin II, jasmolin II, and cinerin II amounts.

returned to the laboratory for pyrethrin extraction. Leaves were ground to a powder in liquid nitrogen. A portion (200–700 mg) of the leaf material was weighed (to 0.1 mg) and extracted in absolute methanol. Another portion of leaf material was weighed, dried at 50°C, and reweighed for percent leaf dry mass measures. Flowers (1–3/plant/sampling) with at least the ray florets expanded but not yet completed anthesis were removed from each plant at the beginning and the end of the experiment, placed on ice, and returned to the laboratory for pyrethrin analysis. Flowers were also extracted in absolute methanol.

Pyrethrin Extraction and Quantification. Pyrethrin leaf and flower extracts

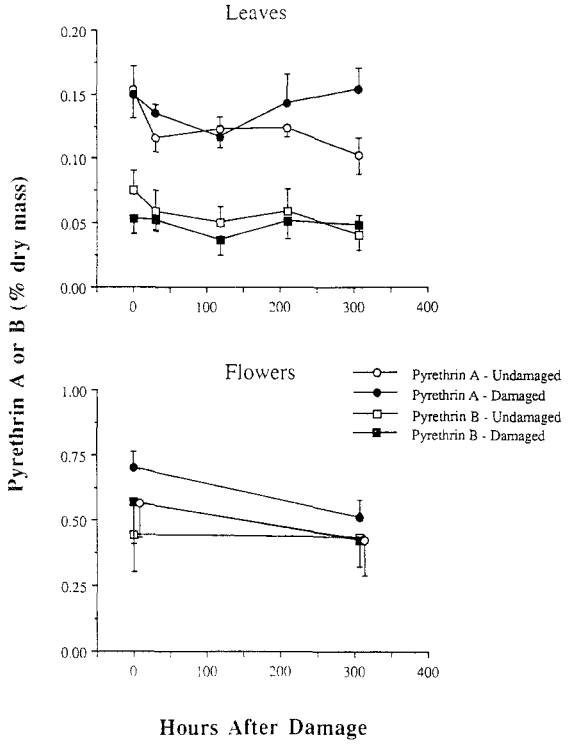

FIG. 2. Mean pyrethrin A or B (±SEM) concentrations of leaves or flowers from five undamaged and five damaged *C. cinerariaefolium* plants grown in a field plot in Amherst, New York. Plants were damaged at time 0.

were kept in the dark at −5°C until analysis. When fresh-frozen leaf material was spiked with 5–100 μg of total pyrethrin, greater than 95% of the added amount was recovered in a single methanolic extraction when the concentration of leaf material was less than 90 mg/ml. Extracts were centrifuged to remove debris before HPLC analysis. Dilutions of a commercial standard (Premium Pyrocide; McLaughlin, Gormely, King Co., Minneapolis, Minnesota) containing 10.98% pyrethrin A and 8.92% pyrethrin B were used as an external standard for the quantification. Pyrethrin A represents a mixture of pyrethrin I, cinerin I, and jasmolin I, whereas pyrethrin B represents a mixture of pyrethrin II, cinerin II, and jasmolin II. Pyrethrin A and B were separated using a reverse-phase HPLC on a Versapack C-18 (250 × 4.1 mm; 10 μm) column (Alltech, Avondale, Pennsylvania) with an LC-18 Pellicular Packing (Supelco, State College, Pennsylvania) containing a guard column and an isocratic mobile phase consisting of 77% aqueous methanol. At a 1.3 ml/min flow rate, pyrethrins A

and B had retention times of 14 and 7 min, respectively. Pyrethins were detected by their absorbance at 254 nm and peak areas were integrated with an HP 3392 integrator. Leaf and flower extracts had peaks that coeluted with the pyrethrins A and B standards; these coeluting peaks were collected separately from the HPLC, rotoevaporated to dryness, taken up in 100 μl of acetonitrile, and subjected to GC-MS analysis (50-m × 0.20-mm HP-5 column, cool on-column injection onto a 1-m × 0.53-mm precolumn, 80–250°C temperature ramp at 10°C/min, 70 eV EI with an HP 5971 Mass Selective Detector). The pyrethrin A HPLC peak contained well-resolved cinerin I, jasmolin I, and pyrethrin I peaks and their mass spectra matched those in the Wiley 138K Mass Spectra Database. The pyrethrin B HPLC peak contained a well-resolved cinerin II peak and broad jasmolin II and pyrethrin II peaks and their mass spectra matched those reported by Kawano et al. (1974). Due to the notorious thermal instability of these esters, we did not attempt to quantify the relative amounts of pyrethrin, cinerin, and jasmolin in the pyrethrin A and B peaks by GC-MS. However, using the straight-phase HPLC technique of Mourot et al. (1978), we separated the pyrethrins, cinerins, and jasmolins in the pyrethrin A and B peaks from the analytical standard, and a pooled flower and leaf extract of damaged and undamaged field-grown plants. In all three samples, pyrethrins I and II represented more than 90% of pyrethrin A and B peak areas, respectively. In expressing the pyrethrin A and B values as percentages of leaf and flower mass, we are assuming that the relative ratios of the three esters in each HPLC peak were not strongly influenced by damage. Given that the molar extinction coefficients of the two cinerins and jasmolins are very similar (ϵ_{229nm} = 21,000–28,000) and that pyrethin I and II represent the majority of the total pyrethrins in both our analytical standard and our plant extracts, we feel that this assumption is justified.

Statistical Methods. Repeated-measures one-way ANOVAs with damage as the main effect were used to analyze the pyrethrin estimates of both field and greenhouse experiments. Analysis was performed with the MGLH ANOVA module from Systat Inc. (Evanston, Illinois).

RESULTS AND DISCUSSION

Pyrethrin concentrations in *C. cinerariaefolium* were not affected by damage (Figures 1 and 2). No statistically significant differences were found in the pyrethrin concentrations of leaves from damaged and undamaged plants for both the greenhouse experiment (pyrethrin A: $F_{1,9} = 0.003$, $P = 0.954$; pyrethrin B: $F_{1,9} = 0.024$, $P = 0.880$) and the field experiment (pyrethrin A: $F_{1,8} = 1.117$, $P = 0.321$; pyrethrin B: $F_{1,8} = 0.270$, $P = 0.617$). Likewise, the effect of leaf damage on flower pyrethrin concentrations was not significant (pyrethrin

A: $F_{1,8} = 0.580$, $P = 0.468$; pyrethrin B: $F_{1,8} = 0.112$, $P = 0.746$). Expanding leaves had significantly higher pyrethrin A and B concentrations than did fully expanded leaves (pyrethrin A: $F_{1,9} = 6.201$, $P = 0.035$; pyrethrin B: $F_{1,9} = 5.541$, $P = 0.043$), which may reflect a higher density of pyrethrin secretory structures in young leaves (Zito et al., 1983).

Foliar and floral pyrethrins are constitutively produced and their biosynthesis is not induced within 13 days of damage. The amount of pyrethrins produced in a leaf may be developmentally constrained by the number of pyrethrin secretory canals or glands produced early in leaf ontogeny. Although the morphological "packaging" of secondary metabolite synthesis and storage may constrain inducibility for pyrethrins, the production of other terpenoid secondary metabolites, such as the oleoresins in *Pinus pinaster* (Walter et al., 1989, Marpeau et al. 1989), are not similarly constrained. The lack of inducibility of pyrethrins may have ecological explanations; for example, the increased pyrethrin concentration above the constitutive level may not deter potential herbivores. Understanding the patterns of inducibility among secondary metabolites may provide important insight into the roles these compounds play in plants.

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DEFENSIVE SECRETION OF TWO NOTODONTID CATERPILLARS (*Schizura unicornis*, *S. badia*)[1]

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Abstract—The cervical gland secretion of larvae of *Schizura unicornis* and *S. badia* (Lepidoptera: Notodontidae) contains formic acid, acetic acid, and a mixture of lipophilic compounds, including 2-alkanones, 2-alkanols, and formates of the alkanols. In *S. unicornis*, the secretion also contains several ω-monounsaturated analogs of the alcohols, ketones, and formates. The absolute configuration of two of the alcohols (2-tridecanol and 2-pentadecanol) was established as *S* in both species. The larvae spray their secretion when physically disturbed, aiming it accurately in the direction of the offending agent.

Key Words—Lepidoptera, Notodontidae, *Schizura*, defensive secretion, formic acid, 2-tridecanone, (*S*)-2-tridecanol, 2-tridecyl formate, 2-pentadecanone.

INTRODUCTION

Two chemical defensive strategies are commonly exhibited by notodontid caterpillars upon disturbance. One, shared with caterpillars of many other families, involves regurgitation of enteric fluid (see, for instance, Figure 7A in Eisner, 1970). The other involves discharge of secretory fluid from a gland, the cervical gland, opening ventrally in the neck region just behind the head. As early as 1887, Poulton investigated the secretion of the cervical gland of one species,

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Dicranura vinula, and found it to contain formic acid. Later investigators confirmed the production of formic acid by this and other species, but found that the secretion could also contain lipophilic components, such as 2-undecanone, 2-tridecanone, decyl acetate, and dodecyl acetate (Monro, Meinwald, and Eisner, cited in Roth and Eisner, 1962; Schildknecht and Schmidt, 1963; Eisner et al., 1972; Weatherston et al., 1979). Moreover, the complement of lipophilic constituents appeared to differ between species, indicating that the secretion was more variable than initially envisioned. We recently had the opportunity to study two notodontid larvae, *Schizura unicornis* and *S. badia* (Figure 1), that had not previously been studied chemically. We here report on the composition of their defensive secretion.

METHODS AND MATERIALS

Biology. The larvae were offspring of single females of *S. unicornis* and *S. badia* taken at light in late June 1990, in Ithaca, New York (the original females are on deposit in the Cornell University Insect Collection, voucher lot # 1203). The two moths were allowed to oviposit and the larvae were reared on cuttings of *Prunus serotina* (*S. unicornis*) and *Viburnum lentago* (*S. badia*), known natural hosts of these *Schizura* species (J.G. Franclemont, Cornell University, personal communication).

For collection of secretion, glands were excised whole from prechilled last-instar larvae (to prevent pupation, these larvae had been kept live at 4°C for approximately three weeks prior to gland removal).

For demonstration of larval spraying behavior, the same technique was used as employed previously with arthropods that discharge acid secretions (Eisner et al., 1961). Individual larvae were released on sheets of deep-red indicator paper (filter paper impregnated with alkaline phenolphthalein solution) and stimulated by pinching their bodies with forceps. If the larvae responded by spraying, the direction of discharge became clearly depicted by the pattern of white dots induced by the secretion on the paper.

Sample Preparation. The excised glands (seven from *S. unicornis*; 10 from *S. badia*) were crushed in prechilled vials after addition of 100 μl of hexane, and the extracts were analyzed directly by GC and GC-MS.

Chemicals. 2-Tridecanone, 2-pentadecanol, 2-heptadecanone, and (*S*)-(+)-lactic acid were purchased from Aldrich Chemical Co. The secondary alcohols were obtained by reducing the corresponding ketones with $LiAlH_4$, and the formates were synthesized by heating the alcohols with 88% formic acid. 2-Tridecyl octanoate and 1-dodecyl octanoate were synthesized by esterifying the corresponding alcohols with octanoic acid. 14-Pentadecen-2-one was a gift of W. Francke (University of Hamburg).

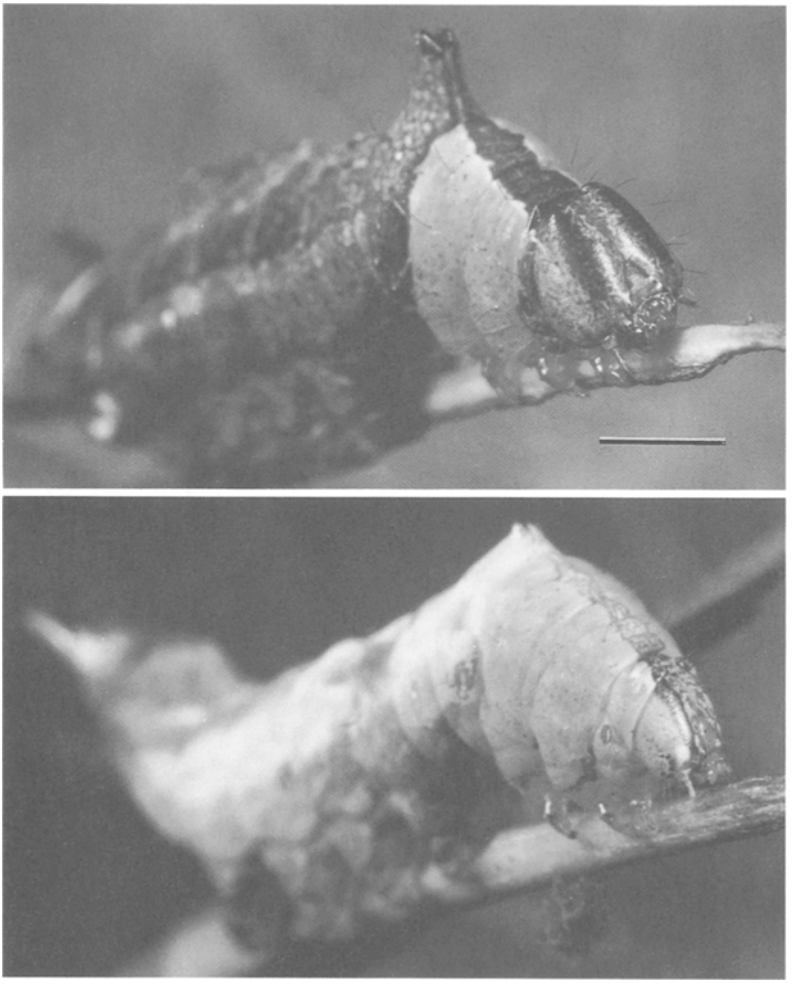

FIG. 1. Last instar larvae of *Schizura unicornis* (top) and *Schizura badia* (bottom). Reference bar, both figures = 2 mm.

Derivatization. Pentafluorobenzyl esters of carboxylic acids and dimethyl disulfide derivatives of unsaturated compounds were prepared as described earlier (Attygalle and Morgan, 1986). The 2-alkanols were derivatized with acetyl (*S*)-lactyl chloride made from (*S*)-(+)-lactic acid (Slessor et al., 1985).

Analytical Procedures. Gas chromatography was performed on a Hewlett-Packard (HP) 5890 instrument equipped with a splitless injector, a flame ionization detector (FID), and an HP 3396A integrator. The following 25-m ×

0.22-mm fused-silica columns were used: (A) DB-1; (B) DB-5; (C) FFAP. A solution prepared from commercial formic acid (91.2%, Mallinckrodt) was used as an external standard for quantitative evaluations by ion-trap detection of acidic components. Similarly, a standard solution of 2-heptadecanone was used to estimate the detector response for the quantification of nonacidic components.

Mass spectra were obtained using the FFAP column installed in an HP 5890 gas chromatograph linked to a Finnigan ion-trap detector (ITD). Chemical ionization mass spectra were obtained on the ITD with methane as the reagent gas.

RESULTS

Biology. Disturbing larvae by hand usually caused them to discharge. Ejections were always accompanied by the unmistakable pungent odor of formic acid. Dissection revealed the gland in both species to be identical to that of the congeneric *Schizura leptinoides* (Figure 2) and *S. concinna* (Detwiler, 1922; Percy and MacDonald, 1979; Weatherston et al., 1979); this resemblance between *S. unicornis* and *S. concinna* had been noted previously (Detwiler, 1922). The gland consists of a large membranous inner sac, and a smaller, somewhat more rigid chamber, interposed between the sac and the gland opening. The opening itself, in the neck region, is a narrow slit.

Pinching larvae with forceps as they crawled on filter paper showed that they aim their discharges. They raised the head and revolved their front end toward the forceps, thereby bringing the gland opening to within near contact of the instrument, and sprayed (Figure 3). The range of initial discharges commonly exceeded 20 cm, attesting to the considerable force of the ejections. Up to five discharges could be elicited from individual larvae. Larvae invariably doused themselves when spraying, as evidenced by the white trail they left on the indicator paper as they crawled away following stimulation.

Chemistry. Analysis of the two extracts by GC-MS, using the FFAP column, revealed the presence of large quantities of formic acid together with small amounts of acetic acid. The presence of acetic acid was confirmed by forming its pentafluorobenzyl ester (mass spectrum and gas chromatographic retention time indistinguishable from those of authentic pentafluorobenzyl acetate). The amount of formic acid per gland in *S. badia* was about 2 mg; acetic acid was present at about 0.1% of this amount.

In addition to the two acids, a number of lipophilic constituents (estimated to total ca. 5% relative to the acidic components in the case of *S. badia*, based on gas chromatographic evidence) were present in the glandular secretion of both species. Gas chromatograms obtained from the nonacidic constituents are shown in Figure 4. The major nonacidic component in *S. unicornis*,

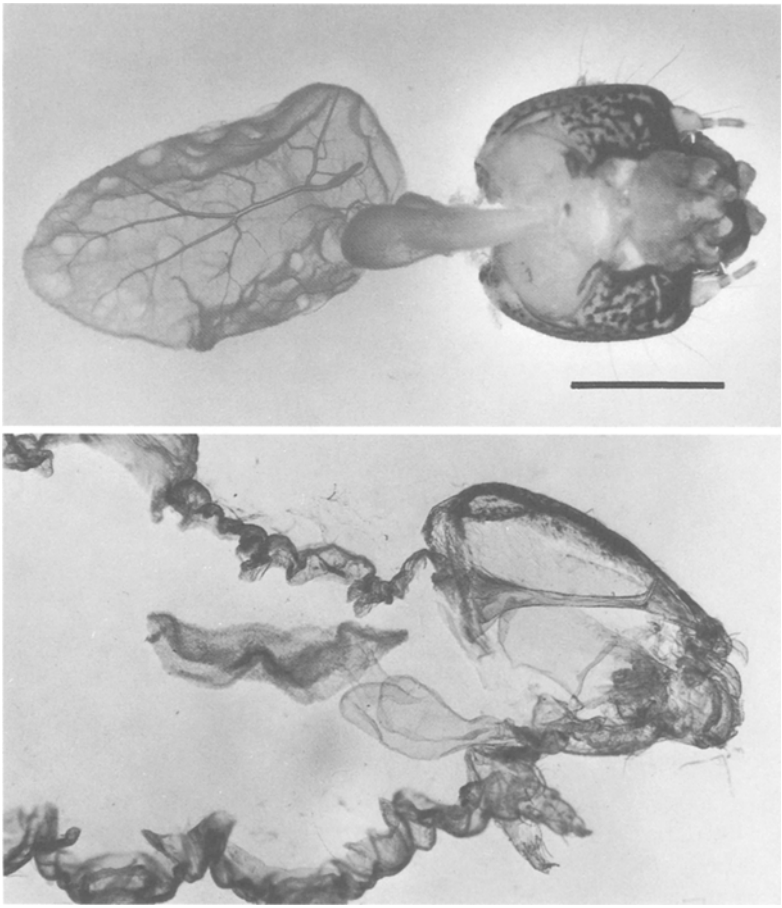

FIG. 2. Defensive glands of *Schizura leptinoides* (the gland in *S. unicornis* and *S. badia* is identical to the one shown here). Top: Head with gland attached, ventral view (penultimate instar). Note the large saclike inner chamber and the smaller outer compartment. Bottom: Front end of larva (last instar) in profile view (KOH-treated preparation, consisting of cuticle only). The inner compartment of the gland is empty and collapsed. The more rigid outer compartment is also empty but has retained its shape. Note that the gland opens ventrally in the neck region immediately behind the head capsule. Reference bar, both figures = 2 mm.

2-tridecanone, was accompanied by two other methyl ketones, 2-pentadecanone and 2-heptadecanone. In *S. badia*, 2-tridecanone and 2-pentadecanone were present in different relative amounts (Table 1). These three ketones were readily recognized by their characteristic mass spectra. The spectra all showed a base

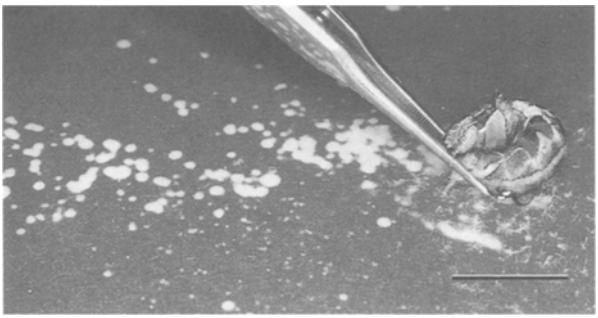

FIG. 3. *Schizura unicornis* larva (last instar) discharging an aimed jet of secretion in response to pinching of its rear with forceps. Note that the larva has rotated its front end so as to direct the head (and therefore the gland opening) toward the forceps. The pattern of the spray is revealed by the white dots on the indicator paper. Reference bar = 1 cm.

peak at *m/z* 43, McLafferty ions at *m/z* 58, and the corresponding molecular ions at *m/z* 198, 226, and 254, respectively.

The alcohols corresponding to 2-tridecanone and 2-pentadecanone were recognized in extracts from both species. In addition, 2-heptadecanol was identified in the extract of *S. unicornis*. These alcohols were recognized by their characteristic mass spectra; the identifications were confirmed by comparison with authentic material. The absolute configurations of 2-tridecanol and 2-pentadecanol were determined by derivatizing the alcohols with acetyl (*S*)-lactyl chloride (Slessor et al., 1985). The two pairs of diastereoisomeric esters obtained from racemic 2-tridecanol and 2-pentadecanol each gave baseline separations on DB-5 and FFAP capillary columns. The acetyl (*S*)-lactyl esters derived from (*R*)-2-tridecanol and (*R*)-2-pentadecanol are known to elute prior to those of the *S* enantiomers on DB-5 (Bartelt et al., 1989; Schaner et al., 1989). This correlation established the enantiomeric compositions of these alcohols from *S. unicornis* and *S. badia* (Table 2). Both *Schizura* alcohols have the *S* configuration. However, in *S. badia*, in which 2-tridecanol is a major component, a small amount (<5%) of the earlier eluting *R* isomer was also observed. We are unable to comment on the significance of this observation as neither the degree of racemization during the derivatization procedure nor the optical purity of the acetyl (*S*)-lactyl chloride acid was determined independently. The identification of these derivatives from *Schizura* extracts was confirmed by GC-MS analysis on the FFAP column. We observed that the elution order of the diastereoisomers on FFAP was identical to that reported on DB-5. The mass spectra of the two diastereoisomers in each pair were very similar to each other. The EI mass spectrum of (*S*)-2-tridecyl acetyl-(*S*)-lactate obtained from the ITD was as follows: *m/z*(%), 182(1), 133(20), 127(5), 115(17), 105(12), 97(8), 88(13),

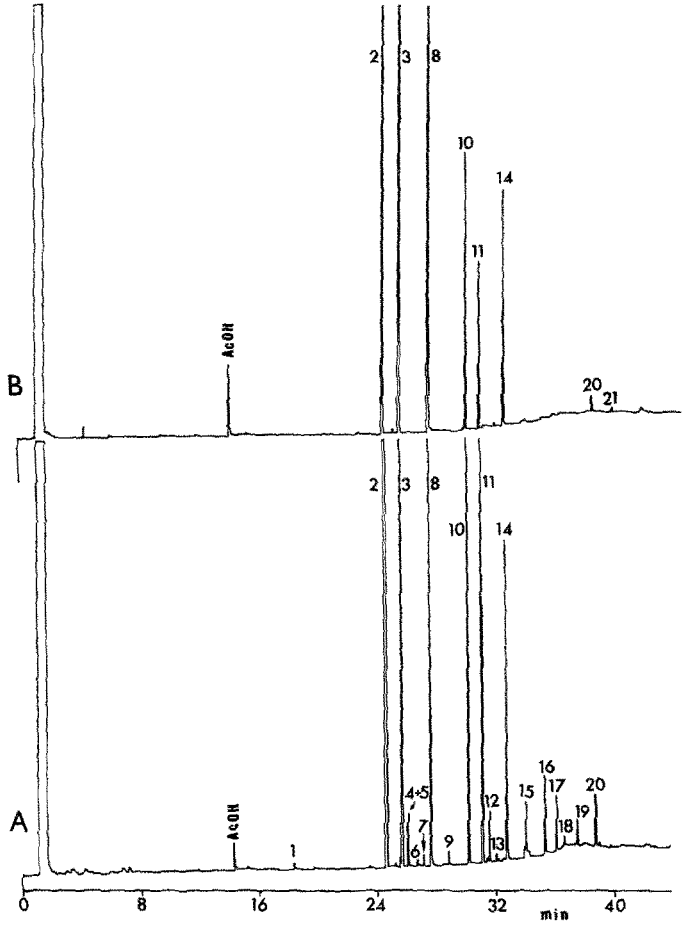

FIG. 4. FID gas chromatograms depicting volatiles in defensive secretion of *S. unicornis* (A) and *S. badia* (B). A 30-m × 0.25-mm fused silica capillary coated with FFAP stationary phase (0.25 μm) was used. The oven temperature was held at 60°C for 4 min and programmed at 4°C/min to 190°C. Numbers identifying peaks correspond to entries in Table 1.

87(13), 85(13), 83(8), 71(16), 70(10), 69(10), 61(7), 57(29), 55(18), 43(100), 41(37). The spectrum of (*S*)-2-pentadecyl acetyl-(*S*)-lactate was: *m/z*(%), 210(1), 133(23), 115(17), 105(16), 97(10), 88(15), 87(13), 85(17), 83(9), 71(22), 70(9), 69(13), 61(9), 57(33), 55(20), 43(100), 41(31).

The formate esters of these alcohols were also present (Table 1), as demonstrated by comparison with synthetic standards.

A number of terminally unsaturated compounds were also detected in the

TABLE I. NONACIDIC COMPONENTS OF DEFENSIVE SECRETIONS OF *S. unicornis* AND *S. badia* AND MASS SPECTRAL EVIDENCE FOR STRUCTURE ASSIGNMENTS

Compound[a]	% by weight[b]		EI mass spectrum (m/z, %)[c]	CI [CH_4] mass spectrum (m/z, %)[c]
	S. unicornis	*S. badia*		
1. 2-Undecanone $[CH_3(CH_2)_8COCH_3]$	0.1		170(M^+, 5), 155(3), 127(2), 113(2), 112(2), 110(3), 85(6), 71(19), 59(20), 58(66), 43(100), 41(32).	171(100)
2. 2-Tridecanone $[CH_3(CH_2)_{10}COCH_3]$	51.3	20.0	198(M^+, 4), 183(1), 85(9), 71(20), 59(28), 58(63), 43(100), 41(38).	199(100)
3. 2-Tridecyl formate $[CH_3(CH_2)_{10}CH(CH_3)OCOH]$	14.8	34.3	228(M^+, 0), 182(M^+-46, 6), 154(5), 139(5), 125(17), 111(29), 97(55), 83(50), 69(61), 57(50), 55(70), 45(76), 43(65), 41(100).	183(57)
4. 12-Tridecen-2-one $[CH_2=CH(CH_2)_9COCH_3]$	0.9		196(M^+, 0), 138(1), 125(6), 111(6), 97(9), 83(6), 82(6), 81(7), 71(17), 69(8), 67(10), 58(30, 55(24), 43(100), 41(28).	197(16), 179(15), 95(100)
5. 2-Tridecyl acetate $[CH_3(CH_2)_{10}CH(CH_3)OCOCH_3]$	0.3		242(M^+, 0), 182(M^+-60, 4), 111(10), 97(14), 83(14), 69(15), 55(21), 43(100).	183(26)
6. Dodecyl acetate $[CH_3(CH_2)_{10}CH_2OCOCH_3]$	0.2		168(M^+-60, 4), 140(5), 111(13), 97(18), 83(25), 69(30), 61(26), 55(35), 43(100), 41(50).	
7. 12-Tridecen-2-yl formate $[CH_2=CH(CH_2)_9CH(CH_3)OCOH]$	0.3		226(M^+, 0), 180(M^+-46, 6), 152(5), 138(9), 124(8), 123(7), 110(18), 109(17), 96(39), 95(35), 82(45), 81(60), 69(28), 68(43), 67(61), 55(81), 45(60), 41(100).	
8. 2-Tridecanol $[CH_3(CH_2)_{10}CH(OH)CH_3]$	7.0	29.7	200(M^+, 0), 185(M^+-15, 2), 182(M^+-18, 1), 154(2), 139(1), 138(1), 125(6), 111(13), 97(21), 85(9), 83(17), 71(11), 70(10), 69(18), 57(23), 55(22), 45(100), 43(28), 41(40).	199(10)

9. 12-Tridecen-2-ol [CH_2=$CH(CH_2)_9CH(OH)CH_3$]	0.2		198(M^+, 0) 183(M^+-15, 1), 180(M^+-18, 2), 151(2), 124(5), 123(5), 109(10), 96(19), 95(21), 82(25), 81(32), 69(23), 67(33), 55(50), 45(100), 43(26), 41(61).	
10. 2-Pentadecanone [$CH_3(CH_2)_{12}COCH_3$]	7.1	6.3	226(M^+, 6), 211(2), 96(9), 85(8), 71(22), 59(35), 58(66), 43(100).	227(100)
11. 2-Pentadecyl formate [$CH_3(CH_2)_{12}CH(CH_3)OCOH$]	8.9	3.8	256(M^+, 0), 210(M^+-46, 7), 182(3), 167(3), 166(2), 153(3), 152(4), 139(7), 138(6), 125(18), 111(35), 97(53), 83(52), 69(52), 57(52), 55(71), 45(64), 43(65), 41(100)	211(13)
12. 14-Pentadecen-2-one [CH_2=$CH(CH_2)_{11}COCH_3$]	0.7		224(M^+, 0), 166(M^+-58, 1), 125(10), 111(6), 97(7), 96(8), 95(7), 85(7), 81(12), 71(22), 58(42), 55(30), 43(100), 41(41).	225(61)
13. 14-Pentadecen-2-yl formate [CH_2=$CH(CH_2)_{11}CH(CH_3)OCOH$	0.2		254(M^+, 0), 208(M^+-46, 4), 166(5), 152(2), 138(7), 124(12), 123(13), 110(19), 96(37), 95(37), 82(43), 81(60), 67(55), 55(85), 45(44), 43(34), 41(100).	
14. 2-Pentadecanol [$CH_3(CH_2)_{12}CH(OH)CH_3$]	4.4	5.2	228(M^+, 0), 227(M^+-1, 0.1), 213(M^+-15, 2), 210(2), 182(1), 152(1), 139(2), 125(8), 111(13), 97(19), 83(18), 71(13), 69(18), 57(23), 55(22), 45(100), 3(28), 41(41).	227(20)
15. 14-pentadecen-2-ol [CH_2=$CH(CH_2)_{11}CH(OH)CH_3$]	0.7		226(M^+, 0), 211(M^+-15, 1), 208(M^+-18, 3), 180(2), 166(3), 152(4), 138(5), 137(5), 124(7), 123(6), 110(12), 109(10), 96(26), 95(23), 83(17), 82(29), 81(34), 69(28), 67(31), 55(58), 45(100), 43(29), 41(64).	225(4)
16. 2-Heptadecanone [$CH_3(CH_2)_{14}COCH_3$]	1.0		254(M^+, 10), 239(3), 236(2), 211(1), 196(1), 194(2), 110(5), 96(14), 85(12), 71(28), 59(44), 58(66), 43(100), 41(43).	255(100)

TABLE I. CONTINUED

Compound[a]	% by weight[b] *S. unicornis*	% by weight[b] *S. badia*	EI mass spectrum (m/z, %)[c]	CI [CH_4] mass spectrum (m/z, %)[c]
17. 2-Heptadecyl formate $[CH_3(CH_2)_{14}CH(CH_3)OCOH]$	0.6		284(M^+, 0), 238(M^+-46, 6), 210(2), 195(2), 194(3), 181(3), 180(2), 166(5), 153(3), 139(10), 138(11), 125(20), 111(40), 97(58), 83(49), 69(50), 57(49), 55(61), 45(51), 43(61), 41(100)	
18. 16-Heptadecen-2-one $[CH_2{=}CH(CH_2)_{13}COCH_3]$	0.2		252(M^+, 0), 194(2), 125(14), 111(7), 96(14), 71(22), 69(14), 67(15), 58(50), 43(100).	253(35)
19. 2-Heptadecanol $[CH_3(CH_2)_{14}CH(OH)CH_3]$	0.4		256(M^+, 0), 241(M^+-15, 3), 238(M^+-18, 1), 195(1), 166(1), 153(2), 152(2), 139(4), 138(3), 125(10), 111(15), 97(24), 85(9), 83(18), 71(12), 69(17), 57(26), 55(24), 45(100), 43(30), 41(43).	255(35)
20. 2-Tridecyl octanoate $[CH_3(CH_2)_{10}CH(CH_3)OCO(CH_2)_6CH_3]$	0.7	0.5	326(M^+-0), 183(8), 182(11), 145(39), 144(25), 127(85), 111(18), 97(27), 83(29), 69(28), 60(12), 57(90), 55(55), 43(69), 41(100).	145(100)
21. Dodecyl octanoate $[CH_3(CH_2)_{10}CH_2OCO(CH_2)_6CH_3]$		0.2	312(M^+, 4) 213(4), 168(8), 145(64), 127(16), 111(19), 97(24), 83(31), 69(30), 61(12), 57(59), 55(51), 43(60), 41(100)	

[a] Numbers refer to Figure 4.
[b] Pooled samples; *S. unicornis* $N = 7$; *S. badia* $N = 10$.
[c] Ion trap mass spectra.

secretion of *S. unicornis*. Interestingly, none of these unsaturated compounds was detectable in the extract of *S. badia* (Table 1). The double-bond positions were established by the DMDS derivatization method (Buser et al., 1983; Francis and Veland, 1981). The mass spectra of these DMDS derivatives are shown in Table 3. That of the derivative of the tridecenone showed a molecular ion at *m/z* 290, and two intense ions at *m/z* 229 $[CH_3-CO-(CH_2)_9-CH=SCH_3]^+$ and 61 $[CH_2=SCH_3]^+$ arising from cleavage between the vicinal thiomethyl groups, thereby identifying the parent compound as 12-tridecen-2-one. Similarly, the other unsaturated ketone peaks were identified as 14-pentadecen-2-

TABLE 2. RETENTION TIMES[a] AND RELATIVE AMOUNTS OF ACETYL (*S*)-LACTYL DIASTEREOMERS OF 2-TRIDECANOL AND 2-PENTADECANOL

	2-Tridecanol				2-Pentadecanol			
	First-eluting diastereomer[b]		Second-eluting diastereomer[b]		First-eluting diastereomer[b]		Second-eluting diastereomer[b]	
Source	R_t (min)	Relative percent	R_t (min)	Relative percent	R_t (min)	Relative percent	R_t (min)	Relative percent
Synthetic racemate	39.86	49	40.13	51	43.69	50	43.91	50
S. badia	39.87	5	40.11	95			43.90	100
S. unicornis			40.15	100			43.94	100

[a] A 30-m × 0.25-mm fused silica capillary column coated with DB-5 (0.25 μm) was used. The oven temperature was held at 40°C for 4 min and programmed at 5°C/min to 260°.
[b] First- and second-eluting diastereomers correspond respectively to the *R* and *S* alcohol configurations.

TABLE 3. EI MASS SPECTRA OF DMDS DERIVATIVES OF UNSATURATED COMPOUNDS IN *S. unicornis* DEFENSIVE SECRETION

Compound	Mass spectrum of DMDS derivative (*m/z*, %)
12-Tridecen-2-one	290(M^+, 26), 229(73), 81(33), 61(50), 43(100)
12-Tridecen-2-yl formate	320(M^+, 38), 259(M^+-61, 36), 213[(M^+-61)-46, 100], 61(36), 45(72)
12-Tridecen-2-ol	292(M^+, 20), 213(53), 61(78), 45(100).
14-Pentadecen-2-one	318(M^+, 13), 257(M^+-61, 100), 109(16), 95(20), 81(17), 61(45), 43(98)
14-Pentadecen-2-yl formate	348(M^+, 24) 287(M^+-61, 39), 241[(M^+-61)-46, 100], 81(42), 61(71), 45(69).
14-Pentadecen-2-ol	320(M^+, 0), 259(35), 241(56), 61(71), 45(100).
16-Heptadecen-2-one	346(M^+, 6), 285(M^+-61, 100), 61(42), 43(78)

one and 16-heptadecen-2-one. The mass spectra of DMDS derivatives of the unsaturated formates 7 and 13 were particularly interesting; while EI-mass spectra of formates rarely show significant molecular ions, those of these DMDS derivatives are particularly strong (Table 3).

Peak number 20 in Figure 4 was identified as 2-tridecyl octanoate from its mass spectrum, although we were unable to observe a molecular ion even under chemical ionization (CI) conditions. Nevertheless, the peak at *m/z* 145, (protonated octanoic acid), was diagnostic for an octanoate ester. A synthetic sample of 2-tridecyl octanoate gave a mass spectrum, as well as retention times on DB-5 and FFAP columns, indistinguishable from those of the natural compound. In addition to 2-tridecyl octanoate, the glandular liquid of *S. badia* also contains 1-dodecyl octanoate, which was identified by comparison with a synthetic sample.

DISCUSSION

The defensive behavior of *Schizura unicornis* and *S. badia* is similar to that of other spraying notodontid caterpillars that have been studied (Poulton, 1887; Herrick and Detwiller, 1919; Eisner et al., 1972; Weatherston et al., 1979). Spraying notodontid larvae probably all aim their ejections, and effect aiming by postural adjustment of their front end. Such postural maneuvering may have evolved in the context of administration of oral effluent. Caterpillars, including notodontids, that regurgitate when disturbed, commonly rotate their front end so as to deliver the disgorged fluid onto the region of their body subjected to assault. We view such behavior, given its widespread occurrence in caterpillars, as being primitive in notodontids, and as having "set the stage" for the later evolution of a defensive behavior making use of an exocrine gland with an anteriorly positioned opening close to the mouth.

Notodontids that spray have not necessarily foregone the tendency to regurgitate when disturbed. We noted both *S. unicornis* and *S. badia* to emit oral fluid when they were stimulated with forceps, but usually only after they had already sprayed one or more times. In our experience, caterpillars that regurgitate but lack defensive glands (including certain notodontids and other species), tend to regurgitate readily, often even after a first poking.

The presence of small amounts of lipophilic compounds in secretions that are fundamentally hydrophilic and acidic, such as those of the two *Schizura* species discussed here, is not unusual. Lipophilic compounds had previously been reported from the secretions of two other notodontid larvae, *Lochmaeus* (*Heterocampa*) *manteo* and *Schizura concinna* (Eisner et al., 1972; Weatherston et al., 1979), and we predict that notodontid cervical gland secretions generally will turn out to contain such components. Lipophilic "additives" are present in

other acidic secretions as well. They occur, for instance, in the spray of certain carabid beetles (Eisner et al., 1968; Attygalle et al., 1992) whose principal component is also formic acid, and in the acetic acid-based spray of a whip scorpion. In the whip scorpion secretion, the lipophilic additive is caprylic acid, which serves as a wetting agent, promoting spreading and penetration of the secretion on target (Eisner et al., 1961). In our view, lipophilic components may generally fulfill such wetting functions in acidic arthropodan defensive secretions. This is not to say that lipophilic components are purely neutral as regards actual repellency of the mixtures. Caprylic acid, for instance, has intrinsic deterrency vis à vis insects (Eisner et al., 1961), as does 2-tridecanone (Eisner et al., 1972), a component not only of the *S. unicornis* and *S. badia* secretions, but also of that of other notodontids (Eisner et al., 1972; Weatherston et al., 1979). 2-Tridecanone is, in fact, secreted as an antiinsectan agent by glandular hairs of wild tomato plants (Williams et al., 1980).

Weatherston et al. (1979) have provided an adaptive explanation for the two-chambered nature of these notodontid glands. Working with *Schizura concinna*, they found formic acid to be produced by the inner saclike chamber, and the lipophilic components (2-tridecanone, decyl acetate, dodecyl acetate), by the smaller outer chamber. We assume such segregation of secretory function to prevail in other notodontids with a two-chambered gland, and that in *S. unicornis* and *S. badia* the acidic and lipophilic components are produced respectively by the inner and outer chambers of the gland. However, larvae of several species of formic acid-spraying notodontids are said to possess single-chambered glands; it is not known whether in these species the secretion also contains lipophilic components (Weatherston et al., 1986).

Overall, the caterpillar defensive sprays characterized in this study reveal little chemical novelty. The presence of small amounts (ca. 0.1%) of acetic acid in formic acid-containing secretions may be a widespread phenomenon. Recently, we found the defensive secretion of a carabid beetle, *Helluomorphoides clairvillei*, to consist of such a mixture (Attygalle et al., 1992). Even the formic acid-based spray of the formicine ant, *Camponotus floridanus*, contains a small fraction of acetic acid (Attygalle, Meinwald, and Eisner, unpublished).

Aliphatic ketones are frequently found in many insect defensive secretions; methyl ketones containing 11–19 carbon atoms are particularly common in secretions of Hymenoptera (Blum, 1981). Terminally unsaturated ketones have been reported only from a small number of insects (Prestwich et al., 1975; Prestwich and Collins, 1982; Tengö et al., 1985; Jackson et al., 1990). To the best of our knowledge, the terminally unsaturated alcohols, their formates, and the heptadecenone we report here have not been described from nature previously, although two incompletely characterized tridecen-2-ol isomers, along with (*Z*)-4-tridecen-2-ol have been reported from *Andrena* bees (Francke et al., 1981).

Methyl carbinols have frequently been reported from both bees and ants

(Luby et al., 1973; Tengö and Bergström, 1977; Blum, 1981; Slessor et al., 1985; Francke et al., 1987; Lloyd et al., 1989; Wittmann et al., 1989), and the finding of two of these alcohols, 2-tridecanol and 2-pentadecanol, as lipophilic constituents from yet another group of insects should come as no surprise.

Formates of 2-alkanols appear to be rare in nature. Trace amounts of 2-undecyl and 2-tridecyl formates have been reported from the ant, *Formica rufa* (Francke et al., 1985). Lardolure, a formate of a methyl carbinol, is known as a pheromone from an acarid mite (Kuwahara et al., 1982). In the *Schizura* secretions, formate esters are among the major components.

While higher fatty acid esters are widespread in insect secretions (Blum, 1981), those of 2-alkanols are not common. Of the few examples known, 2-heptyl octanoate and nonanoate (Wittmann et al., 1989), and octanoates and decanoates of 2-alkanols (Tengö et al., 1985) have been reported from bees. A number of tridecyl esters, including a tridecyl octanoate, have been reported recently from ants in the genus *Myrmecocystus* (Lloyd et al., 1989), although it is not clear from the published data whether the *Myrmecocystus* compounds are 1-tridecyl or 2-tridecyl esters.

The compositions of the defensive secretions of the two *Schizura* reported here are considerably more complex than those from the few other notodontid species previously studied. However, this "complexity" stems from the characterization of constituents present in very small amounts. Evidently, the chemical knowledge gained from the application of current, more powerful analytical techniques can provide a more realistic view of the arthropodan chemical repertory.

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ALLELOPATHIC EFFECT OF PHENOLIC ACIDS FROM HUMIC SOLUTIONS ON TWO SPRUCE MYCORRHIZAL FUNGI: *Cenococcum graniforme* AND *Laccaria laccata*

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Abstract—The aim of this investigation was to determine the impact of *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, catechol, and protocatechuic acid on respiration of two spruce mycorrhizal fungi: *Laccaria laccata* and *Cenococcum graniforme*. These phenols are produced by *Vaccinium myrtillus*, *Athyrium filix-femina*, and *Picea abies*, predominant species of spruce forests in the Alps, and they are also present in humic solutions at 10^{-4} M or 10^{-5} M. Respiration of the two fungi was inhibited by the four phenolic acids, even at concentrations ranging from 10^{-5} M to 10^{-7} M. These data show phenolic acids from humic solutions have biological activity at extremely low concentrations, suggesting a contribution of *V. myrtillus*, *A. filix-femina*, and *P. abies* to allelopathic inhibition of mycorrhizal fungi.

Key Words—*Athyrium filix-femina*, allelopathy, *Cenococcum graniforme*, forest regeneration, humus, *Laccaria laccata*, mycorrhizal fungi, phenolic acid, *Picea abies*, respiration, *Vaccinium myrtillus*.

INTRODUCTION

Phenolic acids produced by plants have been reported to induce phytotoxicity (Rice, 1984). Previous results indicated that natural regeneration failure of subalpine spruce (*Picea abies* L. Karst) forests can be explained by allelopathic phenomena (Pellissier and Trosset, 1989a). They found that germination of spruce seeds and growth of ectomycorrhizal fungi are inhibited by extracts from bilberry leaves (*Vaccinium myrtillus* L.), fern fronds [*Athyrium filix-femina* (L.) Roth], and spruce needles. In other respects, allelopathic effects of vegetation or soil on mycorrhizae have been established (Persidsky et al., 1965).

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The objective of the study reported here was to investigate the role of allelochemicals produced by these plants, and persistent in soil, on respiration of two common ectomycorrhizal fungi of spruce, *Cenococcum graniforme* (Sow.) Ferd. and Winge and *Laccaria laccata* (Scop. ex Fr.) Berk and Br.

METHODS AND MATERIALS

The experimental procedure consisted of: (1) testing the effect of humic solutions on respiration of these two ectomycorrhizal fungi; (2) analyzing the biochemical contents of the plant extracts and humic solutions in order to define which phytotoxins are released from the plants into the soil; and (3) investigating, one by one, the effect of phenolic compounds identified simultaneously in humic solutions and foliar material on fungi respiration. Since one of the essential features in allelopathy is that phytotoxicity of a molecule depends on its concentration (Rice, 1984), we tested five decreasing concentrations in the range of 10^{-3}–10^{-7} M.

Plant Collection and Biochemical Analysis of Potentially Phytotoxic Solutions. Collection of the plant material and humic solutions was done in lots D (*Adenostylo alliariae-Piceetum*) and K (*Homogyno-Piceetum*) (Mayer, 1986) of the common woodland in Cohennoz, Savoie, France. As the biochemical composition of such solutions can vary through the year (Kuiters, 1987), samples were taken from mid-June to the end of August; i.e., during the most intense growing period in high-altitude spruce stands. The living plant material collected for leaf hydrolysates was taken from a variety of areas within the lot. It was air-dried at ambient temperature for three weeks, then ground and mixed to obtain a very fine, flour-type powder. The next stage was aqueous extraction by means of 12-hr agitation in demineralized water at a concentration equivalent to 1% dry matter. Aqueous extraction was selected in order to correspond to ecological conditions as closely as possible. Extraction was followed by filtration at 4°C, and sterilization of the filtrate on a 0.22-μm Millipore membrane before being used. The humic solutions were collected after rainy periods using gutters derived from a system devised by Dambrine (1985) for use in upper mountain region soils. Since these solutions were used for testing on living tissue, hydrochloric acid could not be added to the collection bottle to halt any biochemical changes that might occur (Haider and Martin, 1975). This meant the weather had to be watched closely for rain and the solutions collected as quickly as possible and stored at −18°C until utilized (less than four weeks). Like the leaf extracts, they were sterilized on a 0.22-μm Millipore membrane when finally used.

For hydrolysis, 50 ml aqueous leaf extract was put into 100 ml 2 N hydrochloric acid in a conical flask, in a water bath at 100°C. The humic solution–

HCl volumetric ratio was 1:2. After 40 min, the content was cooled under cold running water.

The mother solution for the dosage of the total anthocyans was successively extracted with 60, 60, and 40 ml ethyl ether. The anthocyans remain in the red acid hypophase, and the flavones, flavonols, and a proportion of the phenolic acids, more lipophilic, pass into the ethereal epiphase. The volume of aqueous solution was recorded and, following filtration on sintered glass, the absorbance spectra were obtained immediately by scanning between 400 and 600 nm.

The ethereal epiphase was evaporated to dryness under a fume hood, then taken up by 20 ml ethanol distilled at 95°C. The reference solution contained 0.1 ml of the alcohol solution to be dosed in the presence of 5 ml ethanol distilled at 95°C. The reactant solution contained 0.1 ml alcohol and 5 ml of 1% aluminum chloride ($AlCl_3$) in ethanol. After a 10-min reaction, a yellow color appeared and the differential spectrum was recorded between 350 and 550 nm. The presence of flavones can be detected by a maximum absorption peak at 390 to 415 nm, and flavonols at 420–440 nm.

High-performance liquid chromatography for separation and subsequent identification of phenolic acids and anthocyans was performed using a Waters apparatus, 5000 A pump and M 440 detector. For the phenolic acids, the liquid phase was a mixture of solvent A: 0.5% acetic acid; and solvent B: 0.5% acetic acid and 99.5% acetonitrile. A linear gradient system ensures optimum separation. The concentration of B in A went from 0 to 35% in 35 min. A methanol–water–acetic-acid (45:55:05) ternary solution was used for the anthocyans, under an isocratic regime. In both cases a μBondapack C18 column (Waters) was used.

Spectrophotometric detection of the phenols was done by multidetection at 250, 260, 270, 280, 290, 300, 310, 320 and 330 nm; the flavonols, at 365 nm. Compound content was estimated by measuring the height of the corresponding peaks, compared to standards (Cutler, 1986).

Phenolic Solutions. Phenolic acids present both in foliar material and humic solutions were selected for investigation of the respiratory effect of specific compounds: *p*-hydroxyacetophenone (fresh weight, FW, 136.2), *p*-hydroxybenzoic acid (FW 138.1), catechol (1,2-benzenediol, FW 110.1) and protocatechuic acid (3,4-dihydroxybenzoic acid, FW 154.12). Aqueous solutions of each of these (from Sigma Chemical Co.) were prepared at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M with demineralized water.

Fungal Material. We measured the respiration of *Cenococcum graniforme* (Sow.) Ferd. and Winge, and *Laccaria laccata* (Scop. ex Fr.) Berk and Br. The equipment used made it necessary to grow the fungus in a liquid medium. An Erlenmeyer flask containing 100 ml of modified Melin-Norkrans nutrient solution (Norkrans, 1949) was inoculated with a 5-mm-diameter agar disk cut from

a fungal colony. The flasks were then placed in a dark incubator at 24°C for five weeks before experimentation.

Oxygen Electrode. Oxygen utilization of fungal mycelia was measured polarographically with a Hansatech instrument (King's Lynn, Norfolk, England), which has a Clarck-type oxygen electrode. The fungal sample (1 ± 0.2 g FW) was set in the measuring cell containing 1 ml of demineralized, oxygen-saturated water (250 nmol/ml at 25°C). After several minutes, oxygen consumption becomes stable at a level corresponding to initial respiration. One milliliter of the solution to be tested (humic or phenolic) was sterilized by Millipore filtration (0.22 μm) and injected into the measuring cell. The oxygen consumption of the mycelium, corresponding to respiration after disturbance, was then observed. The total measurement lasted 60 min, and each experiment was repeated seven times. To demonstrate that no physicochemical reaction was responsible for the reduction of oxygen dissolved in the water, experiments without fungi were performed. One milliliter of the test solution was added to 1 ml of demineralized, oxygen-saturated water. The oxygen concentration in the measuring cell remained unchanged. Therefore, it may be assumed that no oxygen-reducing chemical reactions took place. We ignored the oxygen consumption of the probe because it is negligible (about 0.5 nmol/minute) and the same for all samples. At the end of measure, the sample was dried at 105°C for 48 hr to calculate dry weight. Respiration values reported in Table 2 (below) are expressed as nanomolar O_2 per gram dry weight per minute.

Statistical Analysis. Seven repetitions were carried out for each test (one fungus against one concentration of one phenol). Comparisons between respiration before and after injection were analyzed using the Student's *t* test for two paired groups with one-tail probability (H_0 = respiration after disturbance is not significantly lower than initial respiration).

RESULTS

Biochemical Comparison of Leaf Hydrolysates and Humic Solutions (Table 1). Proanthocyans and flavonols were identified only for the plant extracts. The humic solutions do not contain these two groups of molecules. However, an unidentified compound appeared in the two-solution spectrophotometric profiles, with a maximum absorption at 360 nm. A certain number of phenols were identified only in foliar extracts: caffeic, *p*-coumaric, and vanillic acids. Other phenols were listed among all the hydrolysates and solutions: *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, catechol, and protocatechuic acid. Because of their presence in the humic solution, they were suspected of phytotoxic activity and thus were selected for the respiration experiments.

Effects of humic solutions on respiration of C. graniforme *and* L. laccata

TABLE 1. BIOCHEMICAL COMPOSITION OF LEAF HYDROLYSATES[a]

	Proanthocyans	Flavonoids	Phenolic acids
Athyrium filix-femina	1.14	kaempferol: 0.9 quercetin: 0.6	catechol: 95.2 *p*-hydroxybenzoic: 3.25 protocatechuic: 1.2 caffeic: 0.9 vanillic: 0.88 *p*-coumaric: 0.48
Vaccinium myrtillus	0.23	kaempferol: 0.45 quercetin: 0.03	catechol: 24.8 *p*-hydroxybenzoic: 1.2 protocatechuic: 0.43 caffeic: 0.4 vanillic: 0.3 *p*-coumaric: 0.21
Picea abies	4.32	kaempferol: 0.99 quercetin: 0.56 isorhamnetin: 0.56 myricetin: 0.23	*p*-hydroxyacetophenone: 103.2 catechol: 94.7 *p*-hydroxybenzoic: 47.4 *p*-coumaric: 5.76
Mull	none	none[b]	*p*-hydroxyacetophenone: 1.09 catechol: 0.11 *p*-hydroxybenzoic: 0.03 protocatechuic: traces
Mor	none	none[b]	*p*-hydroxyacetophenone: 5.83 catechol: 0.35 *p*-hydroxybenzoic: 0.28

[a] From *A. filix-femina, V. myrtillus* and *P. abies* (expressed as mg/g dry weight) and mull and mor-type humic solutions (expressed as $\times 10^{-5}$ M).
[b] An unknown, nonflavonic, compound was detected.

(*Table 2*). Initial respiration of *L. laccata* is higher than that of *C. graniforme*, denoting a more active metabolism. The two humic solutions are responsible for a significant ($P < 0.05$) decrease of oxygen consumption in both *C. graniforme* and *L. laccata*. These results justify the necessity of testing each component of the humic solutions singly on the respiration of the two fungi to determine which molecule(s) is (are) responsible for the inhibition observed.

Effects of phenolic acids on respiration of C. graniforme (*Figure 1*) *and* L. laccata (*Figure 2*). Respiration of *C. graniforme* was significantly inhibited by *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, and protocatechuic acid, at all concentrations tested. As regards catechol, the two higher concentrations (10^{-3} M and 10^{-4} M) do not perturb oxygen consumption of *C. graniforme*, whereas 10^{-5} M, 10^{-6} M, and 10^{-7} M are responsible for a significant respi-

TABLE 2. CONSUMPTION OF OXYGEN BY TWO ECTOMYCORRHIZAL FUNGI BEFORE AND AFTER INJECTION OF MULL OR MOR-TYPE HUMIC SOLUTION[a]

	Oxygen consumption (nmol O_2/g dry weight/min		
		After injection of 1 ml	
	Initial	Mull	Mor
C. graniforme	3624 ± 616	2790 ± 711[a]	2969 ± 655[a]
L. laccata	4179 ± 878	3134 ± 757[a]	3092 ± 893[a]

[a]Respiration after injection is lower than initial, for $P < 0.05$ according to Student's t test)

ration decrease. This observation remains unexplained (perhaps because these solutions were not buffered, as suggested by a referee). *L. laccata's* respiration appears significantly affected by the four phenols at each concentration (except for *p*-hydroxybenzoic at 10^{-3} M). These data show that respiration of the two fungal species is inhibited by the phenols present in mull- and mor-type humic solutions.

DISCUSSION

The studies conducted here were short-term, on the order of 1 hr, in which immediate responses were measured. Of course, such an approach is far from the extreme chemical complexity of the soil environment. Nevertheless, such a method can, by its simplicity, provide indications of the allelochemical potential of such common phenolic compounds.

The presence in soils of a wide variety of potentially phytotoxic chemicals released by plants is well documented (Tukey, 1971; Whittaker and Feeny, 1971). Furthermore, the allelopathic influences of compounds such as the phenolic acids have been appreciated for some time (Wang et al., 1967). It is well known that compounds synthesized by plants vary both qualitatively and quantitatively according to various factors: quality of light perceived (Koeppe et al., 1969), hydric nutrients (Gilmore, 1977), plant health (Woodhead, 1981), etc. Consequently, the list of molecules identified in these analyses must in no way be considered as exhaustive, and the data (Table 1) must be viewed as being descriptive for a given moment, i.e., the beginning of summer.

Identical compounds to those identified by Gallet (1988) for *P. abies* and *V. myrtillus*, and by Voirin (1970) for *A. filix-femina* were found in the aqueous extracts analyzed here. The *p*-hydroxyacetophone found in the two humic solutions comes from spruce, as analyses carried out on the other species did not

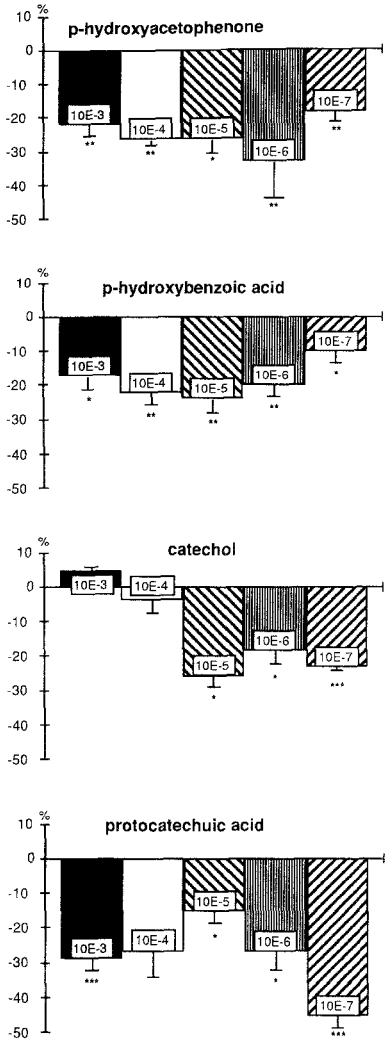

FIG. 1. Effects of phenolic solutions on respiration of *C. graniforme* expressed as percent of initial consumption of oxygen. Each column is the mean of seven repeats ± standard deviation. *, **, *** = significant at 0.05, 0.01, and 0.001 probability levels, respectively, according to Student's *t* test for two paired groups.

bring it to light. Esterbauer et al. (1975) also reported it in this species. Other phenols found among all the hydrolysates and solutions were catechol and *p*-hydroxybenzoic acid. One of the essential features of allelopathic interactions between plants is that phytotoxins must be released from a plant to the soil

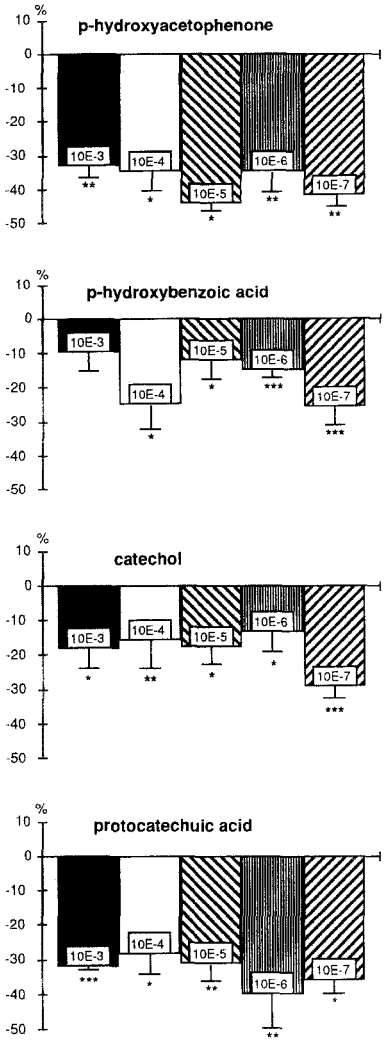

FIG. 2. Effects of phenolic solutions on respiration of *L. laccata* expressed as percent of initial consumption of oxygen. Each column is the mean of seven repeats ± standard deviation. *, **, *** = significant at 0.05, 0.01, and 0.001 probability levels, respectively, according to Student's *t* test for two paired groups.

(Rice, 1984). The data show that *p*-hydroxyacetophone, catechol, *p*-hydroxybenzoic acid, and protocatechuic acid are synthesized by spruce and released in soils. Therefore, they have a role in plant interactions occurring in nature.

Although fungi live in soil, very few studies have dealt with the effects of

humic solutions on mycorrhizal fungi (Acsai and Largent, 1983; Rose et al., 1983). The study conducted here shows that *C. graniforme* and *L. laccata*, two common ectomycorrhizal fungi of spruce (Trappe, 1964; Marx et al., 1991), are sensitive to the humic solutions. These results could explain previous findings of one sporadic formation of ectomycorrhiza by those two fungi in subalpine spruce stands (Pellissier, 1990).

The concentration of allelopathic compounds must also be taken into account. For example, for germination of crop plants, the threshold is set at around 10^{-4}–10^{-5} M; below that, the effect is null (or even, occasionally, stimulating), and above it, inhibition is manifested (Blum et al., 1984). Concerning microbial populations, bacteria and fungi can be stimulated by some phenolic acids at concentrations $\leq 5\mu$M/g soil and reduced at higher concentrations (Blum and Shafer, 1988). In this study, the four molecules appear toxic even at 10^{-5}, 10^{-6}, and 10^{-7} M. Very often, 10^{-7} M was as inhibitory, and often more than 10^{-3} M. Consequently, allelopathic phenomena obey the "all-or-none law": the effect of a phenol is positive, null, or negative, but never more or less toxic according to its concentration. It is also probable that phenolic compounds may have additive or synergistic effects (Einhellig, 1989). Investigations to elucidate this probability are now in progress. In this way, symbiosis with spruce is threatened, as described previously (Pellissier and Trosset, 1989b). Natural conditions are, however, more complicated than glasshouse bioassays. As suggested by Stowe (1979), field experiments (small-scale distribution of *P. abies* seedlings compared to distribution of ectomycorrhizal fungi) are necessary before any final comment is made on allelopathic interference in spruce regeneration.

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FEMALE-TO-MALE SEX PHEROMONES OF LOW VOLATILITY IN THE ASIAN ELEPHANT, *Elephas maximus*

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Abstract—In their natural ecosystems, the sexes of Asian elephants, *Elephas maximus*, live separately. For several weeks prior to ovulation, the urine and cervical mucus of female Asian elephants contain extractable chemical agents of low volatility that elicit a high frequency of flehmen responses from bull elephants as an integral part of mating. Subsequent to flehmen responses, male sexual arousal occurs and, if the female is available, mating results. During the course of our project to determine the agent(s) and describe the responses associated with female to male sexual communication, we have identified an unusual compound. This compound, apparently the sole component of the active fraction, was identified by mass, proton nuclear magnetic resonance, ultraviolet/visible, and infrared spectrometries as indolo-[2,1-b]quinazoline-6,12-dione (tryptanthrine). Exhaustive and repetitive bioassays established that pure authentic (synthetic) tryptanthrine was not the compound responsible for the bioresponse. Rather a coeluting minor component, also of low volatility, elicited the male bioresponse.

Key Words—*Elephas maximus*, Asian elephant, preovulatory pheromone, tryptanthrine indolo[2,1-b]-quinazoline-6,12-dione, novel substance response, estrus.

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INTRODUCTION

Urine from preovulatory female Asian elephants elicits a high frequency of flehmen responses, nonhabituating in nature, from Asian bull elephants (Figure 1) that are an integral part of the mating sequence. The presence of a sex pheromone is suggested by sexual arousal behaviors observed in the males including erection, mounting, and copulation. Initial extraction and fractionation procedures suggested the feasibility of purifying an active pheromone from the preovulatory urine (Rasmussen et al., 1982). However, extensive studies demonstrated that the active principal(s) was either nonvolatile or not detected by capillary column gas chromatography or gas chromatography–mass spectrometry (Rasmussen et al., 1986). Rather, under slightly acidic conditions (pH 4–6) organic solvent extracts of the preovulatory urine exhibited high biological activity (Rasmussen et al., 1982, 1986). Separation procedures were coupled to a quantitative and standardized bioassay using this high frequency (3–35/hr) of flehmen responses by a free-roaming bull elephant who detected randomly placed samples during a 1-hr test session (Rasmussen et al., 1982, 1986). By a series of normal-phase high-performance liquid chromatography (HPLC) fractionations, an active fraction was obtained that exhibited a single HPLC band. This bioactive sample was examined by mass spectrometry (MS), nuclear magnetic resonance spectrometry (NMR), ultraviolet/visible (UV/VIS) spectrometry and Fourier transform infrared spectrometry (FTIR) (Rasmussen and Lee, 1991), and the principal component was identified.

METHODS AND MATERIALS

Separation and Purification Methods. Urine was collected from eight mature female Asian elephants during the appropriate preovulatory days as determined by measurement of serum progesterone concentrations, by assessment of cervical mucus, and by monitoring daily the responses of bulls to cows (Hess et al., 1983).

The active sample was separated and purified from the pre-ovulatory urine as outlined in Figure 2. Preovulatory urine was extracted with dichloromethane using four 5-liter capacity liquid–liquid extractors. The 1000-fold concentrated organic solvent extract (100 liters to 100 ml) was then fractionated by flash chromatography using EM silica gel 60, particle size 0.040–0.063 mm (Rasmussen et al., 1982; Still et al., 1978); the activity was localized in a blue-colored fraction. Further separation by reverse-phase HPLC resulted in the loss of the majority of the bioactivity. Normal-phase HPLC (Figure 2) has proved to be a more reliable technique for fractionation. We used an as-yet-unidentified blue compound present in the urine as an effective marker to standardize fractions because of the variability encountered in retention times during normal-

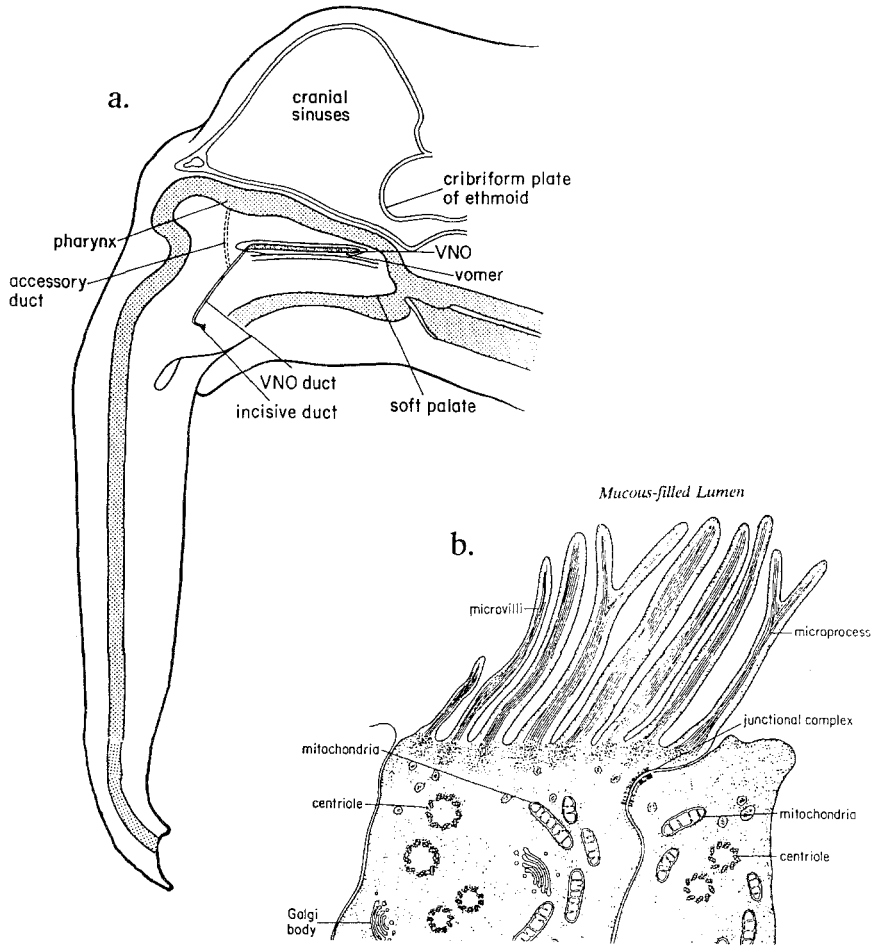

FIG. 1. The flehmen response: (a) Initial olfactory detection is accompanied by audible aspirations and exhalations, followed by placement of the dorsal trunk tip finger in the sample odorant. At the trunk tip mucus from the trunk is mixed either with urine that contains mucus and perhaps pheromones, or with samples. The superficial dermis of the tip contains many free nerve endings and unusual multiinnervated corpuscles (Rasmussen and Munger, 1990). The wetted tip is precisely placed for 1–10 sec on the paired openings of the vomeronasal organ ducts. This is termed a flehmen response. (b) The pheromones apparently reach the central mucus-filled lumen by transiting through the mucus-filled vomeronasal organ ducts, contacting microprocesses of receptor cells where signal transduction is apparently initiated.

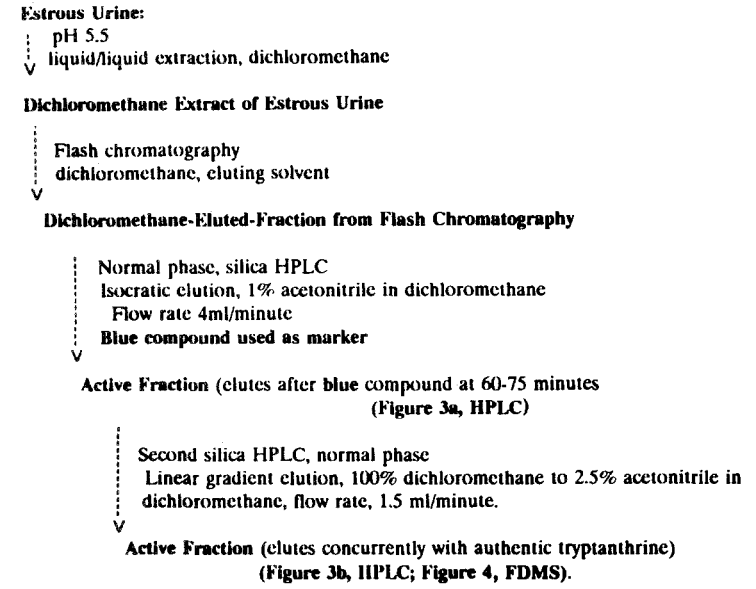

FIG. 2. The extraction, fractionation, and purification methodology is outlined.

phase HPLC. Marker use has also allowed reduction in the number of steps for purification, allowing minimal exposure to oxidants and reducing the potential for contamination associated with the use of large solvent volumes.

The first of two successive normal-phase HPLC separations was carried out on a Whatman microparticulate silica gel, Partisil 10 (Magnum 9) normal-phase 25-cm × 2.5-cm column employing isocratic elution using 1% acetonitrile in dichloromethane. The flow rate, 4 ml/min, was maintained using Waters model 501 dual-piston pumps. A dual-wavelength absorbance detector (Waters 440) set at either 225 or 280 and 340 nm, and a variable wavelength absorbance detector (ISCO) were utilized. The second HPLC separation, using a Partisil 10 analytical column 25 × 1 cm, employed a linear gradient system from 100% dichloromethane to 2.5% acetonitrile in dichloromethane at 1.5 ml/min, programmed by the Waters Data Module 740.

Structural Characterization. The active fraction obtained from the chromatography depicted in Figure 3b was analyzed by infrared, ultraviolet, ^{1}H-proton nuclear magnetic resonance, and mass spectrometries. Both field desorption (FD) and electron ionization (EI) mass spectra were obtained using a Jeol HX100HF mass spectrometer operating at 5-kV acceleration potential and a nominal resolving power of 500 for FD and 5000 for EI spectra. For FD spectra, the sample was dissolved in methanol and the sample loaded onto activated carbon emitters. Spectra were collected over the range of m/z 0–1000 as the

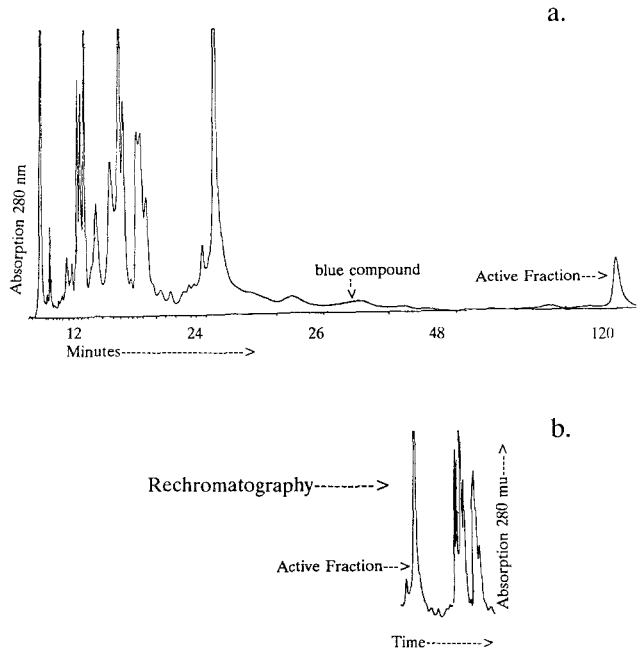

FIG. 3. (a) Initial high-performance liquid chromatography fractionation. Isocratic elution with 1% acetonitrile in dichloromethane. The active fraction eluted at 120 min (x axis) subsequent to a blue marker compound. Absorption at 280 nm is indicated on y axis. (b) Rechromatography by HPLC using gradient elution system, 100% dichloromethane to 2.5% acetonitrile in dichloromethane. The active fraction elutes in the yellow fraction prior to several inactive components. The ordinate represents absorbance at 280 nm.

emitter heating current was ramped from 0 to 25 μamp. For EI spectra, the sample was placed on the outside of a short melting point capillary and inserted into the 150°C ion source using the direct insertion probe. A 70-eV electron beam was used for sample ionization. The reference compound PFA (Pierce) was used as the reference standard for the exact mass measurements.

Infrared spectra were obtained using a Perkin-Elmer 1800 Fourier transform infrared spectrophotometer. Samples dissolved in dichloromethane were spread on a 25-mm-diameter, 4-mm-thick sodium chloride crystal window and dried before scanning from 650 cm^{-1} to 4000 cm^{-1}. Appropriate blanks and controls were scanned.

Ultraviolet/visible (UV/VIS) spectra were obtained using a Perkin-Elmer Lambda-9 spectrophotometer, scanning from 180 nm (UV) through 900 nm (VIS). Samples from HPLC fractionation were dried and dissolved in methanol

or acetonitrile. Appropriate solvent blanks and adjacent chromatographic fractions were scanned as controls. Samples were scanned at several dilutions.

One-dimensional and two-dimensional ^1H NMR spectra were obtained with a Bruker AM-500 spectrometer with a deuterochloroform solution using tetramethylsilane as the internal reference.

Bioassay. The flehmen response (a motor pattern exhibited specifically only during the sensory evaluation of chemicals) by the Asian bull elephant meets the specificity and quantitative requirements for a reliable bioassay. It clearly is a discrete, all-or-none response that can be quantified. The bull elephant apparently utilizes this response not only to detect the presence of a cow elephant and to determine her estrous state, but definitive sexual behaviors occur after the high frequency of flehmen. This high frequency of male flehmen responses has, for a number of years, served as a guide to the fractionation procedures during the purification of a bioactive component (Rasmussen et al., 1986). By testing separated fractions, the bioresponsive region was located and the entire fractionation sequence monitored by bioassays. The distinguishable response involves the vomeronasal organ (Rasmussen and Hultgren, 1990) whose chemoreceptive cells apparently respond to substances of low volatility (Figure 1). The nonhabituating nature of the bull's response in successive bioassays made this flehmen response an invaluable bioassay tool, especially during the long-term purification protocol.

Standard bioassay units were developed (Rasmussen et al., 1986). One bioassay unit was set at a 500-ml equivalent of preovulatory urine demonstrated to elicit seven flehmen responses per hour. The utilization of these standard units during each bioassay step ensured a definitive bioassay result to guide the continued purification.

The procedures for bioassay at the test site, the Washington Park Zoo (WPZ), Portland, Oregon, and the elephants employed have been described in detail in Rasmussen et al. (1982, 1986). Four bulls (between 8 and 30 years old) were available for bioassays several times a week on a random rotation basis. (Three were experienced breeders, but no differences in response frequency was observed among the four bulls.) Samples were presented in 250 ml of appropriate buffered solutions at approximately physiological amounts, estimated from the bioactivity of starting urine and based on calculated bioassay units. The focal-animal sampling technique (Altman, 1974) was employed during a 60-min observation period.

A wide variety of appropriate controls have been tested over the years (Rasmussen et al., 1982, 1986). For the present report, during every bioassay, control urine and the actual extracting solvents were tested. When relevant, other synthetics or combinations of synthetics were also bioassayed. The bioassay had a built-in control; the bulls were routinely let into the exercise yard without any bioassay samples being placed.

Flehmen responses of moderately high frequency (between three and six responses per hour) in the initial bioassay, which decrease to zero with subsequent tests (by test 3 or 4), have been termed novel substance responses (Rasmussen et al., 1986). These responses are generally observed when an elephant is tested with compounds or mixtures that have not been previously encountered either singly or in a particular combination; these components may or may not be naturally occurring compounds in elephant secretions or exudates. Because of this phenomenon, it is necessary to conduct repetitive testing (three to five trials) so that false positives that could result from novel substance responses can be reliably distinguished from sustained positive responses elicited by compounds with apparent biological meaning. Negatives may also require several bioassays to be certain the negativity has not resulted from insufficient concentration (Rasmussen et al., 1986). The standardized bioassay unit utilized was significantly higher than either control, baseline, or novel substance response levels.

Bioassay of Authentic Tryptanthrine. When tryptanthrine was identified as the principal component of the active elephant preparation, we carried out a systematic series of bioassays to determine whether tryptanthrine was responsible for the observed behavioral response. Authentic tryptanthrine (obtained from Aldrich; tested both as received and repurified) and tryptanthrine isolated from elephant preovulatory urine were assayed in replicate tests. Commercially available tryptanthrine was bioassayed at concentrations ranging from 1 ng to 100 mg/250 ml of bioassay media. A wide variety of conditions were employed during bioassays, both singly and in combinations. These included variations in concentration, the use of diverse solubilizing agents including dichloromethane, methanol, or acetone (necessary because of solubility characteristics of tryptanthrine), and the employment of buffers or control (non-estrous) urine. Various temperatures (ranging from 20°C to 60°C), pHs, and mixing effects such as stirring and sonification were also tested.

Preparation and Bioassays of Controls. Nonestrous female urine (100 liters), male urine (50 liters), and the three major food substances, timothy hay, carrots, and grain, were extracted and separated by the same methodology employed for the purification of the active component from estrous urine. We examined nonestrous urine and male urine for tryptanthrine and for artifacts of extraction. The food sources were examined as possible sources of tryptanthrine. In one experiment, 10 liters of estrous and nonestrous urine and a day's collection of cervical mucus were isolated and compared. We extracted the amount of carrots and grain equivalent to a one-day food ration and the amount of hay equivalent to one fourth of a daily ration. Aliquots of these preparations were "spiked" with authentic tryptanthrine to ensure the precise localization of the tryptanthrine fraction. All extracts and fractions were examined for tryptanthrine using analytically HPLC, TLC, FD-MS and UV spectrometries.

Cervical Mucus. Cervical mucus was obtained from the urogenital tract anterior–dorsal to the ureter opening using a specially modified colonscope.

RESULTS

Purification of Active Fractions by HPLC. Our methodology of extraction, flash chromatography, and successive HPLCs, has yielded active fractions with good run-to-run reproducibility and reasonable recovery rates (Table 1).

The activity was localized in a blue fraction obtained from flash chromatography. In the first normal-phase HPLC separation (Figure 2), using 1% acetonitrile in dichloromethane as the eluting solvent, the majority of inactive material eluted first, followed by the inactive blue compound, and finally a late-eluting discrete band containing the bioactivity (Figure 3a). The area of this band was 1–3% of the material (based on UV spectral data) fractionated during this chromatographic separation. The second HPLC separation, using a gradient system from 100% dichloromethane to 2.5% acetonitrile in dichloromethane (Figure 2), resulted in the localization of the bioactivity in a discrete band, yellow in color (Figure 3b). The active fraction was a single peak by HPLC (Figure 3b), a single band by TLC, and gave a single prominent ion by field desorption mass spectrometry (Figure 4).

TABLE 1. PROGRESSIVE PURIFICATION

Fraction	Solutes in bioactive fraction/liter urine	Bioresponse retained (%)
Whole urine	50 g	
Solvent extract	41 mg	90
Flash chromatography	6 mg	80
First HPLC	600 μg	65
Second HPLC	50 μg	50

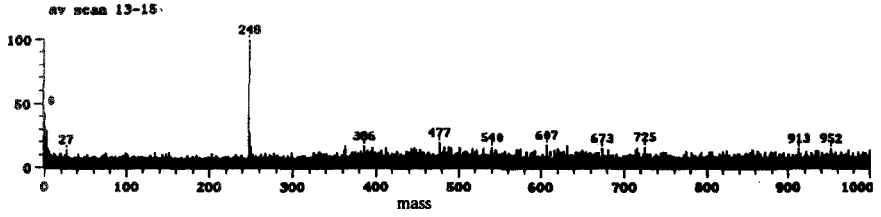

FIG. 4. Field desorption mass spectrum of the active peak indicated in Figure 3b, scans 13–15. Intensity is indicated on *y* axis and mass on the *x* axis.

Assays demonstrated that the active material was effective at microgram or lower levels, but large starting volumes (100 liters) of the chemically complex preovulatory urine were required to provide sufficient material to support the chemical characterization. One hundred liters of urine, in a scaled-up extraction scheme, were required to obtain 2 mg of tryptanthrine.

Identification of Tryptanthrine in Purified Active Fraction. High-resolution EI-MS of the principal component yielded an exact mass for the molecular ion of 248.056, corresponding to a molecular composition of $C_{15}H_8N_2O_2$. Ions at *m/z* 220 and 192 in the spectrum (Figure 5) suggested the sequential loss of two carbonyl groups. The FTIR spectra also indicated two different carbonyls at 1726 cm^{-1} and 1691 cm^{-1}.

Definitive ^1H NMR spectrometry indicated eight aromatic hydrogens (Fig-

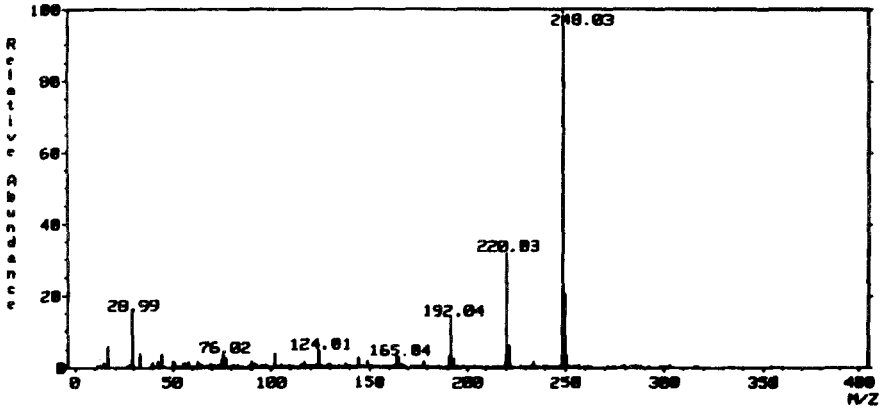

FIG. 5. Electron ionization mass spectrum of the active peak indicated in Figure 3b. Relative abundance is indicated on the ordinate and *m/z* on the abscissa.

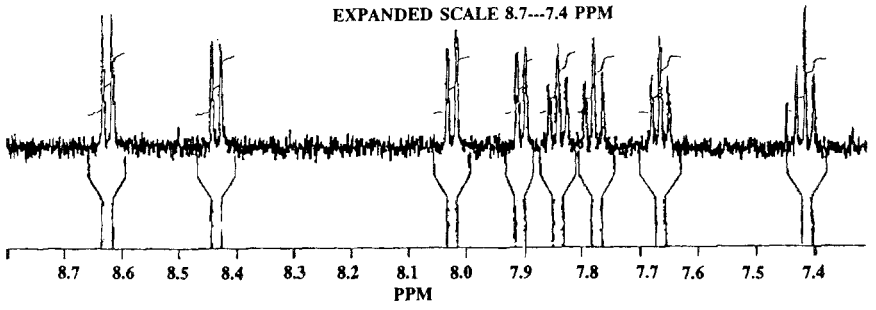

FIG. 6. ^1H nuclear magnetic resonance spectrum of the active peak indicated in Figure 3b. Eight aromatic hydrogens are indicated.

ure 6), assigned on the basis of coupling observed in the 2-D spectrum as hydrogens of two ortho-disubstituted aromatic rings. These spectral data and corresponding infrared and ultraviolet spectra suggested the structure indolo [2,1-b]-quinazoline-6,12-dione (tryptanthrine) (Figure 7), or a closely related isomer. Comparison of the spectral data obtained from the isolated material with the spectra of an authentic sample established unambiguously that the elephant urine isolate is tryptanthrine. Authentic tryptanthrine and tryptanthrine purified from the female urine coeluted when mixed together and fractionated by analytical HPLC.

Bioactivity of Purified Active Elephant Preparations and Authentic Tryptanthrine. We have assayed more than 10,000 samples of preovulatory urine or its fractions during 1527 bioassay sessions. A mean of 14.5 ± 0.7 flehmen/hr was recorded during 310 tests of 1 liter of preovulatory urine and 0.6 ± 0.5 flehmen/hr during 850 tests of anestrous urine. A illustrative bioassay pattern observed during the purification, representative of 20 different preparations, is delineated in Table 2. At each step in purification, 1 liter-equivalent (2 units) was used for bioassay; the other 9 liter-equivalents were used for the chemical purification. Therefore half the starting material was used for bioassays during the chemical isolation procedures.

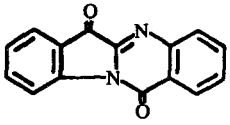

FIG. 7. Structure of tryptanthrine, indolo[2,1-b]-quinazoline-6,12-dione, isolated from preovulatory elephant urine.

TABLE 2. BIOASSAY EFFECTIVENESS DURING FRACTIONATION FROM TEN LITERS OF URINE

Fraction	Units used for bioassay[a]	Flehmen responses/hr
Preovulatory urine		
10 liters	2[b]	14
Solvent extract	2	12.5
Flash chromatography	2	11
First HPLC	2	9
Second HPLC	2	6

[a] One bioassay unit = 7 flehmen responses/hr.
[b] An average liter of active estrous urine contains 2 bioassay units based on bioassays of 500 ml of urine.

When tryptanthrine was identified as the component that was dominant in the active "pure" elephant preparation, we carried out a systematic series of bioassays to determine if indeed the synthetic, authentic tryptanthrine was the responsible pheromone. Flehmen responses by Asian bull elephants could be categorized into five quantitative divisions (Table 3). The negative category included compounds such as 4-*n*-propylphenol, a natural component of elephant urine (Rasmussen et al., 1986); successive tests over months invariably were negative. The elephant fraction was positive during repetitive bioassays. Novel substances, such as compounds isolated from estrous urine (2-*n*-propylphenol) or new mixtures, elicited low, moderate, or high frequency responses on the first one to three bioassays and then diminished to zero. Of the hundreds of compounds bioassayed, only acetic acid showed a consistent pattern of low-level responses. Responses to tryptanthrine, after the third presentation, revealed a lower (one to three) frequency of flehmen responses per hour compared to those observed during tests 1–3 (Table 3). The low-level response to tryptanthrine persisted over several months. After six months, subsequent tests at monthly intervals failed to elicit any bioresponses from any of the WPZ bulls.

A comparison was made between the flehmen responses to equal amounts of elephant preparation and authentic tryptanthrine during 60 bioassay sessions (5–10 samples per session). These tests involved four bulls at Washington Park Zoo and one bull at Taronga Zoo (Sydney, Australia). Tested aliquots of the elephant preparation (containing tryptanthrine) resulted in a dose response dependent on concentration (Table 4). Such results were not obtained for synthetic tryptanthrine. The synthetic only elicited responses during the first several trials and the frequency was not affected by concentration.

TABLE 3. FLEHMEN RESPONSES PER HOUR[a]

Category	Trial 1–3	Trial 4–10	Trial 10–30 (3–4 months)	Trial 30–40 (6 months)
Negative, 4-*n*-propylphenol	0	0	0	0
Positive, elephant fraction (one unit)	5–7	5–7	5–7	5–7
Novel substance, 2-*n*-propylphenol	4	0	0	0
Constant low level, acetic acid	2	2	2	2
Prolonged, tryptanthrine	4	1–3	1	0

[a] Four bulls were tested in 30–40 trials each. Except for the ranges indicated, the results were identical for these bulls.

Possible Sources of Tryptanthrine. We were not able to detect tryptanthrine in 100 liters of nonestrous urine or in male urine or in the three principal food sources. All HPLC peaks in the near-tryptanthrine eluting regions were monitored by UV spectrometry. No ultraviolet/visible spectra characteristic of tryptanthrine were observed.

Bioactivity in Peak Eluting prior to Tryptanthrine. Both the elephant urine preparations subsequent to two HPLC purifications and the tryptanthrine derived from elephant estrous urine were biologically active. During the past three years these preparations consistently always elicited more than five flehmen responses per hour. In contrast, in tests involving synthetic tryptanthrine the response eventually diminished to zero. Subsequently, we were able to obtain repurified, elephant-prepared tryptanthrine that no longer elicited bioresponses, whereas a newly separated, pretryptanthrine eluting peak elicited multiple flehmen responses (Figure 8). This substance has a characteristic ultraviolet absorption spectrum. As assessed by its absorption maxima at several wavelengths, its

TABLE 4. DOSE RESPONSE[a]

Absorption at 251 nm	Flehmen responses/hr
0.5	5
1.0	8
2.5	13

[a] One aliquot of HPLC-purified elephant preparation per milliliter; sample bioassayed was 10 aliquots.

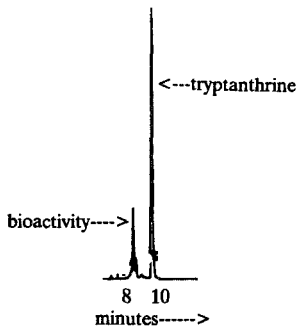

FIG. 8. Rechromatography of tryptanthrine purified from elephant estrous urine (active peak, in Figure 3b). The eluting solvent, 10% hexane in dichloromethane, was employed in the isocratic mode. Tryptanthrine is the large peak on the right. The smaller peak on the left contains the bioactivity. Absorbance at 220 nm is on the *y* axis; time is indicated on *x* axis.

concentration may be only about 1/100th that of tryptanthrine. We are diligently obtaining sufficient quantities of this low-volatile, active material for chemical and structural identification.

DISCUSSION

There is a paucity of chemical knowledge about specific pheromones operational prior to or during mating in animals of high intelligence. We have isolated from the chemically complex urine of elephants a highly bioactive fraction. Such isolation was accomplished using a highly reliable bioassay to guide the chemical fractionation.

The principal constituent of the highly concentrated active fraction was a component, indolo[2,1-b]-quinazoline-6,12-dione (tryptanthrine), lacking bioactivity. Interestingly, we were unable to detect tryptanthrine in nonestrous female urine or male urine. Tryptanthrine has previously been found only in plants such as the cannonball tree *Couroupita guaianensis*) (Nelson and Wheeler, 1937) and yeasts *Candida* sp. (Bergman et al., 1977; Bird, 1963; Friedlander and Roschestwensky, 1915). We did not find tryptanthrine in the three major food items of the elephants, although it could be present at very low levels. To our knowledge, this is the first time this compound has been reported in any animal.

The bioassay of synthetic tryptanthrine, the apparently sole component of the active purified elephant preparation, and its negativity in bioassays after its low-level positivity demonstrated the necessity of extensively testing authentic compounds after their identification in an apparently homogeneous active fraction. The prolonged novel substance response we have observed is intriguing. Perhaps during the experiments to isolate the active pheromone, which was in the same fraction as tryptanthrine, the bull learned to associate tryptanthrine with the pheromone, or with other novel compounds, or possibly tryptanthrine is part of an active sex pheromone set.

Our results indicate that: (1) tryptanthrine is the dominant molecule in the active, low-volatile fraction from the preovulatory urine, (2) tryptanthrine is inactive in bioassay and is not a singly active pheromone, and (3) the bioactive, tryptanthrine-containing elephant preparation contains a minor, coeluting substance that is bioactive.

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Joffrey assisted in the mass spectral analyses. We thank William R. Anderson, Jr., for the nuclear magnetic resonance spectrometry.

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ENANTIOMERIC COMPOSITION OF GRANDISOL AND GRANDISAL PRODUCED BY *Pissodes strobi* AND *P. nemorensis*[1] AND THEIR ELECTROANTENNOGRAM RESPONSE TO PURE ENANTIOMERS

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Abstract—Grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol) and its corresponding aldehyde, grandisal, were previously isolated and identified as aggregation pheromone components for *Pissodes strobi* (Peck) and *P. nemorensis* Germar, but the enantiomeric ratios produced by these insects were not previously determined. We isolated grandisol and grandisal from males of both *P. strobi* and *P. nemorensis*. The insect-produced grandisol was derivatized with trifluoroacetic anhydride, and the enantiomeric composition was determined by gas chromatography on an optically active cyclodextrin glass capillary column. The insect-produced grandisal was first reduced to grandisol before derivatization. *P. nemorensis* produced nearly 100% (1*R*,2*S*)-grandisol and nearly 100% (1*S*,2*R*)-grandisal. *P. strobi* produced 99% (1*R*,2*S*)-grandisol and approximately 60% (1*R*,2*S*)-grandisal. In electroantennogram (EAG) studies with live *P. nemorensis* and *P. strobi*, no significant differences were found between the responses of males and females to racemic grandisol, racemic grandisal, or the 1*R*,2*S* and 1*S*,2*R* enantiomers of grandisol and grandisal, which is consistent with previous assertions that these compounds are aggregation pheromones. Although no studies to date with *P. strobi* have demonstrated a behavioral response to grandisol and grandisal, *P. strobi* antennae detected all enantiomers of grandisol and grandisal tested in EAG tests. The antennae of *P. nemorensis* responded significantly more to (1*R*,2*S*)-grandisal than to (1*S*,2*R*)-grandisal, despite producing only (1*S*,2*R*)-grandisal.

Key Words—*Pissodes strobi*, *Pissodes nemorensis*, grandisol, grandisal,

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[1] Coleoptera: Curculionidae.

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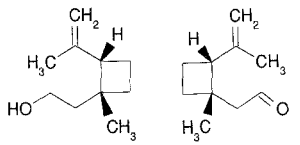
electroantennogram, enantiomers, Coleoptera, Curculionidae, white pine weevil, aggregation pheromone.

INTRODUCTION

Pissodes strobi (Peck) breeds in the terminal leaders of white pine (*Pinus strobus* L.), Engelmann spruce (*Picea engelmannii* Parry), Sitka spruce (*Picea sitchensis* Carr.), and to a lesser extent, other pines and spruces (Smith and Sugden, 1969; Phillips and Lanier, 1983a). Development of the larvae kills the apical meristem and at least two years' growth at the top of the host tree. As a result of terminal damage, the recoverable volume of the tree is reduced, and the lumber quality is degraded. *P. nemorensis* Germar, which was recently synonymized with *P. approximatus* (Phillips et al., 1987), breeds in wind breakage, stumps, cut logs, and moribund trees. This species causes little economic damage, but high population levels may result in damage to reforestation and Christmas tree projects (Finnegan, 1958), and *P. nemorensis* may serve as vectors of procerum root disease (Nevill and Alexander, 1992). *P. strobi* and *P. nemorensis* breed in different habitats, and artificially produced hybrids display reduced reproductive fitness (Phillips and Lanier, 1983b). Although the two sibling species have vastly different life histories, methods to distinguish between them with certainty based on morphological characters alone have not been developed (Godwin et al., 1982).

Booth and Lanier (1974) presented the first evidence of an aggregation pheromone for *P. nemorensis*, and Booth et al. (1983) reported the isolation and identification of *cis*-2-isopropenyl-1-methylcyclobutaneethanol, grandisol (see Figure 1), and its corresponding aldehyde, grandisal, as pheromone components of *P. nemorensis* and *P. strobi*. Racemic mixtures of grandisol and grandisal in conjunction with host odors were highly attractive for *P. nemorensis* and had limited field activity for *P. strobi*. However, Booth et al. (1983) did not perform a discrimination analysis (Godwin et al., 1982) of the trapped individuals to determine which species was captured in their study. *P. strobi* and *P. nemorensis* are sympatric in northeastern North America, and they assumed that all *Pissodes* trapped on white pine leaders were *P. strobi*. Sub-



A B

FIG. 1. Structure of (1*R*,2*S*)-grandisol (A) and (1*S*,2*R*)-grandisal (B).

sequent field and laboratory experiments with *P. strobi* have failed to demonstrate that live *P. strobi* males produce an aggregation pheromone when feeding on white pine leaders and have failed to demonstrate behavioral activity of racemic or optically pure grandisol and grandisal toward *P. strobi* (Booth, 1978; Phillips, 1984; Phillips and Lanier, 1986; T.W. Phillips, unpublished data).

Pheromone specificity at the enantiomeric level has been demonstrated to be an isolation mechanism for several sympatric sibling species (Silverstein, 1979, 1988; Birch et al., 1980). Because *P. nemorensis* produce grandisol and grandisal as part of an aggregation pheromone and because *P. strobi* also produce grandisol and grandisal, we reasoned that *P. nemorensis* and *P. strobi* may be using an enantiomeric blend as part of an isolation mechanism. Booth et al. (1983) did not determine which enantiomers of grandisol and grandisal are produced by male *P. strobi* and *P. nemorensis*. We set out to determine which enantiomers of grandisol and grandisal *P. nemorensis* and *P. strobi* produce. In addition, since behavioral activity of grandisol and grandisal for *P. strobi* has not been demonstrated, we set out to determine if *P. strobi* could detect grandisol and grandisal. Thus, we determined the electroantennogram responses of male and female *P. strobi* and *P. nemorensis* to a range of doses of racemic grandisol, racemic grandisal, and the pure enantiomers of grandisol and grandisal.

METHODS AND MATERIALS

Insect Colony. P. strobi and *P. nemorensis* were field-collected from white pine leaders and downed red pine (*Pinus resinosa* Ait.) logs, respectively, in central New York in late July and early August 1989. Adults emerged in the laboratory, and a colony of each species was maintained according to the methods of Phillips (1981). Approximately 30 insects of each sex were placed in screened gallon jars with three to six bolts (logs 2–5 cm diam.). Freshly cut white pine was used to rear *P. strobi*, and red pine and/or Scotch pine (*Pinus sylvestris* L.) were used to rear *P. nemorensis*. The bolts were capped with hot paraffin wax to seal in moisture for the developing larvae. Lighting was set at 16:8 hr light–dark to simulate a spring reproductive photoperiod, and temperature was set at 25.5°C:20°C (light–dark). The bolts in the adult containers were changed every week for *P. strobi* and every two weeks for *P. nemorensis*. Emerging adults were either added to the colony or were separated by sex and placed in different growth chambers for later aerations and/or electroantennogram assays.

Isolation of Grandisol and Grandisal. After a minimum of one month in a spring photoperiod and isolation from females, between 50 and 300 live *P. strobi* or *P. nemorensis* males were aerated on freshly cut red pine bolts. The aeration chamber was a glass vessel (12 cm diam. × 50 cm tall) with side arms

on opposite sides of the top and bottom. Room air was pulled through 40 g of activated charcoal and then through the glass vessel containing the males. Volatiles emanating from the males were adsorbed on 30 g of Porapak Q (Alltech Associates, Inc., Deerfield, Illinois). Flow through the system was measured with Gilmont flowmeters before and after the insect-holding vessel and was held constant at 100 ml/min. A number of aerations were conducted, and these ranged from three to five days. The Porapak was extracted with 50 ml of pentane, which was then dried over sodium sulfate and concentrated to 0.5 ml by fractional distillation. Insect-produced grandisol and grandisal were collected in pure form from a Varian 2700 gas chromatograph equipped with a flame ionization detector, a 99 : 1 effluent splitter, and a thermal gradient collector (Brownlee and Silverstein, 1968). A 5.4-m × 4-mm ID glass column packed with 4% Carbowax 20 M on Chromosorb G 60–80 mesh was used isothermally at 200°C for the separations. Under these conditions, grandisal had a retention time of 10.7 min and grandisol had a retention time of 26.3 min.

Synthetic Compounds. Synthetic grandisol (racemic) was obtained from Chemical Samples Co. (division Albany International, Inc., Albany, New York). Synthetic grandisal (racemic) was obtained by oxidation of grandisol with pyridinium chlorochromate in dichloromethane in the presence of sodium acetate (1 equivalent); the reaction time was 80 min at room temperature (Webster et al., 1987). The reaction mixture was then filtered through a 5 × 1.5-cm Florisil (Fisher Scientific, Pittsburgh, Pennsylvania) column, and after evaporation of the solvents, the purity of the aldehyde was determined on a 50-m DB-1 methyl silicone capillary column (0.25-mm ID × 0.25-μm film) programmed from 45°C to 200°C at 5°C/min with an initial time of 1 min with a column head pressure of 140 kPa in a Hewlett-Packard 5890 gas chromatograph with a flame ionization detector. The pure enantiomers of grandisol were synthesized using the methods of Webster and Silverstein (1986), and the pure enantiomers of grandisal were synthesized from the grandisol enantiomers as described above.

Synthesis of Trifluoroacetate Derivatives of Grandisol. In order for grandisol enantiomers to be separated on the cyclodextrin column, derivatization was required. For the racemic material, 1.5 mg of grandisol was added to 10 μl of dry dichloromethane. A solution of 20 μg dimethylaminopyridine in 2 μl pyridine was then added along with 5 μl trifluoroacetic anhydride. After 2 hr, the solution was diluted in 0.5 ml of pentane and washed with 0.5 ml water, 0.5 ml saturated sodium bicarbonate, 0.5 ml copper sulfate (dilute), 0.5 ml water (discarding the water layer each time), and the solution was then dried over sodium sulfate. Insect-produced grandisol was treated as described above in the quantities that were isolated. Because only grandisol derivatives could be separated on the cyclodextrin column, insect-produced grandisal was reduced to grandisol with lithium aluminum hydride (LAH) before derivatization (Fieser and Fieser, 1967).

Determination of Enantiomer Proportions Produced by P. nemorensis *and* P. strobi. An optically active cyclodextrin glass capillary gas chromatography (GC) column (50 m × 2 mm ID, Lipodex C, Macherey-Nagel, DHren, Germany) was used to determine the enantiomeric composition of the trifluoroacetate derivative of grandisol. A Hewlett-Packard 5890 gas chromatograph was used with a column head pressure of 140 kPa, an initial temperature of 45°C, programmed to a final temperature of 83°C by ramping 3°C/min, after an initial time of 0.5 min. Retention time of derivatives of insect-produced material was compared to that of pure enantiomers and racemic material under identical operating conditions. A minimum of two useful chromatographic runs was conducted with the derivatives of insect-produced grandisol and grandisal from each species.

Electroantennogram Response to Grandisol and Grandisal. Antennal receptivity to pure enantiomers of grandisol and grandisal as well as racemic mixtures was determined with the electroantennogram (EAG) technique. This technique is a simple method for electrophysiological detection of the responses of insect antennae to volatile semiochemicals (Roelofs, 1984). We used the EAG apparatus described by Bjostad (1988).

The thorax and abdomens of live male and female *P. strobi* and *P. nemorensis* individuals were first wrapped in Teflon tape to immobilize the insects legs. A specially modified pair of forceps with a semicircle ground into each tong was used to hold the antennae exposed and away from the snout. The forceps was modified to allow the snout through, but not to allow the antennae back out (without the forceps, the antennae would be held tight under the snout when disturbed). The insect was secured to the forceps with tape, and the forceps was connected to a micromanipulator. The indifferent glass capillary electrode was placed over the snout of the insect, and the recording glass capillary electrode (30 μl ID at tip) was connected to the tip of the antennae with a micromanipulator under a dissecting microscope.

Presentation of chemical stimuli was accomplished by injecting a puff of pheromone-laden air into a pure airstream (500 ml/min) that continuously passed over the antennae. To minimize background contamination, airstreams were filtered through molecular sieves and activated charcoal. The air was then rehydrated by bubbling through distilled water. Stimulation pipets were prepared by depositing 5 μl of the desired solution onto a strip of 100% cellulose paper (4 × 25 mm). Doses were tested in 10-fold increments from 0.05 ng to 500 ng for racemic grandisol, racemic grandisal, (1*R*,2*S*)- and (1*S*,2*R*)-grandisol, and (1*R*,2*S*)- and (1*S*,2*R*)-grandisal. Each of the six treatments was tested with a minimum of 12 insects (half male and half female) of both *P. strobi* and *P. nemorensis* and each insect was tested with different doses (starting with the lowest) of only one of the treatments. The solvent was allowed to evaporate, and the stimulation pipet was immediately tested for electrophysiological activ-

ity. The control was a strip of 100% cellulose paper (4 × 25 mm) to which 5 μl of pentane (the solvent in which the treatments were dissolved) was added and allowed to evaporate.

Statistical Analysis. The statistical program BMDP (BMDP Program Librarian, Department of Biomathematics, University of California, Los Angeles, California 90024) was used for EAG data analysis. Since preliminary statistical analysis indicated no significant differences between males and females of either species, these data were pooled in the analysis. In the EAG studies, the differences of principal interest were between treatments, not doses within a treatment, and each insect was used for all five doses of one treatment. Because sequential samples were taken from the same insect (each dose of one treatment), EAG data were analyzed in a repeated measures design. When significant F values were found in the analysis of variance, Duncan's (1955) multiple-range test was performed.

RESULTS AND DISCUSSION

Separation of Synthetic Grandisol Derivatives. The cyclodextrin column gave near-baseline separation of the 1*R*,2*S* and 1*S*,2*R* enantiomers of the trifluoroacetate derivatives of grandisol. In our initial experiment, the retention time for the 1*R*,2*S* enantiomer was 58.72 min, and the retention time for the 1*S*,2*R* enantiomer was 59.45 min (Figure 2A).[3] The purity of our synthetic grandisol-TFA enantiomers was also verified (Figure 2B and C).

The reported methods for determining enantiomeric composition are (1) polarimetry; (2) NMR (^{1}H and ^{13}C) with chiral shift reagents, chiral derivatizing agents, or chiral solvating agents; (3) achiral chromatography (gas or liquid) of diastereomeric derivatives; (4) chiral chromatography (gas or liquid) of the enantiomers directly; (5) X-ray crystallography of a derivative prepared with a chiral reagent; and (6) bioassay of synthetic enantiomers (Silverstein, 1985). The only method that allows a small sample size and no need for pure reference samples is chiral chromatography (Silverstein, 1988). In the current study, we collected the compounds of interest from the insects, purified the compounds by gas chromatography, performed a simple derivatization, and injected them onto an optically active column. This procedure was much simpler and required much less of the insect-produced compound than other options.

Separation of Derivatives of Insect-Produced Grandisol and Grandisal. Male *P. nemorensis* produced nearly 100% of the (1*R*,2*S*)-grandisol and nearly 100% (1*S*,2*R*)-grandisal when a number of individuals were aerated together

[3]The chromatograms in Figure 3 were collected using identical temperature regimes and head pressure as in Figure 2, but were collected six weeks later. The differences in retention time of racemic grandisol-TFA cannot be explained, but is accounted for with the standards.

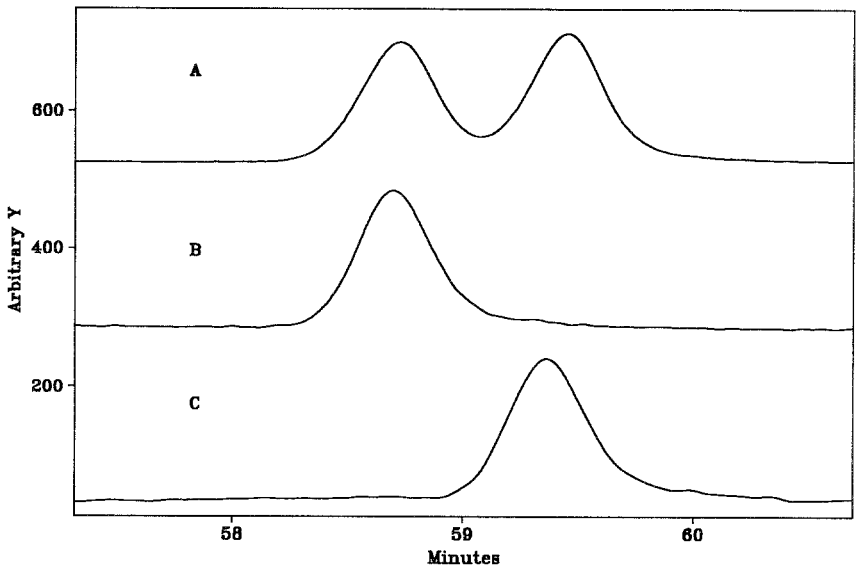


FIG. 2. Chromatograms from an optically active cycodextrin-C glass capillary column in the retention time region of synthetic racemic grandisol-TFA (A), synthetic (1*R*,2*S*)-grandisol-TFA (B), and synthetic (1*S*,2*R*)-grandisol-TFA (C).

(Figure 3). Male *P. strobi* produced approximately 99% of the (1*R*,2*S*)-grandisol and approximately 60% (1*R*,2*S*)-grandisal when a number of individuals were aerated together. Although the amount of variation between individuals is unknown, results were consistent between different aerations. The amount of grandisal isolated from *P. strobi* for this analysis was easily distinguishable from noise, but was quite small (10–100 ng per collection).

Racemic grandisol and grandisal have been demonstrated to be highly attractive to *P. nemorensis* (Booth et al., 1983), yet *P. nemorensis* produces nearly 100% of the 1*R*,2*S* enantiomer of grandisol, and nearly 100% 1*S*,2*R* enantiomer of grandisal. Apparently, enantiomers not produced by the insects are not inhibitory for *P. nemorensis*. Numerous tests in the laboratory and in the field with *P. strobi* have failed to demonstrate any behavioral effects for grandisol, grandisal, or combinations of the two (both racemic and optically pure) (Booth, 1978; Phillips, 1984; Phillips and Lanier, 1986; T.W. Phillips, unpublished data). However, since *P. strobi* produces neither racemic material nor pure enantiomers, the correct synthetic enantiomeric blend was never tested in the field. Moreover, we collected pheromone from a group of between 50 and 300 male (depending on the number available for a particular aeration). It is possible that aerations of large (unnatural) groups of males in crowded conditions may have affected levels of pheromone production.

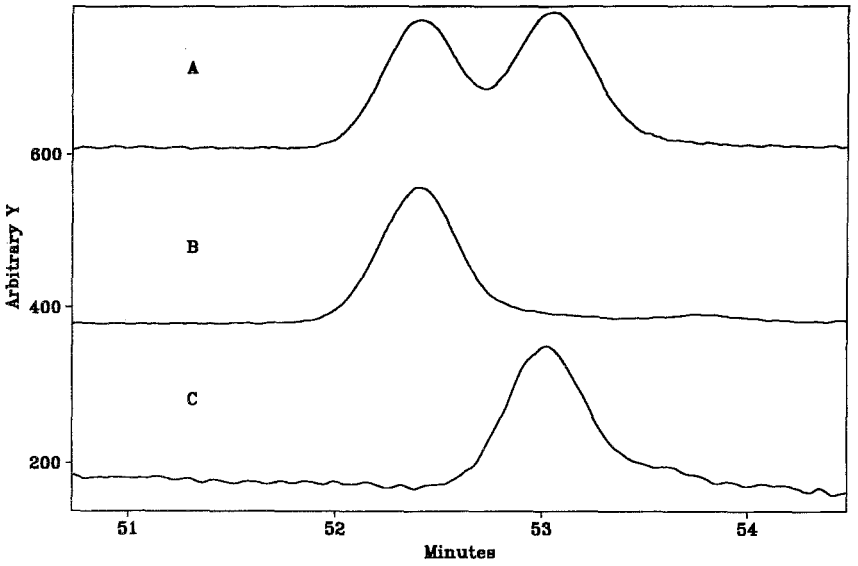


FIG. 3. Sample chromatograms from an optically active cycodextrin-C glass capillary column in the retention time region of synthetic racemic grandisol-TFA (A), *P. nemorensis*-produced grandisol that was derivatized to grandisol-TFA (B), and *P. nemorensis*-produced grandisal that was reduced to grandisol and derivatized to grandisol-TFA (C).

The biosynthesis of pheromones has been well studied in several species of Coleoptera (Vanderwel and Oehlschlager, 1987). Studies with the boll weevil have demonstrated that this insect can produce grandisol and its other pheromone components de novo (Mitlin and Hedin, 1974), but boll weevils feeding on their host plant exhibit enhanced pheromone production (Hedin et al., 1975), possibly due to host monoterpene alcohols (Thompson and Mitlin, 1979). Many coleopterans produce sex pheromones of the same chirality as their precursors (Vanderwel and Oehlschlager, 1987 and references therein). Because grandisol and grandisal have two chiral centers, one of which is a quaternary carbon center that cannot epimerize, conversion of (1*R*,2*S*)-grandisol to (1*S*,2*R*)-grandisal and vice versa is impossible. However, we have demonstrated that *P. nemorensis* produced nearly 100% of the (1*R*,2*S*)-grandisol and nearly 100% (1*S*,2*R*)-grandisal. One possibility is that two separate biosynthetic pathways are involved in the synthesis of grandisol and grandisal in *P. nemorensis*. Our data do not necessarily conflict with the dogma that structurally similar compounds arise from a common biosynthetic route. Another possibility is that both enantiomers of grandisol are biosynthesized, but one of them is selectively converted to the aldehyde (or to the alcohol from the aldehyde if both enantiomers of grandisal are biosynthesized).

Electroantennogram Response to Grandisol and Grandisal. The electroantennogram technique demonstrated that male and female *P. nemorensis* and *P. strobi* are sensitive to very low levels of grandisol and grandisal. Both species were able to detect puffs of air delivered from pipets containing 50 pg of either compound on a small piece of 100% cellulose paper (Figures 4 and 5). Previous behavioral data were ambiguous as to whether *P. strobi* were capable of detecting grandisol and grandisal. The EAG response increased with increasing doses for all compounds tested for both *P. nemorensis* and *P. strobi*, and there was no significant difference between males and females of either species. Previous evidence indicated that grandisol and grandisal acted as aggregation pheromones for both sexes *P. nemorensis* (Booth et al., 1983). Our data are consistent with

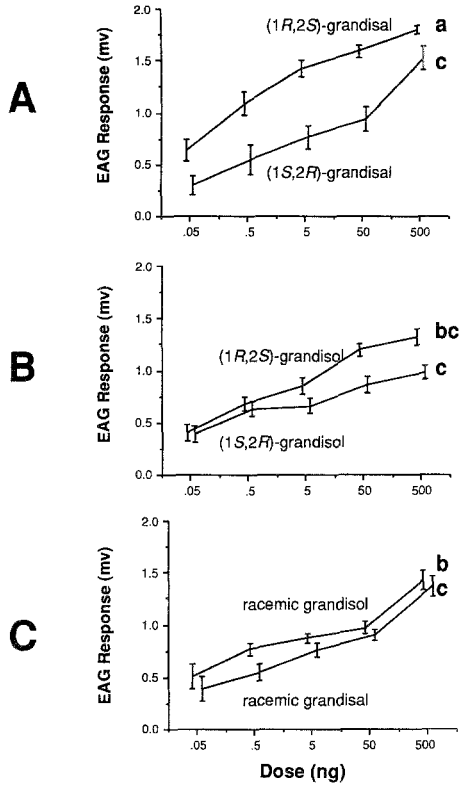


FIG. 4. Electroantennogram response of *P. strobi* to (A) (1*R*,2*S*)-grandisal and (1*S*,2*R*)-grandisal, (B) (1*R*,2*S*)-grandisol and (1*S*,2*R*)-grandisol, and (C) racemic grandisal and racemic grandisol. Significant differences between curves are indicated by different lowercase letters (all values were significantly different than the solvent control EAG response of 0.03 $\pm$ 0.08). Bars above and below the mean represent standard error.

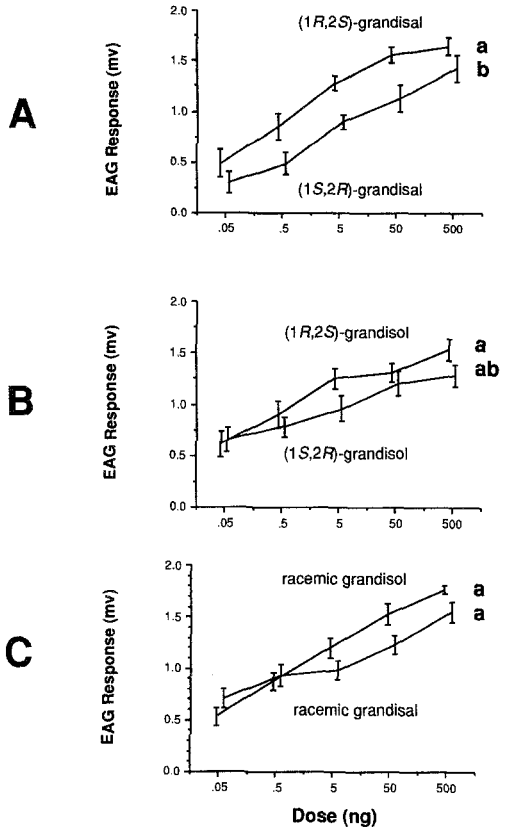


FIG. 5. Electroantennogram response of *P. nemorensis* to (A) (1*R*,2*S*)-grandisal and (1*S*,2*R*)-grandisal, (B) (1*R*,2*S*)-grandisol and (1*S*,2*R*)-grandisol, and (C) racemic grandisol and racemic grandisal. Significant differences between curves are indicated by different lowercase letters (all values were significantly different than the solvent control EAG response of 0.02 ± 0.07). Bars above and below the mean represent standard error.

this assertion. However, EAG data do not indicate what type of behavioral response, if any, the insect would elicit in response to the compound. It indicates only that sensilla on the insects' antennae are receptive to the compounds tested.

The EAG response of *P. strobi* to (1*R*,2*S*)-grandisal was the highest overall, and significantly higher ($P < 0.05$) than the response of *P. strobi* to all other treatments (Figure 4). The EAG response of *P. nemorensis* was significantly higher to (1*R*,2*S*)-grandisal than to (1*S*,2*R*)-grandisal (Figure 5), yet *P. nemorensis* males produce nearly 100% (1*S*,2*R*)-grandisal. Unfortunately, since EAG data do not provide information on behavior, the ecological significance

of *P. nemorensis* antennae responding more strongly to the grandisal enantiomer it does not produce than to the grandisal enantiomer it does produce is unknown.

Both *P. strobi* and *P. nemorensis* produce grandisol and grandisal, but only *P. nemorensis* has been demonstrated to use these compounds as part of an aggregation pheromone. There are several possible explanations for the production of grandisol and grandisal by *P. strobi* and its lack of behavioral response to it: (1) *P. strobi* antennae may not be capable of responding to grandisol and grandisal, (2) the enantiomeric composition of grandisol and grandisal presented to *P. strobi* to date may be different from what they produce, and (3) the production of grandisol and grandisal by *P. strobi* may be a vestige from the evolutionary line from which *P. strobi* and *P. nemorensis* originated. We have demonstrated that *P. strobi* antennae respond to grandisol and grandisal. We have also demonstrated that *P. strobi* produce a different enantiomeric blend of grandisol and grandisal than has been tested to date for behavioral activity. The current data still do not explain why *P. nemorensis* responds behaviorally to a racemic mixture of grandisol and grandisal, and its sibling species, *P. strobi*, does not. There are a number of reasons to expect that *P. strobi* are producing these compounds as a vestige of their ancestral line from which the two species originated. First, these morphologically indistinct species have evolved quite recently (Phillips, 1984). Second, although the number of females that contribute eggs to a given leader is unknown, the amount of host material available in an average host leader is suitable for only the offspring of one to two *P. strobi* females given their reproduction potential. Large aggregations of *P. strobi* on one leader may not be adaptive. Third, *P. strobi* produce something (not enantiomers of grandisol and grandisal) that interrupts the response of *P. nemorensis* to natural pheromone and to racemic synthetic material (Phillips and Lanier, 1986). Lastly, the mating site for *P. strobi* is the leader of its host plant. Although chemical and visual cues are often integrated in attraction, a silhouette of a host leader may be easy to locate visually and may not require an aggregation pheromone to find it. If the above is correct, the sibling species pair of *P. nemorensis* and *P. strobi* may represent a unique system in pheromone biology.

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RECOGNITION OF APHID PARASITOIDS BY HONEYDEW-COLLECTING ANTS: THE ROLE OF CUTICULAR LIPIDS IN A CHEMICAL MIMICRY SYSTEM

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Abstract—The aphidiid wasp *Lysiphlebus cardui* parasitizes in ant-attended *Aphis fabae cirsiiacanthoidis* colonies without causing aggressive behavior in the ant *Lasius niger*. By contrast, *Trioxys angelicae*, another aphidiid parasitoid of aphids, is rapidly recognized and vigorously attacked by the ants. *L. niger* workers also responded differently to dead individuals of *L. cardui* and *T. angelicae*. Dead *L. cardui* parasitoids were often ignored when encountered by *L. niger*, whereas dead *T. angelicae* individuals were immediately grasped by ants that discovered them. However, hexane-washed parasitoids caused a similar reaction pattern in the ants, in that both aphidiid species were tolerated in the aphid colony. Lure experiments demonstrated that chemical stimuli on the cuticle are major cues for the ants to distinguish between the parasitoids. The hexane extract of *L. cardui* transferred to washed individuals of *T. angelicae* resulted in ant responses characteristic towards *L. cardui*, and *L. niger* workers displayed the typical removal pattern they normally showed towards *T. angelicae* when *T. angelicae* extract was applied to *L. cardui* individuals. Both parasitoid species treated with the hexane extract of *A. fabae cirsiiacanthoidis* were similarily treated by the ants as were aphid control individuals. The suggestion that the aphidiid wasp *L. cardui* uses chemical mimicry is discussed.

Key Words—*Aphis fabae cirsiiacanthoidis*, Homoptera, Aphidae, ants, ant–parasitoid interactions, Hymenoptera, Formicidae, Aphidiidae, parasitoids, *Lysiphlebus cardui*, *Trioxys angelicae*, cuticular lipids, chemical mimicry.

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INTRODUCTION

Insects that are associated with ants in a symbiotic relationship often provide sugar-rich secretions to the ants. In return, ants can act as effective guards in warding off predators and parasites (Banks, 1962; El-Ziady, 1960; El-Ziady and Kennedy, 1956; Flanders, 1951; Way, 1963). However, this ant protection is not perfect. Many predators and parasitoids of the nutrition-providing insects manage to integrate in a mutualistic association to gain access to their prey or host organism (Eisner et al., 1978; Mason et al., 1991; Pontin, 1959). Some species of the family Aphidiidae successfully overcome the defense mechanism of honeydew-collecting ants. Takada and Hashimoto (1985) described species-specific interactions between the root aphid *Sappaphis piri* Matsumura, the ant species *Lasius niger* (L.) and *Pheidole fervida* F. Smith, and the aphid parasitoids *Aclitus sappaphis* Takada & Shiga and *Paralipsis eikoae* (Yasumatsu). *A. sappaphis* was tolerated by the ant *P. fervida*, but vigorously attacked by *L. niger*. By contrast, *P. eikoae* was tolerated by *L. niger* and attacked by *P. fervida*.

In the aphid–ant system *Aphis fabae cirsiiacanthoidis* Schrank and *Lasius niger* on creeping thistle, *Cirsium arvense* (L.) Scop., the aphidiid wasp *Lysiphlebus cardui* (Marshall) that displays a cryptic behavior when searching in aphid colonies is either disregarded or tapped with the antennae by honeydew-collecting ants (for quantitative data see Völkl, 1992; and Völkl and Mackauer, 1993). In contrast, the aphidiid wasp *Trioxys angelicae* (Haliday) showing a "nervous" searching behavior in aphid colonies is readily attacked by ants; in some encounters the wasp manages to escape by flying off the plant, in others the parasitoid is seized by worker ants and killed (Völkl, 1992; Völkl and Mackauer, 1993). Völkl and Mackauer (1993) could demonstrate by using lightly anesthetized living females of both parasitoid species that behavioral cues of the wasps were not primarily responsible for the difference in the response of the ants. Furthermore, ants of different and unrelated genera responded in a similar pattern towards the parasitoid species. These authors suggested that *L. cardui* mimics the cuticular lipid profile of the aphid host, *A. fabae cirsiiacanthoidis*, to avoid attack by the ants. Such a strategy of being chemically "invisible" is known from a number of inquilines of social insects (e.g., Howard et al., 1980, 1982, 1990a, b; Vander Meer and Wojcik, 1982; Vander Meer et al., 1989).

The aim of this study was to test the hypothesis of Völkl and Mackauer (1993) that chemical cues on the cuticular surface rather than behavioral or morphological features prevent *L. cardui* from being recognized and attacked by honeydew-collecting ants. Experiments with dead nontreated and hexane-treated individuals of *L. cardui*, *T. angelicae*, and *A. fabae cirsiiacanthoidis*, as well as lure experiments with these parasitoids and aphids, were carried out

to demonstrate the major role of epicuticular lipids as recognition cues in this aphid–ant–parasitoid system.

METHODS AND MATERIALS

Source of Parasitoids and Aphids. L. cardui parasitoids were obtained by collecting *A. fabae cirsiiacanthoidis* mummies on *C. arvense* in the vicinity of Bayreuth, Germany, at the beginning of July 1991 by cutting off whole thistle plants. In the laboratory, the mummies were removed from the plants with forceps and placed in a gauze-covered cage (28 × 20 × 36 cm), where the parasitoid females could eclose. Thus, *L. cardui* individuals had no contact to either living aphid hosts or to the host plant of the aphids. The females were tested the day after eclosion.

From a laboratory culture of *T. angelicae* parasitizing *A. fabae cirsiiacanthoidis* on *C. arvense* in a climate chamber (20°C ± 1°C, 16:8 hr light–dark), aphid mummies were removed from the plants and kept in a gauze-covered glass vial (8 cm diam., 12 cm high) in the laboratory until adult eclosion. Similar to *L. cardui*, *T. angelicae* parasitoids did not experience either their host aphids or the aphid host plant. For the experiments, the females were tested the day after eclosion.

To gain unparasitized *A. fabae cirsiiacanthoidis* individuals, potted thistle plants infested with this aphid species were kept in a gauze-covered cage (100 × 50 × 110 cm) in the field to exclude aphid parasitoids. During the whole season, no mummy could be detected.

General Aspects of the Experiments. Field experiments were conducted in a roof garden between early July and the end of August 1991. Four potted *C. arvense* plants, each infested with a mixed-age colony of about 500 individuals of *A. fabae cirsiiacanthoidis*, were placed close to a naturally established colony of *L. niger*. Within a few days of exposure, each aphid colony was visited by honeydew-collecting workers of this ant species. The trials were carried out during periods when ant activity was at a maximum at temperatures ranging between 18°C and 28°C. Four experiments with *L. cardui*, *T. angelicae*, and *A. fabae cirsiiacanthoidis* were performed to test the hypothesis that cuticular lipids on the surface of the parasitoid species were the major recognition cues for the ants.

Experiment 1: Introduction of Dead Parasitoids and Aphids. Living females of both parasitoid species (*L. cardui*: $N = 31$; *T. angelicae*: $N = 35$) were transferred singly in small glass tubes (1 cm diam., 3 cm high) and killed by freezing at −50°C for 2 min. After freezing, the dead parasitoids were kept at room temperature at least for 1 min before testing began. For the bioassay, a dead parasitoid was carefully placed into a leaf axil of the plant that was fre-

quently passed by *L. niger* workers, without disturbing the foraging behavior of the ants. The same procedure was conducted with dead aphids of different developmental stages ($N = 39$). When encountering the test individual, the ants either ignored it or removed it from the plant. The ant that finally removed the individual was recorded.

Experiment 2: Introduction of Dead Hexane-Washed Parasitoids and Aphids. To test the significance of chemical stimuli on the surface of the parasitoids for triggering removal behavior of the ants, an organic solvent was used to wash off the epicuticular lipids of the test individuals (Gilby, 1980; Jackson and Blomquist, 1976; Lockey, 1988). Dead females of both parasitoid species previously killed by freezing were twice immersed in 250 μl hexane for 1 min each. Subsequently, the insects were transferred on filter paper and kept in the laboratory for 24 hr for drying. The next day, the hexane-washed parasitoids (*L. cardui*: $N = 22$; *T. angelicae*: $N = 21$) were tested by carefully placing an individual into a leaf axil of the aphids' host plant. The same procedure was carried out with dead hexane-washed aphids of different developmental stages ($N = 20$). The recording of the ants' behavior was the same as in experiment 1.

Experiment 3: Introduction of Dead Parasitoids Treated with Hexane Soak of Other Parasitoid Species. Freeze-killed females of *T. angelicae* and *L. cardui* were twice soaked in 250 μl hexane for 1 min each. The extract portions of one species were combined and kept for the subsequent transfer procedure. The hexane-washed individuals were allowed to air dry for about 5 min. Subsequently, conspecific individuals were placed into a glass vial and covered with the hexane soak of the other parasitoid species; that is, *T. angelicae* parasitoids ($N = 23$) received the hexane extract of *L. cardui*, while the *T. angelicae* extract was deposited on *L. cardui* parasitoids ($N = 22$). The solvent was removed under nitrogen, and as a result, the lipids should coat the test individuals. After the treatment, the test individuals were allowed to air dry for 24 hr at room temperature. To check whether the technique of transferring epicuticular lipids was successful, control experiments were conducted to demonstrate that the specific reaction of *L. niger* ants towards the parasitoid species did not change. For this purpose, control individuals (*L. cardui*: $N = 24$; *T. angelicae*: $N = 23$) were prepared by treating the washed parasitoids with the hexane soak of conspecific females. Each parasitoid lure was covered with the equivalent of an extract of three parasitoids either from conspecific females or from alien females. Preliminary experiments with lower cuticular lipid concentrations showed that the recovery of the lipids was too low to result in a clear behavioral response of the ants. The introduction of the parasitoid individuals into an aphid colony and the recording of the behavioral response of the ants followed experiment 1. Each lure was used only once in an experiment.

Experiment 4: Introduction of Dead Parasitoids Treated with Hexane Soak

of A. fabae cirsiiacanthoidis. Freeze-killed unparasitized *A. fabae cirsiiacanthoidis* of different developmental stages were solvent-washed as explained for the parasitoids in experiment 3. The hexane soak of the aphids was applied on both hexane-washed *L. cardui* ($N = 20$) and *T. angelicae* ($N = 20$) females. As control individuals, washed aphids ($N = 22$) received their own extract. Each lure and control individual was covered with the equivalent of the extract of four aphids. After the transfer procedure, the insects were allowed to air dry for 24 hr at room temperature. The next day, the parasitoid lures and the aphid control were tested in the same way as described in experiment 1. Each lure was only used once in an experiment.

Statistical Analysis. The performance of a three-way table analysis with the factors species, treatment, and ant behavior resulted in a significant three-factor interaction term. In this case, according to Sokal and Rohlf (1981) we analyzed the data by employing separate two-way table tests of independence (chi^2 test, two-tailed) with the Yates' correction for continuity for small sample sizes (Sokal and Rohlf, 1981). Moreover, due to the small sample sizes of the test series, we pooled the removal events of the second and subsequent ants for each species and compared them with the respective number of removals by the first ant coming across the test individual.

RESULTS

Introduction of Dead Parasitoids and Aphids (Experiment 1). L. niger workers responded significantly different towards dead individuals of both parasitoid species and aphids. Figure 1 shows the distribution of *L. niger* ants that removed the test individuals. About 40% of *L. cardui* and 80% of *T. angelicae*

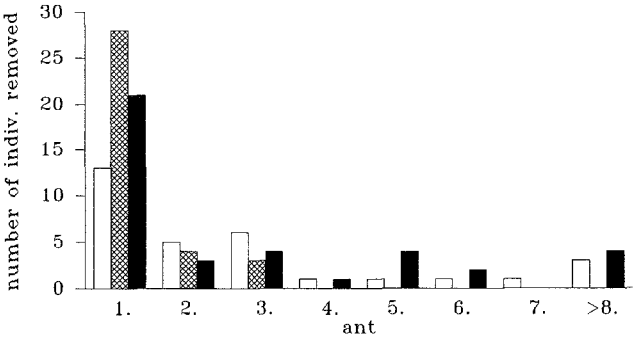

FIG. 1. Frequency distribution of the removal of dead nontreated test individuals by *Lasius niger* workers. Open bars: *Lysiphlebus cardui* ($N = 31$); cross-hatched bars: *Trioxys angelicae* ($N = 35$); shaded bars: *Aphis fabae cirsiiacanthoidis* ($N = 39$).

were seized by the first ant encountering the wasps (Figure 2); all *T. angelicae* individuals were finally removed by the third ant, in contrast to about 75% of *L. cardui* females. Half of the dead *A. fabae cirsiiacanthoidis* were immediately carried off by the ants after discovery. The response of *L. niger* workers towards dead individuals of both parasitoid species was significantly different when comparing the data of removal by the first ant to the pooled removal events of the subsequent ants ($chi^2 = 8.57$; $P = 0.003$; $N = 66$). In addition, ants showed also a significantly different response towards aphids and *T. angelicae* ($chi^2 = 4.53$; $P = 0.03$; $N = 74$). *L. cardui* females and aphids released a similar removal behavior in the ants ($chi^2 = 0.56$; $P = 0.45$; $N = 70$).

Introduction of Dead Hexane-Washed Parasitoids and Aphids (Experiment 2). Dead *L. cardui* and *T. angelicae* parasitoids immersed in hexane prior to the experiment induced similar removal reactions of *L. niger* workers (Figure 2, B: $chi^2 = 0.64$; $P = 0.425$; $N = 43$). Half of the *L. cardui* females and about one third of the *T. angelicae* females were taken by the first ant encountering the test individual. For *A. fabae cirsiiacanthoidis* only every fifth dead individual was removed by the first ant. The statistical analysis revealed no difference in ant behavior towards hexane-washed parasitoid species and aphids (*L. cardui–A. fabae cirsiiacanthoidis*: $chi^2 = 2.90$; $P = 0.09$; $N = 42$; *T. angelicae–A. fabae cirsiiacanthoidis*: $chi^2 = 0.37$; $P = 0.54$; $N = 41$).

A comparison of the results prior to solvent-washing and after the hexane treatment within a test group showed that *L. niger* workers displayed a similar

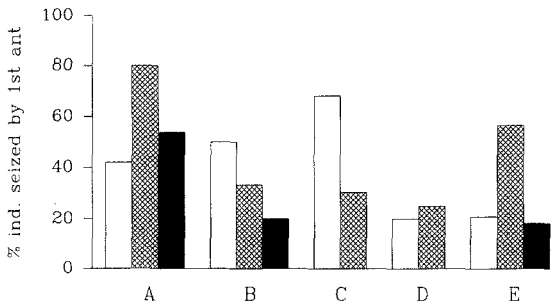

FIG. 2. Response of *Lasius niger* to nontreated and differently treated aphid parasitoids of *Lysiphlebus cardui* and *Trioxys angelicae*, and *Aphis fabae cirsiiacanthoidis*. The bars represent the proportions of test individuals that were removed by the first ant when encountering the individual in the aphid colony. (A) Nontreated; (B) hexane-washed; (C) treated with the hexane soak of the other parasitoid species; (D) treated with the hexane soak of *Aphis fabae cirsiiacanthoidis*; (E) control individuals (treated with the hexane soak of conspecifics). Open bars: *Lysiphlebus cardui*; cross-hatched bars: *Trioxys angelicae*; shaded bars: *Aphis fabae cirsiiacanthoidis*. Numbers of tested individuals were between 20 and 39 (for the exact numbers see Methods and Materials).

removal response when encountering dead *L. cardui* females in the aphid colony ($chi^2 = 0.09$; $P = 0.76$; $N = 53$). By contrast, dead nontreated and solvent-washed *T. angelicae* individuals induced a significantly different reaction of the ants ($chi^2 = 10.29$; $P = 0.0013$; $N = 56$). Similarily, the ants' response towards dead nontreated and hexane-washed individuals of *A. fabae cirsiiacanthoidis* differed significantly ($chi^2 = 8.33$; $P = 0.004$; $N = 79$). This could probably be due to the release of alarm substances from the siphons when removing the live aphids with forceps from the colony before freezing. These signals could be still present on the siphons during the experiments, causing the first ant to remove the dead nontreated aphids immediately (Nault et al., 1976).

Introduction of Dead Parasitoids Treated with Hexane Soak of Other Parasitoid Species (Experiment 3). Control individuals of *L. cardui* (females treated with the hexane soak of conspecifics) were removed significantly less often by the first ant than control individuals of *T. angelicae* (Figure 2, E: $chi^2 = 4.91$; $P = 0.03$; $N = 47$). Moreover, *L. niger* workers displayed a similar removal response towards nontreated and control individuals of both parasitoid species (Figure 2, A and E: *L. cardui*: $chi^2 = 1.86$; $P = 0.17$; $N = 55$; *T. angelicae*: $chi^2 = 2.65$; $P = 0.10$; $N = 58$). These results showed that the transfer of cuticular lipids was successful.

The behavior of *L. niger* workers differed significantly between *T. angelicae* and *L. cardui* females covered with the cuticular lipid extract of the other species ($chi^2 = 4.99$; $P = 0.003$; $N = 45$). In almost 70% of the trials, *L. cardui* parasitoids treated with the hexane soak of *T. angelicae* induced an immediate removal by the first ant (Figure 2, C). This response did not significantly differ from the behavior the ants showed towards the *T. angelicae* control group ($chi^2 = 0.25$; $P = 0.62$; $N = 45$). When encountering dead test individuals of *T. angelicae* with the solvent extract of *L. cardui* parasitoids, *L. niger* workers removed them with a similar probability as *L. cardui* control individuals (Figure 2, C: $chi^2 = 0.18$; $P = 0.67$; $N = 47$).

Introduction of Dead Parasitoids Treated with Hexane Soak of A. fabae cirsiiacanthoidis *(Experiment 4).* When *A. fabae cirsiiacanthoidis* individuals treated with the solvent extract of conspecifics were offered in an aphid colony, foraging *L. niger* workers rarely responded immediately at discovery. Only a fifth of the tested individuals ($N = 22$) were removed by the first ant. However, a comparison of the ants' response to nontreated (experiment 1) and control individuals of *A. fabae cirsiiacanthoidis* revealed a significant difference (Figure 2, A and E: $chi^2 = 6.00$; $P = 0.01$; $N = 61$). This result might be due to alarm substances present on the nontreated aphids (see explanation above). A possible contamination of test individuals with such alarm substances in the aphid hexane extract could be ruled out because these lures were dried for 24 hr, during which time these alarm substances should evaporate. *L. niger* responded to dead parasitoids of *L. cardui* ($N = 20$) and *T. angelicae* ($N = 20$)

treated with the hexane soak of *A. fabae cirsiiacanthoidis* (Figure 2, D) in a similar way. Eighty percent of the *L. cardui* lures and 75% of the *T. angelicae* lures were ignored by the discovering ant. The observations did not reveal any difference in the removal behavior of the ants towards the tested parasitoid individuals compared to the aphid control (*L. cardui–A. fabae cirsiiacanthoidis*: $chi^2 = 0.0$; $P = 1.0$; $N = 42$; *T. angelicae–A. fabae cirsiiacanthoidis*: $chi^2 = 0.03$; $P = 0.87$; $N = 42$).

DISCUSSION

To gain access to nests or to resources of social insects, predators and parasitoids may use several strategies. Morphological adaptations are used by myrmecophilous staphylinid beetles to integrate into ants' nests (Kistner, 1966, 1979). Behavioral mechanisms play a role in the beetle *Amphotis marginatus* Fabr. (Nitidulidae) begging for food from ants of the species *Lasius fuliginosus* (Latreille) (Hölldobler and Wilson, 1990). A similar behavior is also known from aphid parasitoids of the genus *Paralipsis* Förster (Aphidiidae) (Maneval, 1940; Takada and Hashimoto, 1985). In addition, chemical cues are involved in many inquilines of social insects either in using chemical secretions as appeasement and integrating substances (Hölldobler and Wilson, 1990) or in mimicking the specific cuticular lipid pattern of the host species (Howard et al., 1980, 1982, 1990a, b; Vander Meer and Wojcik, 1982; Vander Meer et al., 1989). Chemical mimicry was supposed to play a role in the relationship between the aphidiid wasp *L. cardui* and the ant *L. niger* in colonies of the aphid *A. fabae cirsiiacanthoidis* (Völkl and Mackauer, 1993). Völkl and Mackauer (1993) assumed that *L. cardui* mimics the cuticular lipid profile of its host aphid, thus being tolerated in the host colony by honeydew-collecting ants.

The present results demonstrate that recognition of *L. cardui* and *T. angelicae* parasitoids by ants is based on chemical cues located on the cuticular surface of the wasps. Dead nontreated *L. cardui* and *T. angelicae* offered in an aphid colony elicited a different removal response of the ants when being discovered (experiment 1). While *L. niger* workers rarely cared for dead *L. cardui* parasitoids in the aphid colony, dead *T. angelicae* individuals were readily removed by ants encountering them. This specific ants' reaction is comparable to that ants showed towards living parasitoids when searching in an aphid colony: *T. angelicae* females are immediately attacked by honeydew-collecting ants, while *L. cardui* wasps search unmolested for appropriate aphid hosts (Völkl and Mackauer, 1993). Moreover, the ants' removal response towards dead *L. cardui* resembled their response when encountering dead aphids in the colony. Consequently, ants were able to distinguish between dead individuals of the two parasitoid species, that is, the different searching behavior of the wasps is not the major cue for triggering aggressive reactions of *L. niger* ants.

The removal of the cuticular lipids from the surface of the parasitoids by immersion in an organic solvent (e.g., Gilby, 1980; Jackson and Blomquist, 1976; Lockey, 1988) resulted in an identical removal response of the ants when hexane-washed individuals of *L. cardui* and *T. angelicae* were offered (experiment 2). Consequently, *L. niger* ants could not discriminate between two aphidiid species when deprived of their species-specific lipid layer. Hexane-washed aphids also elicited a tolerant ant response. The significant difference in the behavior of the ants towards nontreated and hexane-washed *T. angelicae* suggests that these individuals lost their specific recognition cues after the solvent extraction, which obviously triggered the aggressive behavior of the ants. The hexane immersion of *L. cardui* individuals did not alter the response of *L. niger* compared to its reaction towards nontreated conspecific parasitoids. These findings also show that any possible morphological feature of the parasitoid species important for species recognition by the ants could be ruled out.

To confirm the presumption that chemical cues on the cuticular surface are responsible for the recognition of the parasitoids by *L. niger*, the lure experiments were conducted. The results showed that the cuticular lipids caused them to be ignored or removed by the ants. The transfer of *L. cardui* extract to individuals of *T. angelicae* had the effect that *L. niger* ants treated *T. angelicae* lures similar to *L. cardui* controls. The removal behavior of the ants towards *L. cardui* individuals covered with the hexane extract of *T. angelicae* also was in accordance with the results of the *T. angelicae* control. Similarily, the transfer of *A. fabae cirsiiacanthoidis* soak onto *L. cardui* and *T. angelicae* parasitoids caused the same response of *L. niger* as towards the aphid control.

The experiments confirm the hypothesis that the aphidiid wasp *L. cardui* uses chemical cues located on the cuticular surface to avoid attacks by aphid-guarding ants (Völkl and Mackauer, 1993). These recognition cues are hexane-soluble and can be artificially transferred to other nonconspecific parasitoids. Moreover, the almost identical removal behavior of *L. niger* workers towards dead aphids and *L. cardui* suggests that *L. cardui* mimics the cuticular lipid profile of its aphid host. The use of epicuticular lipids to escape attack is known from a number of inquilines of ants and termites (Howard et al., 1980, 1982, 1990a, b; Vander Meer and Wojcik, 1982; Vander Meer et al., 1989). Most of these intruders known by now mimic the cuticular hydrocarbon pattern of their host species. Another strategy to incorporate into a host's nest is by producing the typical fatty acid bouquet of the host. This could be demonstrated with the death's head hawkmoth, *Acherontia atropos* L. (Moritz et al., 1991). The detailed chemical background of the adaptation of *L. cardui* to avoid attacks from honeydew-collecting ants is still unknown. However, the cuticular lipids of the species involved in this aphid–ant–parasitoid system are already under chemical investigation to find out why *L. cardui* is chemically invisible to ants.

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BACTERIAL–BARNACLE INTERACTION: POTENTIAL OF USING JUNCELLINS AND ANTIBIOTICS TO ALTER STRUCTURE OF BACTERIAL COMMUNITIES

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Abstract—In preparation for studies using natural products to probe interactions between bacterial consortia and settlement stage barnacles, we isolated 16 strains of bacteria associated with barnacles and examined: (1) effects of films of bacterial isolates on barnacle settlement, and (2) bacteriostatic effects of juncellins and standard antibiotics. Bacteria were isolated from the biofilm associated with *Balanus amphitrite*. On the basis of morphological and biochemical characteristics, bacteria were classified into five major groups: *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, and *Vibrio*. Barnacle settlement was inhibited by all *Vibrio* films and 64% of the other isolates. No film stimulated barnacle settlement. Juncellins were approximately as potent as standard antibiotics for all bacterial species tested. *Vibrio* spp. were most resistant to juncellins.

Key Words—Marine bacteria, fouling, *Balanus amphitrite*, natural products, antibiotics, microbial, *Vibrio* spp., ecology, larval settlement.

INTRODUCTION

Among marine ecologists a consensus is emerging that classical concepts of succession, such as those for terrestrial environments, do not apply to marine fouling communities (Sutherland and Karlson, 1977; Sutherland, 1984; Maki et

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al., 1988; Clare et al., 1992). Part of the successional argument in question is that bacterial fouling is a requisite for macrofouling. Although larvae of some invertebrates settle preferentially on microbial films (Scheltema, 1974; Kirchman et al., 1982), recent work in the field (Roberts et al., 1991) and the laboratory (Maki et al., 1992; Gerhart et al., 1992) shows that microbial fouling is not a requisite for macrofouling. Work with common organisms such as barnacles (Maki et al., 1988), bryozoans (Maki et al., 1989), hydroids (Roberts et al., 1991), and ascidians (Gerhart et al., 1992) shows that microbial films are not required.

Wahl (1989) reiterated the classic paradigm of succession in colonization of a surface in which molecular films led to microbes and these were followed in turn by macrofoulers. Wahl states that the jury is still out as to whether the pattern is due to successional conditioning events or simply to numbers of propagules of each type in the water column. Roberts et al. (1991) provided evidence that larvae of three species of common macrofouling organisms settle at the same rate as surfaces are colonized by bacteria. These observations led to the idea that initial patterns of common foulers may be due in large part to the number of competent larvae contacting a surface (Clare et al., 1992).

However, larval settlement is affected by microbial films (Maki et al., 1989; Hadfield et al., 1993; Holmström et al., 1992). For example, films composed of individual species of bacteria exert negative effects on settlement of *Balanus amphitrite* larvae (Maki et al., 1992).

Thus, although bacteria are not required for settlement of many invertebrate larvae, they still play an important role in determining patterns of macrofouling. Furthermore, since virtually all submerged surfaces are filmed with bacteria (Baier, 1984; Little et al., 1986) bacteria–macrofouler interactions may be important determinants of community development.

We hypothesize that bioactive natural products such as antibiotics and juncellins (Vitalina et al., 1991) can be used to probe bacterial–macrofouler interactions by systematically altering the composition of the bacterial consortium and determining responses of settlement stage macrofouling larvae. Here we report isolation and characterization of bacteria found associated with the common macrofouler *Balanus amphitrite* Darwin. Films of isolated bacteria are tested for their effects on barnacle settlement. Then the effects of juncellins and antibiotics on bacterial isolates are determined.

METHODS AND MATERIALS

Isolation and Characterization of Bacteria Found in Biofilm Associated with Macrofoulers. Bacteria were isolated from surface fouling (10 g) by scraping pilings at the site of *Balanus amphitrite* attachment. The sample was homog-

enized in a sterilized mortar and pestle, suspended in 99 ml of sterile aged seawater, and serial dilutions prepared.

Pour Plate Culture. A pour plate method was used for the isolation of pure species of bacteria (Rodina, 1972). In this method, 1.0 ml of the inoculum was transferred to sterilized Petri dishes followed by approximately 15–20 ml of 3% agar in 2216E marine peptide broth. Petri dishes were thoroughly mixed by rapid clockwise and counterclockwise rotation and then allowed to solidify. The inverted plates were incubated at 28°C.

Isolation and Characterization of Bacteria. Pour plate cultures contained many types of microbial colonies. Isolated bacterial colonies were grown individually in Dibco 2216 marine peptone broth. Morphological characteristics (form size, margin and color of the colony) of bacteria were observed in pour plate and agar slant cultures. The growth form in nutrient broth (flocculent, membranous, and pellicle) was noted. Gram staining identified gram-positive and gram-negative bacteria. Motility was observed (yes or no) in hanging drop culture. Bacterial isolates were also characterized by catalase activity, indole production, nitrate reduction, cholera-red test, methyl red test, Voges-Proskauer test, starch hydrolysis, gelatinase liquefication, and Hugh and Leifso's test (Holt, 1977). For each test log-phase bacteria were inoculated in prescribed conditions and observations were made after the prescribed incubation.

Bacterial Film Formation. Polystyrene (50 × 9-mm Falcon No. 1006) Petri dishes were filmed with bacteria and used in settlement assays. Bacterial strains were grown in the dishes to stationary phase (~24 hr) in Zobell's marine broth (Difco 2216) at room temperature. Marine broth was discarded, and the dishes were rinsed three times with sterilized seawater and then refilled with 5 ml of fresh filtered and sterilized seawater. The series of filmed dishes in triplicate and controls (containing sterile broth for 24 hr) were rinsed with sterile seawater and used in settlement assays.

Biofilm Settlement Assays. Single-choice assays (settle vs. not settle) were conducted in Falcon No. 1006 polystyrene Petri dishes (Branscomb and Rittschof, 1984; Maki et al., 1992). Approximately 50 larvae were introduced into each Petri dish containing bacterial film. Timing of the assay began with larval addition, and the assay interval was 24 hr at 27°C ± 2°C. Larvae permanently attached to test substratum were counted as settled, and larvae not attached were counted as not settled. Statistical comparisons between test and control were done by frequency analysis (G statistic), with Bonferroni's method for multiple comparisons (Seber, 1977).

Collection and Extraction of Active Material from Juncella juncea. The octocoral *J. juncea* was collected and estimated as described previously (Vitalina et al., 1991). The procedure yields juncellin 1 and juncellin 2. Extracts were solubilized in methanol, dispensed to treatments, and the methanol removed by

drying before tests were initiated (after Rittschof et al., 1985). The fractions containing juncellins are approximately 90% pure.

Antimicrobial Assays. Antimicrobial assays were conducted to test juncellins using standard agar diffusion techniques. Bacterial isolates were grown on antibiotic agar medium (agar 2.0 g and peptone 1.0 g in 100 ml sterilized seawater). Four concentrations of juncellin fractions—7, 70, 700, 7000 ng/disk—based on fraction dry weight, were loaded on disks (6.5 mm). Agar diffusion techniques were used to test the sensitivity of bacterial isolates to commercial antibiotics chloramphenicol, malachite green, penicillin, and streptomycin. The concentration of 0.01 mg/disk was placed on a paper disk.

RESULTS

Bacteria in Biofilm Associated with Macrofouler Balanus amphitrite. On the basis of morphological characteristics of bacterial colonies, a total of 16 bacterial isolates from *B. amphitrite* was isolated (Table 1). Of these, five bacterial species were sensitive to vibriotatic compound 0/129 (2,4 diamino-6,7-diisopropyl pteridine). This test is specific in confirming *Vibrio* sp.; classification of other bacterial isolates required an integration of biochemical test results with morphological observations (Tables 2 and 3). Bacterial identification was based on Bergey's Manual of Determinative Bacteriology (Holt, 1977). The 16 bacterial isolates were placed into five major groups: *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, and *Vibrio* (Table 4).

Bacterial Effect on Cyprid Settlement. Effects of bacterial films upon barnacle settlement were determined using polystyrene as the substratum and 3-day-old cyprid larvae (Maki et al., 1988). Films of all bacterial isolates inhibited barnacle settlement in all biofouling sequential tests at $P < 0.05$, demonstrating that individual isolates of bacteria were inhibitory on cyprid settlement when compared to control (Figure 1). *Vibrio* isolates were the most potent in inhibiting barnacle settlement.

Antimicrobial Assays. Antimicrobial assays with juncellins demonstrated that juncellins are potent bacteriostatic compounds. Juncellins inhibited the growth of the bacterial isolates associated with macrofoulers. For juncellin 1, 10 ng/disk arrested the growth of 50% of the bacteria associated with *Balanus amphitrite*. With 10,000 ng/disk, growth of all 16 species tested was inhibited (Table 4). By comparison, 10 ng/disk of juncellin 2 inhibited the growth of 50% of the bacteria, and 10,000 ng/disk inhibited 75% of the bacteria (Table 4). Zones of inhibition ranged from 6 to 12 mm in radius.

The sensitivity of bacterial isolates to commercial antibiotics, chloramphenicol, malachite green, penicillin, and streptomycin, was determined (Table 3). All 16 bacterial isolates were sensitive to standard antibiotics at 0.01 mg/

TABLE 1. CULTURAL AND MORPHOLOGICAL CHARACTERISTICS OF ISOLATED BACTERIA ASSOCIATED WITH *Balanus amphitrite variegatus*

Strain	Colonial appearance			Growth on marine broth	Gram stain		Motility	Shape	Growth in agar slant
	Elevation	Margin	Color		Pos.	Neg.			
S_1	Raised	Lobate	Dull white	Pellicle		−ve	+[a]	Long rods	Filiform
S_2	Flat	Lobate	Orange	Pellicle		−ve	+	Short rods	Echinulate
S_3	Flat	Undulate	Dull white	Pellicle		−ve	+	Short rods	Filiform
S_4	Raised	Entire	White	Pellicle		−ve	+	Small curved rods	Rhizoidal
S_5	Convex	Entire	Yellow	Pellicle		−ve	+	Short rods	Echinulate
S_6	Raised	Undulate	Dull white	Pellicle		−ve	+	Small rods	Filiform
S_7	Flat	Lobate	White	Membranous		−ve	+	Short rods	Arborescent
S_8	Raised	Entire	Bluish green	Flocculent		−ve	+	Short rods	Beaded
S_9	Raised	Lobate	Dull white	Pellicle		−ve	+	Short rods	Filiform
S_{10}	Flat	Entire	Bluish green	Membranous		−ve	+	Short rods	Echinulate
S_{11}	Flat	Undulate	Dull white	Membranous		−ve	+	Long rods	Filiform
S_{12}	Raised	Entire	Dull white	Pellicle		−ve	+	Short rods	Arborescent
S_{13}	Flat	Undulate	Dull white	Flocculent		−ve	+	Long rods	Echinulate
S_{14}	Raised	Lobate	White	Flocculent		−ve	+	Small rods	Echinulate
S_{15}	Raised	Undulate	Yellow	Pellicle		−ve	+	Short rods	Rhizoidal
S_{16}	Flat	Undulate	Brown	Membranous		−ve	+	Short rods	Echinulate

[a] + = motile.

TABLE 2. BIOCHEMICAL CHARACTERISTICS OF BACTERIA ASSOCIATED WITH *Balanus amphitrite variegatus*

	Name of test	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}	S_{13}	S_{14}	S_{15}	S_{16}
1.	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.	Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3.	Indole production	+	+	+	+	+	−	−	−	−	+	+	−	−	−	+	+
4.	Carbohydrate metabolism																
i.	Fermentative	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−
ii.	Oxidative	−	−	−	−	−	+	+	+	+	−	−	+	+	+	−	−
iii.	Gas production from glucose	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
5.	Starch hydrolysis	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+
6.	Gelatin hydrolysis	+	+	+	+	+	−	−	−	−	+	+	−	−	−	+	+
7.	Nitrate reduction	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−
8.	H_2S production	−	−	−	−	−	+	+	+	+	+	+	−	−	−	+	+
9.	Methyl red test	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
10.	Voges-Proskauer test	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+
11.	Cholera red test	−	−	−	−	−	+	+	+	+	−	−	+	+	+	−	−

TABLE 3. ANTIBIOTIC SENSITIVITY TEST WITH BACTERIA ASSOCIATED WITH *B. amphitrite variegatus*

Antibiotic (concentration 0.01 mg)	Bacteria number															
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}	S_{13}	S_{14}	S_{15}	S_{16}
1. Chloremphenicol	+	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+
Zone of inhibition in (mm)	13.0	8.5	–	9.0	13.0	14.0	12.5	15.0	15.0	9.5	9.5	19.0	3–3.5	–	13.0	13.5
2. Malachite Green	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Zone of inhibition in (mm)	10.0	10.5	8.0	7.5	6.0	4.5	6.0	7.5	6.0	8.0	5.5	7.0	–	9	15.0	5.0
3. Penicillin	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–
Zone of inhibition in (mm)	2.0	2.5	3.0	3.0	1.5	2.5	1.5	1.3	5.5	–	–	–	5.0	3	5.5	–
4. Streptomycin	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–
Zone of inhibition in (mm)	4.0	3.5	3.5	3.5	4.0	3.5	9.5	9.5	6.0	8.5	7.5	7.5	6.0	8	7.5	–

TABLE 4. EFFECT OF JUNCELLINS 1 AND 2 ON BACTERIAL ISOLATES ASSOCIATED WITH *Balanus amphitrite*

	10 mg/10 μl		1 mg/10 μl		0.1 mg/10 μl		0.01 mg/10 μl	
Strain	J1	J2	J1	J2	J1	J2	J1	J2
Ae_1[a]	++	++	−	++	−	++	−	+
Ae_2	+++	−	−	−	−	−	−	−
Al_1	+++	+++	+++	+++	++	++	++	−
Al_2	++	−	++	−	++	−	++	−
Al_3	++	+++	++	++	++	++	++	++
F_1	++	+++	++	++	++	−	++	−
F_2	++	++	++	++	++	++	++	++
P_1	++	+++	−	+++	−	++	−	++
P_2	++	++	++	−	++	−	++	−
P_3	+	+++	−	−	−	++	−	+
P_4	++	+++	++	−	++	−	++	−
V_1	++	+++	−	+++	−	+++	−	++
V_2	++	+++	−	++	−	++	−	+
V_3	+	−	−	−	−	−	−	−
V_4	+++	−	+++	−	−	−	−	−
V_5	+++	+++	+++	+	++	−	+	++

[a] +, 4–6 mm radius; ++, 6–8 mm radius; +++, 8–12 mm radius.

disk. Zones of inhibition ranged from 4 to 15 mm radius. Juncellins are approximately as potent as standard antibiotics. Juncellins are natural bacteriostatic compounds.

DISCUSSION

Theoretically, there are three ways that organisms such as octocorals prevent fouling (Davis et al., 1989; Wahl, 1989; Vrolijk et al., 1990). They can physically slough mucus or use secondary compounds in one of two ways. One way to employ secondary compounds would be to target both macrofoulers and microfoulers. The second way would be to control macrofoulers by regulating the growth of microfoulers such as bacteria that inhibit macrofouler larval settlement. Juncellins appear to work by the first option because they inhibit barnacle settlement and are bacteriostatic.

The lack of fouling on *Juncella juncea* by common fouling organisms may result from secondary compounds (the juncellins), as hypothesized for the unfouled nature of most gorgonians (Ciereszko et al., 1960; Targett et al., 1983; Standing et al., 1984; Rittschof et al., 1985; Vitalina et al., 1991). In antimi-

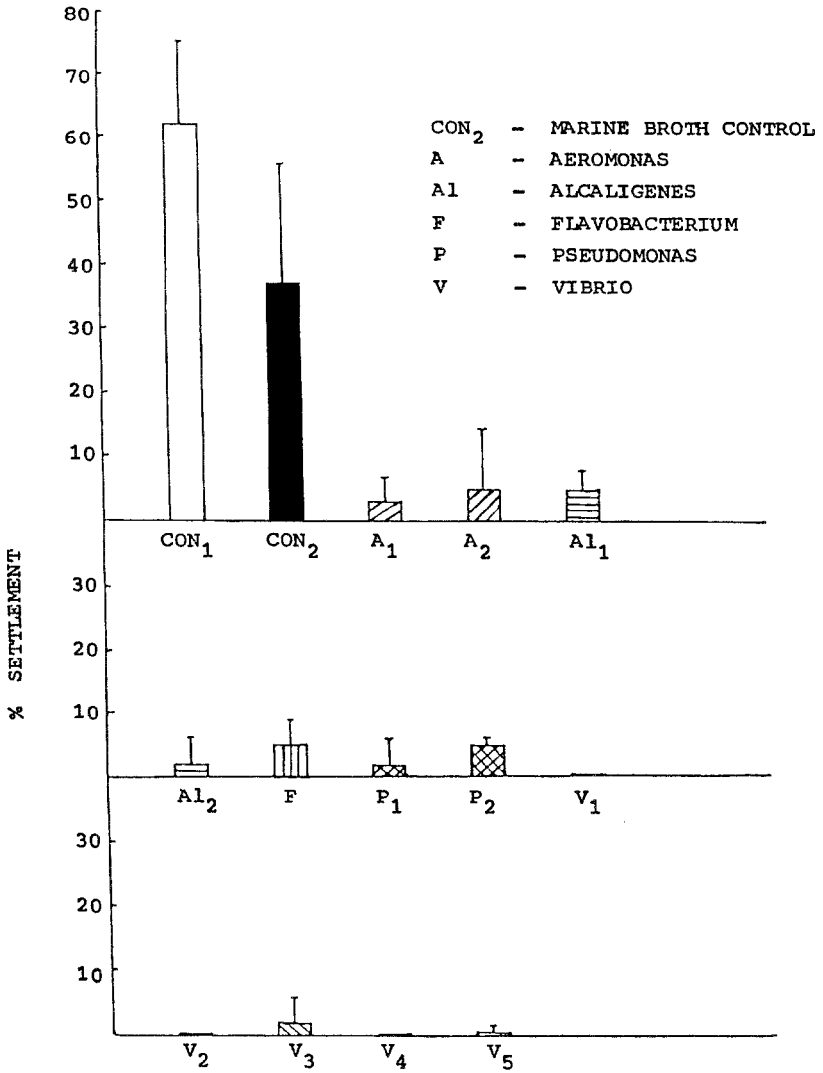

FIG. 1. Inhibition of larval barnacle settlement by films of isolated bacteria. Polystyrene Petri dishes were filmed in triplicate with bacteria isolated in the vicinity of barnacles. Settlement stage barnacles were added and settlement determined. CON_1 = settlement in a polystyrene dish exposed only to filtered seawater; CON_2 = settlement on dishes incubated with sterile marine broth; A = *Aeromonas*; Al = *Alcaligenes*; F = *Flavobacterium*; P = *Pseudomonas*; V = *Vibrio*. Bars indicate variation between replicates.

crobial assays, juncellins cause large inhibition zones with sharp boundaries at 7 ng/disk. Thus, in addition to inhibiting barnacle settlement (Vitalina et al., 1991) juncellins are bacteriostatic.

Few bioactive compounds have been examined for both antimicrobial and antifouling activities. The juncellins are the first of the nontoxic antibarnacle compounds to be tested in this way. We hypothesize that other antibarnacle compounds with structures similar to juncellin, such as the renillafoulins (Keifer et al., 1986) will also be bacteriostatic. The bacteriostatic activity is interesting because these compounds are not toxic to crustacean larvae or human cell lines (Keifer et al., 1986) and are potentially important in effective delivery of the compounds at the surface of the animal.

Bacteria isolated from biofilms associated with tropical *Balanus amphitrite* were gram-negative and motile. Five genera of bacteria, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, and *Vibrio*, were found associated with *B. amphitrite*. Colwell and Liston (1960) determined that microorganisms associated with *Crassostrea gigas* comprised mostly *Achromobacter*, *Glavobacterium*, *Pseudomonas*, and *Vibrio*. Lovelace et al. (1968) reported a greater diversity of bacteria, nine taxa being associated with *Crassostrea virginica*: *Achromobacter*, *Bacillus*, coryneforms, *Cytophaga*, *Flavobacterium*, Enterobacteriaceae, *Micrococcus*, *Pseudomonas*, and *Vibrio*. Although there is always the question of bias due to differential growth of bacteria on the particular medium selected, our findings are more similar to those of Colwell and Liston (1960) than to Lovelace et al. (1968).

That attachment of *B. amphitrite* is inhibited on bacterial filmed surfaces is well established (Maki et al., 1985). Effects could be due to substratum surface chemistry (Rittschof and Costlow, 1989a,b; Roberts et al., 1991) or to bioactive compounds released by the bacteria. Maki et al. (1990) found bacterial isolates in general inhibited barnacle settlement. We found some isolates in all five genera of bacteria inhibited settlement. In both studies, films of *Vibrio* sp. strongly inhibited barnacle settlement. Bacteria that strongly promoted settlement were not observed in either study.

The lack of stimulatory effects of bacteria on barnacle settlement is in marked contrast to effects of bacteria on the settlement of other invertebrate larvae. For example, the exopolymers of *Deleya marina* biofilms have been hypothesized to stimulate the settlement and metamorphosis of the polychaete worm *Janua brasiliensis* (Kirchman et al., 1982). Oysters (Weiner et al., 1985) attach to surfaces upon which specific microorganisms are growing. Most recently, Hadfield et al. (1993) reported a direct correlation between bacterial films and settlement of *Hydroides elegans*.

Marine bacteria produce a wide range of bioactive extracellular molecules (Okami, 1986; Staley and Stanley, 1986). The surface is changed by the extra-

cellular molecules (Baier, 1984) and the nature of larval responses depends upon the capabilities of the larvae as well as the nature of the extracellular products.

The ability of barnacle larvae to respond to both stimulatory (Crisp and Meadows, 1962, 1963; Rittschof et al., 1984, 1985) and inhibitory (Standing et al., 1984; Rittschof et al., 1985, 1986; Johnson and Strathmann, 1989; Vitalina et al., 1991) compounds is well documented. Observations of bacterial film and cyprid settlement interactions demonstrate that although microfouling is not a prerequisite to barnacle settlement, bacterial films can be very important.

Little is known of the stability of marine bacterial assemblages or of their role in the initial development and structuring of marine fouling communities. It is likely that the use of chemical signals from bacterial communities to cue settlement by macrofoulers is similar to that observed for diatoms (Strathman et al., 1981) and for crustose coralline algae (A.N.C. Morse, 1992; D.E. Morse, 1992). Documented effects of mixed films and monospecific bacterial films on macroinvertebrate settlement (cf. Mitchell and Kirchman, 1984; Weiner et al., 1985; Maki et al., 1992; Holmström et al., 1992) enable prediction of productive studies of the effects of specific bacterial consortia on settlement of macrofouling organisms. Future work will probe bacteria–barnacle interactions by selectively modifying the species composition of bacterial films and challenging them with competent settlement stage invertebrate larvae.

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GEOMETRICAL AND OPTICAL ISOMERISM OF PHEROMONES IN TWO SYMPATRIC *Dryocoetes* SPECIES (COLEOPTERA: SCOLYTIDAE), MEDIATES SPECIES SPECIFICITY AND RESPONSE LEVEL

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Abstract—In a field-trapping experiment, western balsam bark beetles, *Dryocoetes confusus* Swaine, were highly attracted to a 5:1 mixture of (±)-*exo*- and (±)-*endo*-brevicomin. Beetles in the sympatric species *D. affaber* (Mann.), were best attracted to a 1:1 blend of these semiochemicals [either (±):(±) or (+):(+)], suggesting that both geometrical isomers are pheromone components in these species. In laboratory bioassays and further field experiments, attraction of *D. confusus* was greatest when the (+) enantiomers of both geometrical isomers of brevicomin were presented in a 9:1 ratio. Responses by male *D. confusus* to attractive mixtures were reduced in the presence of (−)-*exo*-brevicomin. Exploitation of the complete range of variability in pheromone structure (both geometrical and optical isomerism) would allow for optimization and regulation of response levels within a species and also could maintain reproductive isolation among sympatric congeneric species primarily through production and response to species-specific blends.

Key Words—Semiochemicals, pheromones, *Dryocoetes confusus*, *Dryocoetes affaber*, Coleoptera, Scolytidae, enantiomers, diastereoisomers, *exo*-brevicomin, *endo*-brevicomin.

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INTRODUCTION

Species specificity of chemical communication signals in insects is an important mechanism for resource partitioning (Rudinsky, 1969; Birch and Wood, 1975; Byers, 1983; Prokopy et al., 1984) and reproductive isolation (Wood, 1970; Vité et al., 1978; Cardé, 1986; Merrill, 1991). Insects that use pheromones with a common molecular structure could theoretically achieve species specificity of olfactory signals by exploiting the geometrical or optical isomerism of these compounds. Specific isomers could then act alone or in blends with other isomers or compounds at particular ratios, usually within narrow margins of variation (Tumlinson, 1988; Linn and Roelofs, 1989).

The relationship between the stereochemistry of pheromones and their biological activity has been a major issue since 1956 when it was found that bombykol was one of four possible geometrical isomers (Hecker, 1958; Butenandt et al., 1961). In the 1970s, extensive research demonstrated that sensitivity to $E:Z$ isomer ratios and to other diastereoisomers is a general mechanism for pheromonal specificity, particularly in moths and bark beetles (Silverstein et al., 1968; Renwick et al., 1976; Richerson and Payne, 1979; Borden, 1985; Byers et al., 1989; Baker, 1989). Kafka et al. (1973) demonstrated the capability for chiral discrimination in olfaction by bees and locusts; Riley et al. (1974) documented the first case of enantioselective response to a semiochemical in the ant, *Atta texana* (Buckley). Chiral sensitivity has now been reported for many insect species (Silverstein, 1979, 1988; Mori, 1984; Löfquist, 1986) and is considered to confer a high degree of specificity in chemical communication among scolytid beetles (Borden et al., 1976, 1980; Birch et al., 1980; Brand et al., 1979; Payne et al., 1982; Birch 1984; Byers, 1989).

While either geometrical or chiral specificity in animal olfaction could allow the utilization of numerous, distinct pheromone communication channels, even more specificity could theoretically be achieved by exploiting combinations of these two types of isomerism. This level of specialization for congeneric sympatric species has been demonstrated only in dermestid beetles. Khapra beetles, *Trogoderma granarium* Everts, use the (*R*)-(−) enantiomers of (*Z*)- and (*E*)-trogodermal in a 92:8 ratio, while beetles in three other *Trogoderma* spp. respond to the (*R*)-(−) enantiomer of either (*E*)- or (*Z*)-trogodermal (Cross et al., 1976; Silverstein et al., 1980).

We report that the combined effect of the ratio of geometrical and optical isomers of brevicomin (7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) determines both level of response and pheromonal specificity in the western balsam bark beetle, *Dryocoetes confusus* Swaine, and the sympatric species *D. affaber* (Mannerheim).

The western balsam bark beetle is a major pest of subalpine fir, *Abies lasiocarpa* (Hook.) Nutt (Stock, 1981, 1991). It is sympatric in British Columbia

with *D. affaber*, which primarily colonizes weakened or damaged spruce trees, *Picea* spp., but can also attack *Abies* spp. (Wood, 1982). Male *D. confusus* produce an aggregation pheromone that attracts beetles of both sexes (Stock and Borden, 1983; Borden et al., 1987). In abdominal extracts of feeding males (+)-*exo*- and (+)-*endo*-brevicomin were 99.2% and 81.6% optically pure, respectively (Schurig et al., 1983). (Hereafter, exoB is used for *exo*-brevicomin and endoB for *endo*-brevicomin).

Racemic and (+)exoB have been shown to be attractive to *D. confusus* of both sexes, while (−)exoB was initially considered inactive (Borden et al., 1987) or even additive when combined with (+)exoB (Stock et al., 1993). Field tests showed that (±)endoB in a 1:1 ratio with (±)exoB inhibited attraction and (−)endoB did not inhibit attack by *D. confusus*. Thus Stock et al. (1990) concluded that (±)- or (+)endoB was an antiaggregation pheromone. The semiochemical system of *D. affaber* has not been reported.

METHODS AND MATERIALS

Collection of Insects and Hosts. Subalpine firs infested with *D. confusus* were felled near Merritt, British Columbia and sawn into 70-cm-long bolts, which were placed in cages in the laboratory. Emerging adults were sexed and kept on moistened paper at 5°C until used.

Analysis of Volatiles. To determine the natural ratio of exoB to endoB released into the air, volatiles from fresh subalpine fir bolts infested with male *D. confusus* were captured on Porapak-Q (Byrne et al., 1975). Frass from boring males was collected and steam distilled through a 30-cm glass Dufton column. The volatiles from the infested log aerations and frass were analyzed by gas chromatography (GC) using Hewlett Packard 5830A and 5880A instruments equipped with capillary inlet systems and flame ionization detectors. Samples were analyzed on open tubular glass columns coated with SP-1000 (Supelco, Bellefonte, Pennsylvania). Temperature program was 70°C for 2 min, then 4°C/min to 180°C, holding for 20 min. Coupled gas chromatography–mass spectrometry (GC-MS) was performed with a Hewlett Packard 5895A GC-MS-DS fitted with a 30-m × 0.32-mm-ID fused silica column coated with SP-1000 (J & W Scientific, Inc., Folsom, California). Helium was the carrier gas for GC and GC-MS.

Synthetic Pheromones. The (±)exoB (96.3% pure, 2.5% endoB) and (±)endoB (96.4% pure, 0.4% exoB), were obtained from Phero Tech Inc., Delta, British Columbia. Chiral brevicomins were synthesized by B.D. Johnston (Department of Chemistry, Simon Fraser University), according to procedures developed by Johnston and Oehlschlager (1982) and Oehlschlager and Johnston (1987). Formulations included (+)exoB (98.1 and 93.05% chemically and opti-

cally pure, respectively), (−)exoB (97.0 and 95.0% chemically and optically pure, respectively), (+)endoB (98.8 and 90.15% chemically and optically pure, respectively). Blends of exoB and endoB were prepared on a weight-to-weight basis.

Laboratory Bioassays. Determination of the optimal ratio of exoB to endoB for attraction of *D. confusus* was conducted using walking beetles in an open arena olfactometer (Wood and Bushing, 1963; Stock and Borden, 1983). Groups of 10 beetles were exposed for 2.5 min to an airstream (700 ml/min) containing volatile stimuli applied in 10 μl of pentane to a filter paper wick. The solvent was used as a control, room lighting was diffuse, and room temperature was 20–21°C.

A series of 1-pg stimuli consisting of (+)exoB, (+)endoB, and blends of these two compounds at ratios of 9:1, 7:1, 4:1, 2.3:1, and 1:1 were tested. Thereafter, we carried out a dose–response bioassay using the 9:1 blend of (+)exoB and (+)endoB at five doses (0.1, 1, 10, 100, and 1000 pg); pentane (10 μl) and (+)exoB (1 pg) were included as control stimuli.

Field Experiments. Trapping experiments were conducted in a subalpine forest located 40 km west of Merritt, British Columbia. In all experiments multiple funnel traps (Lindgren, 1983) (Phero Tech Inc.), were placed 15 m apart in randomized complete blocks, with 10–20 replicates. As a standard procedure in all field experiments, volatile stimuli were released either as single compounds or in blends, at approximately 0.2 mg/24 hr from a glass capillary tube (1.0 mm ID) sealed at one end (Borden et al., 1987).

Field experiment I was performed before the laboratory bioassays. In late summer, 1990, we tested the attractiveness of (±)exoB and blends of (±)exoB and (±)endoB at the following ratios: 11:1, 5:1, 1:1.

Field experiment II and later experiments were conducted using synthetic (+)exoB and (+)endoB (the naturally occurring enatiomers). In the summer of 1991, the first of two field experiments tested mixtures of (+)exoB and (+)endoB at three different ratios: 5:1, the most attractive blend in the previous field experiment with racemic brevicomins; 9:1, which elicited the best response in laboratory bioassays, and the 1:1 blend, which was unattractive for *D. confusus* (Stock et al., 1990), but attractive in the field for *D. affaber*. Also included were (±)exoB and an unbaited control.

In field experiment III, having investigated the role of ratios of geometrical isomers of pheromones in mediating the aggregation response by *D. confusus*, we then tested for enantioselectivity. Because (−)exoB was known not to be attractive (Borden et al., 1987; Stock et al., 1990), only combinations of (+)- and (±)exoB and endoB were tested at a 9:1 ratio.

For field experiment IV, to investigate the role of (−)exoB further, we conducted a final field experiment near the end of the summer of 1992. We tested the following stimuli: the attractive mixture of (+)exoB and (+)endoB

at the 9:1 ratio released at approximately 0.2 mg/day; (−)exoB:(+)endoB at the 9:1 ratio, and also the (+):(+), 9:1 stimulus with another glass capillary containing (−)exoB in the same trap.

Statistical Analysis. Laboratory bioassay results were subjected to chi-square analysis using the frequencies of positive responders; independence of response for individual beetles has been previously demonstrated (Stock, 1981). Data from field experiments were analyzed by two-way ANOVA and the Ryan-Einot-Gabriel-Welsch multiple F or REGWF test (Schlotzhauer and Littell, 1987) on numbers of beetles captured transformed by $x' = \log(x + 1)$. If data were not then normally distributed, we used the nonparametric Friedman test (Friedman, 1937; Conover, 1980). All analyses employed SAS computer software (SAS Institute, 1990).

RESULTS AND DISCUSSION

Analysis of Volatiles. GC analysis (confirmed by MS) of volatiles emanating from males in logs disclosed exoB and endoB in a ratio of 11.4:1 (Figure 1); frass volatiles contained the isomers in a 5.4:1 ratio. Schurig et al. (1983) analyzed crushed abdomens of feeding male *D. confusus* and determined an 18:1 average ratio of exoB to endoB mostly as (+) enantiomers. The consistent presence of these two compounds from the start of volatile production by male

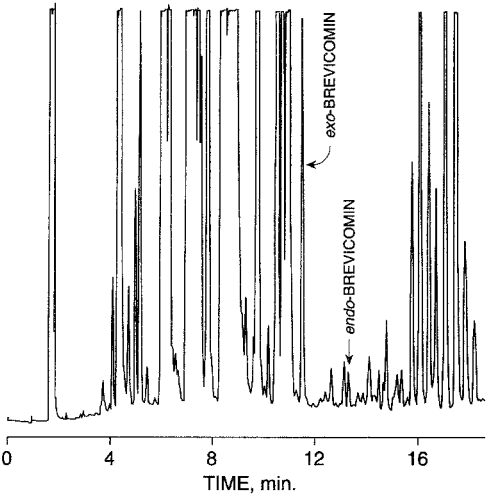

FIG. 1. Gas–liquid chromatogram of Porapak Q-trapped volatiles produced by male *Dryocoetes confusus* feeding inside fresh bolts of *Abies lasiocarpa*, showing *exo*-brevicomin and *endo*-brevicomin in an 11.4:1 ratio.

beetles suggested that they both have a role in aggregation of *D. confusus*, even though high levels of (±)endoB were known to inhibit response (Stock et al., 1990). This hypothesis was tested in further experiments. In addition, the variable ratios of the two compounds in different analyses (see above) necessitated that the optimal ratio be determined experimentally.

Field Experiment I. The 5:1 blend of (±)exoB and (±)endoB was most attractive to *D. confusus* females (Figure 2); males responded poorly and did not discriminate between treatments. On the other hand, *D. affaber* responded optimally to the 1:1 blend, lower catches were obtained with the 5:1 blend, and no *D. affaber* responded to the other treatments.

Laboratory Bioassays. The highest response by *D. confusus* of both sexes was elicited by the (+)exoB–(+)endoB blend at a 9:1 ratio (Figure 3). Since field experiment I indicated that the 11:1 ratio was less attractive than the 5:1 ratio (Figure 2), we did not test ratios >9:1. The high responses to picogram

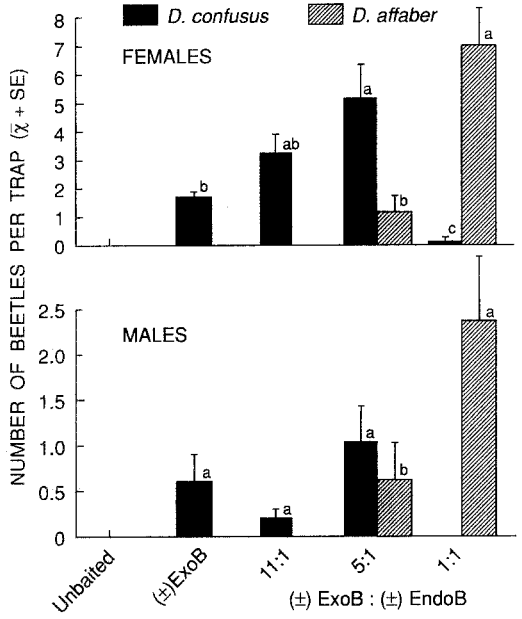

FIG. 2. Numbers of *Dryocoetes confusus* and *D. affaber* caught in field experiment I to traps baited with blends of (±)-*exo*-brevicomin (ExoB) and (±)-*endo*-brevicomin (EndoB) at several ratios, 10 replicates, 25 August–22 September 1990. Bars with the same letter within each species are not significantly different, two-way ANOVA and REGWF test on data transformed by $x' = \log(x + 1)$, $P < 0.05$. Treatments with zero catches were excluded from statistical analysis. More females than males were flying during this experiment.

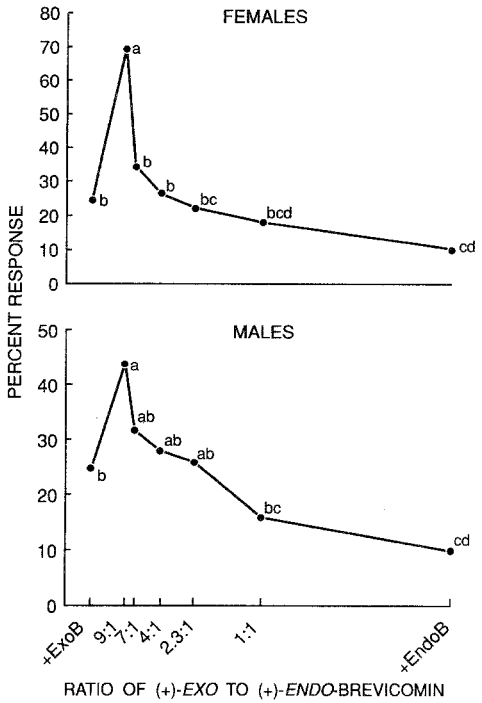

FIG. 3. Response of *Dryocoetes confusus* in laboratory olfactometer bioassays to 1 pg stimuli of (+)-*exo*-brevicomin (ExoB), (+)-*endo*-brevicomin (EndoB), and five blends of the two isomers in various ratios. Fifty beetles of each sex tested per stimulus. Response to pentane controls: males 4%; females 6%. Percents with the same letter are not significantly different, $P < 0.05$..

level stimuli demonstrate the remarkable sensitivity of *D. confusus* to its pheromone. This sensitivity was further exemplified in the dose–response laboratory experiment in which there was a significantly higher response from females to the 9:1 blend at five doses (from 0.1 to 1 000 pg) than to (+)exoB at 1 pg (Table 1). Females showed the highest level of response at 1 pg and 10 pg with decreasing levels of response at other doses. Males did not show any statistically significant effect of concentration, but the highest numerical response was also to 1 pg stimuli.

Field Experiment II. As in the laboratory bioassays, the 9:1 blend of (+)exoB and (+)endoB was the most attractive for *D. confusus* of both sexes in the field (Figure 4). The response to the 1:1 mixture was lower than to (±)exoB. In agreement with the previous field experiment (using racemic blends) (Figure 2), *D. affaber* responded best to the 1:1 mixture of (+) enantiomers (Figure 4).

TABLE 1. RESPONSE BY *Dryocoetes confusus* IN LABORATORY OLFACTOMETER BIOASSAYS TO DIFFERENT DOSES OF 9:1 BLENDS OF (+)-*exo*-BREVICOMIN (exoB) AND (+)-*endo*-BREVICOMIN (endoB)[a]

Stimulus	Dose (pig)	Percent Response[b]	
		Males	Females
Pentane (10 μl)	0	4 b	6 d
(+)exoB	1	24 a	24 c
exoB 9:1 endoB	0.1	28 a	54 b
exoB 9:1 endoB	1	38 a	84 a
exoB 9:1 endoB	10	22 a	68 ab
exoB 9:1 endoB	100	26 a	56 b
exoB 9:1 endoB	1000	24 a	54 b

[a] Fifty beetles of each sex tested per stimulus.
[b] Percents within a column followed by the same letter are not significantly different, contingency tables of chi-square. $P < 0.05$.

Field Experiment III. The response of male and female *D. confusus* to chiral combinations of the 9:1 exoB–endoB blend demonstrated that the (+) enantiomers were responsible for attraction (Figure 5). Two hypotheses could account for the reduction of catches to the combination of (±)exoB and (+)endoB. One is that the release rate of the active (+)exoB was approximately half the rate achieved when optically active materials were deployed. The other is that the response was partially inhibited by the presence of (−)exoB. The presence of both (−)exoB and (−)endoB in the (±):(±) combination lowered the catches to a level not significantly different from those to the unbaited controls. This dramatic reduction of catches suggests an inhibitory or blocking effect by the (−) enantiomers when they are combined. A similar reduction in response was observed by Kohnle and Vité (1984) for European *D. autographus* (Ratzeburg).

Field Experiment IV. Despite low catches due to cold weather near the end of the flight period in 1992, the results of this experiment again indicated that for *D. confusus*, the presence of (−)exoB inhibited the response of males to the attractive (+):(+) combination; females were not affected (Table 2). This result is consistent with those observed in field experiment I (Fig. 2), in which males responded so poorly to (±):(±) blends that they failed to discriminate among ratios of exoB–endoB. Even though we used a 9:1 exoB–endoB blend, the chiral combination (−):(+) was not attractive for *D. confusus*. This combination elicited a response from *D. affaber*, which suggests that the main pheromonal component for *D. affaber* is (+)endoB, while high proportions of (+)exoB inhibit the response in this species.

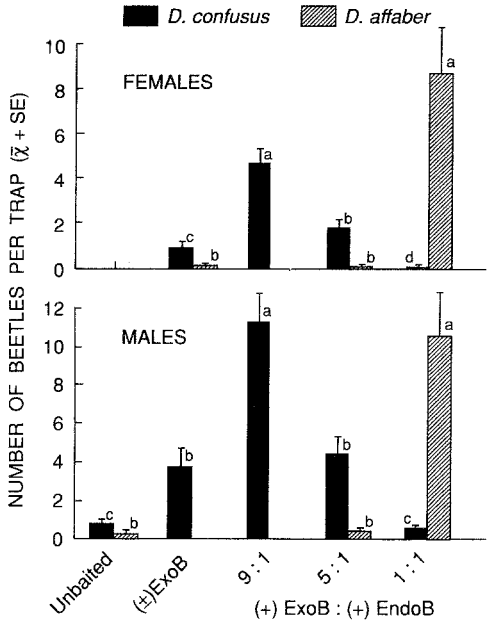

FIG. 4. Numbers of *Dryocoetes confusus* and *D. affaber* caught in field experiment II to traps baited with blends of (+)-*exo*-brevicomin and (+)-*endo*-brevicomin in three ratios, 10 replicates, 4–17 July 1991. Bars with the same letter within each species are not significantly different, two-way ANOVA and REGWF test on data transformed by $x' = \log(x + 1)$, $P < 0.05$. Zero catches to unbaited traps were excluded from statistical analysis. More males than females were flying during this experiment.

Our results do not agree with those of Stock et al. (1993), who found that the unnatural (−)exoB enhanced the attraction of *D. confusus* to (+)exoB. The inhibition of response to the 9:1 blend of (+)exoB and (+)endoB, caused by (−)exoB (Table 2) suggests that this compound could play a role as an antiaggregant in a similar way to that reported for (−)-*trans*-verbenol in the western pine beetle, *Dendroctonus brevicomis* LeConte (Byers, 1983).

Physiological, Ecological, and Systematic Implications. Our results demonstrate that a combined effect of geometrical and optical isomers of brevicomin determines species specificity and level of response to semiochemicals in two *Dryocoetes* spp.

Specifically, they elucidate three principal phenomena: (1) The ratio of geometrical isomers of exoB and endoB, either as racemic compounds or as (+) enantiomers, determines both the level of response and the species specificity of the blend. (2) As disclosed through varying the ratio of exoB to endoB, both compounds are multifunctional. ExoB can act with endoB in a 9:1 ratio as an

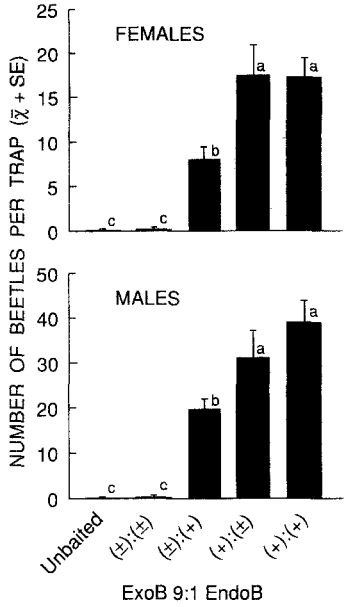

FIG. 5. Numbers of *Dryocoetes confusus* caught in field experiment III to traps baited with chiral combinations of *exo*-brevicomin (ExoB) and *endo*-brevicomin (EndoB) all in a 9:1 ratio, 20 replicates, 4–26 July 1991. Bars with the same letter are not significantly different, two-way ANOVA and REGWF test on data transformed by $x' = \log(x + 1)$, $P < 0.05$.

aggregation pheromone for *D. confusus* and apparently serves as a synomone (Nordlund, 1981) that deters *D. affaber* from responding to the endoB produced by *D. confusus*. EndoB, on the other hand, acts at comparatively low levels in combination with exoB as an aggregation pheromone for *D. confusus* and acts at higher levels as an antiaggregation pheromone for *D. confusus* (Stock et al., 1990). As the relative amount of (+)endoB in the blend increases, it also functions as an attractant for *D. affaber*, although its hypothesized role as a pheromone remains to be verified. (3) The chiral nature of the pheromones is also of major importance. Optimal response by *D. confusus* to the "correct" 9:1 ratio of geometrical isomers occurs only when the (+) enantiomers of both pheromones are present. Even when high proportions of the "right" geometrical isomer are present, the effect of the "wrong" enantiomer can result in loss of attractiveness and specificity of the chemical message.

The presence of both exoB and endoB as pheromones in *D. confusus* (Schurig et al., 1983; Borden et al., 1987; Stock et al., 1990), *D. autographus* (Kohnle and Vité, 1984; Kohnle, 1985), and *D. affaber* (unpublished data)

TABLE 2. NUMBERS OF *Dryocoetes confusus* AND *D. affaber* CAUGHT IN FIELD EXPERIMENT IV TO TRAPS BAITED WITH TWO CHIRAL COMBINATIONS OF BLENDS OF *exo*-BREVICOMIN (exoB) AND *endo*-BREVICOMIN (endoB) AT 9:1 RATIO IN A SINGLE RELEASE DEVICE AND WITH (−)-*exo*-BREVICOMIN (SEPARATE RELEASE DEVICE) TOGETHER WITH (+):(+) BLEND[a]

Stimulus	Number of Beetles Captured ($\bar{X} \pm$ SE)[b]			
	D. confusus		*D. affaber*	
	Males	Females	Males	Females
Unbaited	0	0	0	0
(+)exoB:(+)endoB	1.9 ± 0.2 a	2.2 ± 0.4 a	0	0
(−)exoB:(+)endoB	0.2 ± 0.1 b	0.1 ± 0.1 b	4.0 ± 1.2	4.4 ± 1.2
(+)exoB:(+)endoB with (−)exoB	0.6 ± 0.2 b	1.9 ± 0.4 a	0	0

[a] Approximate release rate 0.2 mg/day/device. Ten replicates 16 July–20 August, and six replicates 20 August–2 October 1992.
[b] Means within a column followed by the same letter are not significantly different, Friedman test, $P < 0.05$. Zero catches in unbaited traps excluded from statistical analysis.

suggests that speciation in this genus has been accompanied by a process of fine tuning of pheromone production and response mechanisms that provide species specificity of semiochemical channels, especially under sympatric conditions.

The use of multicomponent pheromone blends is a widespread mechanism that provides uniqueness of chemical signals in insects (Silverstein, 1981; Cardé, 1986; Linn and Roelofs, 1989). When one considers the possible combinations resulting from two or more chemicals of varying chirality, mixed in different ratios of geometrical isomers, it appears that insects with such types of pheromones have virtually unlimited means for partitioning of pheromone communication channels. There may be some tolerance to changes in component ratios (Schlyter, 1987; Byers, 1988). However, overlapping of chemical signals between species can be avoided by very small shifts in these ratios (Figures 2 and 4). Optimal responses within a species can be mediated by even smaller shifts (Figure 3). Thus, as exemplified by our results with two sympatric *Dryocoetes* spp., pheromone systems can provide a highly effective mechanism of reproductive isolation.

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SECONDARY AMINES ISOLATED FROM VENOM GLAND OF DOLICHODERINE ANT, *Technomyrmex albipes*

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Abstract—A series of unsaturated secondary amines have been isolated from the dolichoderine ant *Technomyrmex albipes* (F. Smith). The major components of the mixture have been shown by spectroscopic procedures to be dinon-8-enylamine, and *N*-hept-6-enylnon-8-enamine, and these structural assignments have been confirmed by synthesis. Mass spectrometry indicates the presence of trace amounts of the bis C_{11} amine and the C_9–C_{11} amine. The four amines, present in total at approximately 2.8 μg/ant, are located in the gaster of the insect in a gland that is considered to be the venom gland although it is atypical from a morphological standpoint.

Key Words—Hymenoptera, Formicidae, ant, *Technomyrmex albipes*, unsaturated secondary amines, venom gland, GC-MS.

INTRODUCTION

The ant subfamily, Dolichoderinae, has not provided a rich source of nitrogen-containing extractives, furnishing so far only the iridoid, actinidine **(1)**, from *Iridomyrmex* species, e.g., *I. nitidiceps* (Cavill et al., 1982), and a range of pyrazines isolated from the heads of *I. humilis* (Cavill and Houghton, 1974), the mandibular glands of *I. purpureus* (Cavill et al., 1984), and the heads of other dolichoderine species (Brophy, 1989). Hence, it was of interest to observe that the black house ant, *Technomyrmex albipes*, which can be something of a household pest in the Sydney area, furnished, by dichloromethane extraction,

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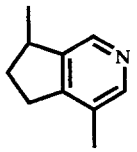
two major products (present in the current extraction in approximately equal amounts), which, from their odd molecular weight and the even mass of their mass spectrometric ions, were likely to contain nitrogen. Two other nitrogenous products, present in only trace amounts, were also detected. The two major extractives were recorded in *T. albipes* collected from several different sites in the eastern suburbs of Sydney and from the New England Tableland of New South Wales, although the proportions of the two compounds varied.



(1)

This paper describes the isolation, structural determination, and synthesis of these novel compounds and discusses their likely glandular origin.

METHODS AND MATERIALS

Chemical Analyses. ^{1}H and ^{13}C NMR data were obtained on a Bruker AC 300F spectrometer with chemical shifts quoted on the δ scale relative to $CHCl_3$ as internal standard. Gas chromatography was obtained on either an OV-1 column (SCOT, 30 m $\times$ 0.5 mm) or a DB5 column (FCOT, 30 m $\times$ 0.32 mm). Both were programmed from 60°C to 250°C at 5°C/min. Combined gas chromatography–mass spectrometry (GC-MS), both EI and CI, were performed either on: (1) an AEI MS12 mass spectrometer, (2) a Finnigan 4000 mass spectrometer, or (3) a VG Quattro mass spectrometer. In all cases the GLC column conditions were as detailed above. Accurate mass measurements were obtained on either an AEI MS 902 mass spectrometer under CI conditions, using isobutane as reagent gas, by a peak timing method (Brophy et al., 1979) or on a VG Autospec mass spectrometer.

Extraction of Ants. The ants (*Technomyrmex albipes*) were collected on this occasion over several weeks from a location in the eastern suburbs of Sydney and were stored in twice-distilled dichloromethane. The filtration of this suspension of ants in dichloromethane yielded approximately 4500 of the ants (1.58 g) which were ground with anhydrous sodium sulfate (5 g); the solid was transferred to a Soxhlet thimble in which the mixture was extracted with dichloromethane for 24 hr. This dichloromethane extract was combined with the dichloromethane solution obtained from the original ant collection and the total extract (100 ml) was washed with saturated aqueous sodium hydrogen carbonate (2 $\times$ 50 ml) and brine (2 $\times$ 75 ml). The organic phase was dried (Na_2SO_4) and

evaporated under reduced pressure to give a brown oil (136 mg). A pentane solution (10 ml) of this oil was extracted with aqueous sulfuric acid (0.5 M; 4 × 10 ml); the combined aqueous extracts were made alkaline with sodium hydrogen carbonate and extracted with dichloromethane (4 × 10 ml). Evaporation of the dried (Na_2SO_4) solution left a gum (12.5 mg) from which the following spectroscopic data were obtained: NMR δ_H: 1.25–1.45 (m, CH_2), 1.50–1.60 ($C\underline{H}_2CH_2CH{=}CH_2$), 2.00–2.10 (m, $C\underline{H}_2CH{=}CH_2$), 2.20 (broad s, NH), 2.65 (t, J = 7 Hz, $C\underline{H}_2NH$), 4.90–5.05 (m, $CH{=}C\underline{H}_2$), 5.75–5.90 (m, $C\underline{H}{=}CH_2$). δ_C: 26.81, 27.29, 28.78, 28.88, 29.07, 29.36, 29.73, 33.70, 33.79, 49.61 (CH_2), 114.21, 114.41 ($=CH_2$), 138.91, 139.16 ($-CH=$).

GC/MS (EI) indicated the presence of two major amines and two other amines in trace amounts (Figure 1). The main components showed the following principal ions: **(3)** *m/z*(%) 126(100%), 154(87), 196(18), 237(8); **(2)** *m/z*(%), 154(100%), 224(15), 264(3), 265(2); **(4)** *m/z*(%), 154(100%), 182(55), 293(0.5); **(5)** *m/z*(%) 182(100%), 252(5), 321(0). Accurate mass measurements, under CI (isobutane) conditions gave the following results for the protonated parent ions of the two principal compounds: **(3)** found 238.2518 ($C_{16}H_{32}N$ required 238.2534); **(2)** found 266.2856 ($C_{18}H_{36}N$ required 266.2847).

Non-8-enoic Acid ***(6)***. Magnesium turnings (514 mg) were suspended in sodium dried ether (10 ml) and 8-bromoct-1-ene (3.8 g) in dried ether (10 ml) was added portionwise. With the first addition, the mixture was stirred vigorously, with warming, to initiate the reaction. After the addition was complete, the mixture was refluxed for 1 hr, diluted with ether (20 ml) and cooled to −10°C before excess Dry Ice was added slowly over about 10 min. The mixture was stirred for 15 min, after which aqueous sulfuric acid (50%; 10 ml) was added slowly with cooling, followed by water (50 ml). The product was isolated by extraction with ether (3 × 100 ml), the ethereal phase concentrated (100 ml), and the acid purified by extraction into aqueous sodium hydroxide solution (10%; 2 × 50 ml). The aqueous phase was acidified to pH 1 with hydrochloric acid (10 M) and the acid returned to ether (3 × 100 ml). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give the acid (1.98 g; 64%) as a pale pink liquid, bp 133–134°C at 3 torr (Ansell and Whitfield, 1971) (bp 110–114°C at 2.5 torr); NMR δ_H: 1.25–1.40 (6H, m, 3 × CH_2), 1.59 (2H, m, $C\underline{H}_2CH_2CH{=}CH_2$), 2.00 (2H, m, $C\underline{H}_2CH{=}CH_2$), 2.29 (2H, t, J = 7.5 Hz, $C\underline{H}_2COOH$), 4.85–4.98 (2H, m, $CH{=}C\underline{H}_2$), 5.74 (1H, qt, J_{cis} = 10.2 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, $CH_2C\underline{H}{=}CH_2$). δ_C: 24.42, 28.54, 28.56, 33.55, 28.73, 33.89, (CH_2), 114.11 ($=CH_2$), 138.57 ($=CH-$), 180.44 (CO).

Hept-6-enenitrile ***(7)***. A solution of potassium cyanide (3.7 g) in water (7 ml) was added to 6-bromohex-1-ene (6.3 g) dissolved in ethanol (95%; 28 ml). The mixture was refluxed for 20 hr, cooled, and diluted with ether (100 ml). The mixture was washed successively with water (2 × 50 ml), hydrochloric

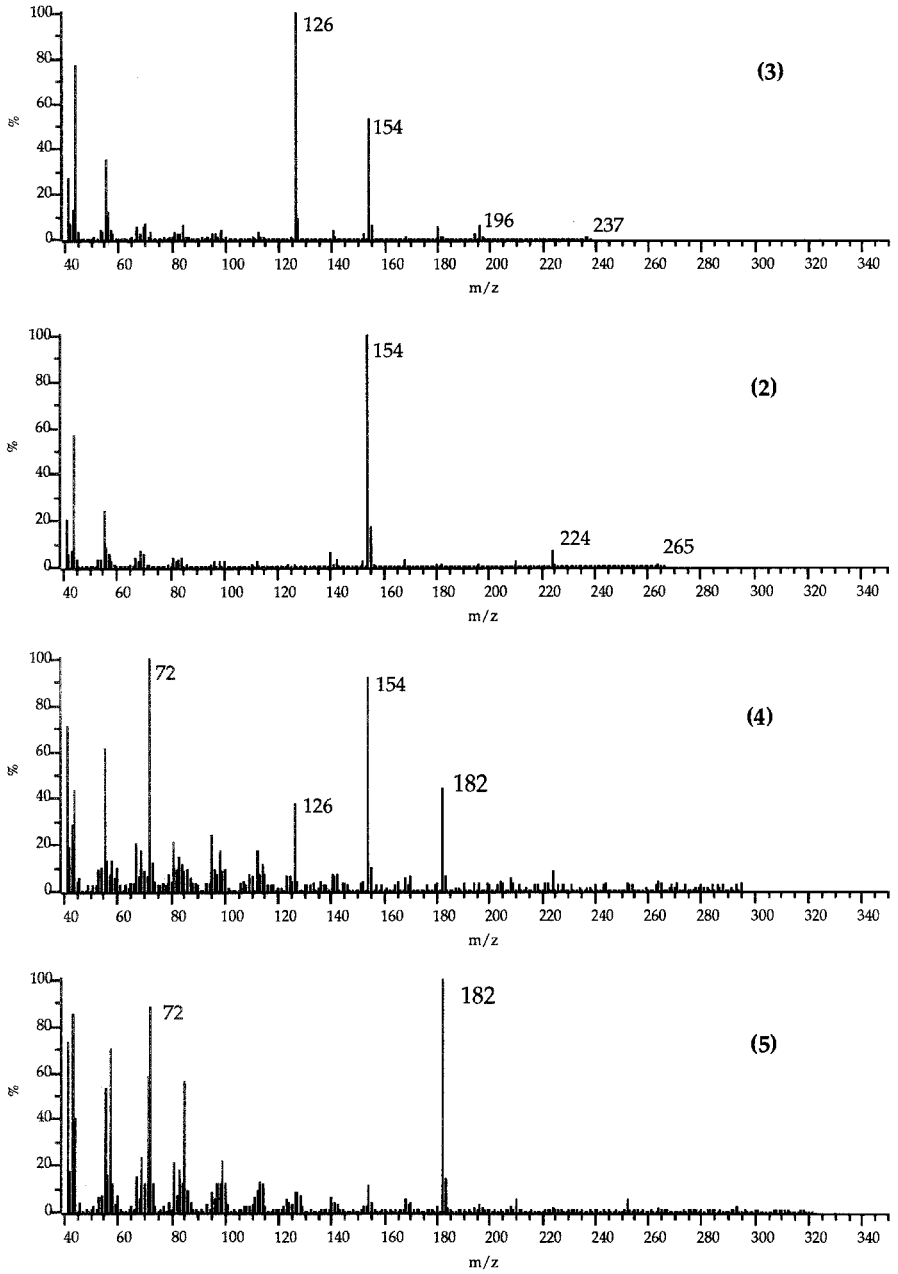


FIG. 1. Mass spectra of the four amines detected in the venom gland of *Technomyrmex albipes*.

acid (2 M; 2 × 50 ml), saturated aqueous sodium hydrogen carbonate solution (2 × 50 ml), and finally water (2 × 50 ml). The organic phase was dried (Na_2SO_4) and the solvent removed under reduced pressure to leave the nitrile (3.5 g; 83%) as a pale yellow liquid, bp 109–112°C at 73 torr (Vasil'eva and Freidlina, 1966) (bp 82°C at 20 torr); NMR δ_H: 1.40–1.60 (4H, m, 2 × CH_2), 1.98–2.08 (2H, m, $C\underline{H}_2CH{=}CH_2$), 2.28 (2H, t, J = 7 Hz, CH_2CN), 4.88–5.00 (2H, m, $CH{=}C\underline{H}_2$), 5.71 (1H, qt, J_{cis} = 10.2 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, $CH_2C\underline{H}{=}CH_2$). δ_C: 16.60, 24.39, 27.36, 32.44 (CH_2), 114.93 ($=CH_2$), 119.42 (CN), 137.30 ($-CH=$).

Non-8-enenitrile ***(8)***. The nitrile (2.9 g; 85%) was obtained as a yellow liquid, bp 133–135°C at 48 torr, by refluxing a solution of 8-bromoct-1-ene (4.77 g) in aqueous ethanol containing potassium cyanide (2.32 g). The procedure followed that outlined for the lower homolog. NMR δ_H: 1.22–1.48 (6H, m, 3 × CH_2), 1.55–1.66 (2H, m, $C\underline{H}_2CH_2CH{=}CH_2$), 1.95–2.05 (2H, m, $C\underline{H}_2CH{=}CH_2$), 2.29 (2H, t, J = 7.1 Hz, CH_2CN), 4.88–5.00 (2H, m, $CH{=}C\underline{H}_2$), 5.75 (1H, qt, J_{cis} = 10.3 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, $CH_2C\underline{H}{=}CH_2$). δ_C: 16.88, 25.13, 27.98, 28.28, 28.32, 33.36 (CH_2), 114.30 ($=CH_2$), 119.59 (CN), 138.48 ($CH=$).

Hept-6-enamine ***(9)***. A well-stirred mixture of sodium (3.25 g) and toluene (40 ml) was heated to reflux whereupon the sodium liquefied. A solution of hept-6-enenitrile (3.5 g) in a mixture of absolute ethanol (15 ml) and toluene (15 ml) was then added slowly over 30 min to the refluxing mixture of sodium in toluene. Further absolute ethanol was added to destroy excess sodium, after which the mixture was refluxed for 1 hr, cooled, and water (50 ml) added, followed by hydrochloric acid (10 M; 25 ml). The mixture was extracted with ether (3 × 80 ml) and the aqueous phase evaporated to dryness under reduced pressure to free the amine salt of residual ethanol. The residue was then treated with aqueous sodium hydroxide (10%; 30 ml) and the free base returned to ether by extraction (3 × 80 ml). The ethereal layer was dried (Na_2SO_4) and the solvent removed to give the amine (1.53 g; 42%) as a pale yellow liquid, bp 74–78°C at 48 torr. MS 113.1194 ($C_7H_{15}N$ requires 113.1205) NMR δ_H: 1.10–1.30 (8H, 3 × CH_2, NH_2), 1.88 (2H, m, $C\underline{H}_2CH{=}CH_2$), 2.50 (2H, t, J = 7 Hz, $\underline{CH}_2NH_2$), 4.73–4.83 (2H, m, $CH{=}C\underline{H}_2$), 5.62 (1H, qt, J_{cis} = 10.3 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, $CH_2C\underline{H}{=}CH_2$). δ_C: 26.00, 28.40, 33.27, 33.36, 41.76 (CH_2), 113.91 ($=CH_2$), 138.48 ($-CH=$).

Non-8-enamine ***(10)***. The amine (0.69 g; 32%), bp 130–136°C at 76 torr, was obtained by reduction of non-8-enenitrile (2.08 g) in absolute ethanol–toluene (1 : 1, 24 ml) with sodium (2.0 g) suspended in toluene (25 ml) according to the procedure detailed for the lower homolog. MS 141.1515 ($C_9H_{19}N$ requires 141.1518) NMR δ_H: 1.00–1.50 (12H, 5 × CH_2, NH_2), 1.92 (2H, dt, $\underline{CH}_2CH{=}CH_2$), 2.55 (2H, t, J = 7 Hz, $\underline{CH}_2NH_2$), 4.78–4.90 (2H, m, $CH{=}\underline{CH}_2$), 5.68 (1H, qt, J_{cis} = 10.3 Hz, J_{trans} = 17.3 Hz, J_{CH_2} = 6.7 Hz,

$CH_2C\underline{H}=CH_2$). δ_C: 26.65, 28.67, 28.89, 29.14, 33.58, 42.00, (CH_2), 113.95 ($=C\underline{H}_2$), 138.88 ($-CH=$).

N-Hept-6-enylnon-8-enamide ***(11)***. Thionyl chloride (2.5 ml; freshly distilled) was added over 10 min to non-8-enoic acid (1.10 g) heated on a water bath maintained at 50–60°C. After gas evolution was complete, the temperature of the water bath was raised to 80–90°C and kept at this temperature for 30 min. Excess thionyl chloride was removed under reduced pressure and the residual acid chloride (1.03 g) was dissolved in anhydrous ether (10 ml). A solution of hept-6-enamine (1.06 g) in anhydrous ether (5 ml) was added portionwise to the acid chloride, after which the reaction mixture was washed successively with water, aqueous sodium hydroxide solution, water, dilute hydrochloric acid, and water. The ethereal solution was dried (Na_2SO_4) and the solvent removed to give a yellow oil (1.31 g) which, after chromatography on silica (60H, Merck Art 7736; eluting with dichloromethane), yielded the amide (1.02 g; 69%) as a white solid mp < 20°C. MS (EI) 251.2251 ($C_{16}H_{29}NO$ requires 251.2249), 252(MH^+, 3%), 251(1), 210(27), 196(8), 182(6), 168(58), 140(85), 126(30), 114(50), 112(65), 100(48), 69(68), 55(100). NMR δ_H: 1.25–1.55 (12H, m, 6 × CH_2), 1.60–1.70 (2H, m, $C\underline{H}_2CH_2CH=CH_2$), 2.00–2.10 (4H, m, 2 × $C\underline{H}_2CH=CH_2$), 2.16 (2H, t, J = 7.5 Hz, $COCH_2$), 3.20–3.28 (2H, m, $C\underline{H}_2NH$), 4.90–5.03 (4H, m, 2 × $CH=C\underline{H}_2$), 5.50 (1H, br s, NH), 5.78 (1H, qt), 5.79 (1H, qt) (J_{cis} = 10.3 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, 2 × $CH_2C\underline{H}=CH_2$). δ_C: 25.71, 26.28, 28.43, 28.64, 28.72, 29.05, 29.41, 30.50, 33.58, 36.67, 39.32 (CH_2), 114.15, 114.35 ($=CH_2$), 138.59, 138.83 (2 × $-CH=$), 173.07 (CO).

N-Non-8-enylnon-8-enamide ***(12)***. Non-8-enoic acid (517 mg) was converted into its acid chloride by reaction with freshly distilled thionyl chloride (2 ml) as described previously, and a solution of this derivative (455 mg) in anhydrous ether (10 ml) was treated portionwise with non-8-enamine (561 mg) in anhydrous ether (5 ml). The mixture was worked up as described for the lower homolog to give the amide (424 mg, 46%), mp < 25°C. MS (EI) 279.2565 ($C_{18}H_{33}NO$ requires 279.2562), 280(MH^+, 10%), 279(9), 250(5), 238(13), 210(8), 196(45), 168(20), 156(8), 142(20), 121(18), 100(40), 86(30), 69(55), 55(100). NMR δ_H: 1.20–1.40 (14H, m, 7 × CH_2), 1.40–1.50 (2H, m), 1.50–1.70 (2H, m) (2 × $C\underline{H}_2CH_2CH=CH_2$), 2.02 (4H, m, 2 × $C\underline{H}_2CH=CH_2$), 2.14 (2H, t, J = 7.5 Hz, $COCH_2$), 3.22 (2H, dt, $C\underline{H}_2NH$), 4.80–5.00 (4H, m, 2 × $CH=C\underline{H}_2$), 5.60 (1H, br s, NH), 5.78 (1H, qt), 5.79 (1H, qt) (J_{cis} = 10.3 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, 2 × $CH_2C\underline{H}_2=CH_2$). δ_C: 25.76, 26.83, 28.68, 28.74, 28.95, 29.09, 29.59, 33.65, 33.69, 36.77, 39.47 (CH_2), 114.18, 114.22 ($=CH_2$), 138.94, 139.01 (2 × $-CH=$), 173.19 (CO).

N-Hept-6-enylnon-8-enamine ***(3)***. A solution of *N*-hept-6-enylnon-8-enamide (266 mg) in anhydrous ether (5 ml) was added to a suspension of lithium aluminium hydride (65 mg) in anhydrous ether (10 ml), maintained over nitro-

gen. The mixture was refluxed for 5 hr, cooled, and excess lithium aluminium hydride destroyed by the careful addition of water. Hydrochloric acid (5 M) was then introduced to acidify the reaction mixture, which was extracted with ether (3 × 30 ml). The combined ethereal extracts were dried (Na_2SO_4) and evaporated to yield the amine hydrochloride (272 mg) as a white solid. The solid was made alkaline with aqueous sodium hydroxide (10%; 20 ml) and the aqueous mixture extracted with ether (3 × 40 ml); evaporation of the solution left the amine (231 mg; 73%) as a pale yellow liquid, bp 164–167°C at 3 torr, which was identical by gas chromatography and mass spectrometry with the natural material. MS (EI) 237(M^+, 10%), 208(7), 196(16), 180(11), 154(85), 140(10), 126(100), 55(15). NMR δ_H: 1.25–1.50 (16H, m, 8 × CH_2), 1.70 (1H, br s, NH), 1.98–2.08 (4H, m, 2 × $C\underline{H}_2CH{=}CH_2$), 2.56 (2H, t, J = 7 Hz), 2.57 (2H, t, J = 7 Hz) ($C\underline{H}_2NHC\underline{H}_2$), 4.88–5.02 (4H, m, 2 × $CH{=}C\underline{H}_2$), 5.78 (2H, qt, J_{cis} = 10.3 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, 2 × $CH_2C\underline{H}{=}CH_2$). δ_C: 26.85, 27.32, 28.81, 28.83, 29.02, 29.37, 29.92, 30.05, 33.67, 33.73, 49.98, 50.03 (CH_2), 114.10, 114.25 ($=CH_2$), 138.90, 139.08 ($-CH=$).

Dinon-8-enylamine ***(2).*** A solution of *N*-non-8-enylnon-8-enamide (340 mg) in anhydrous ether (15 ml) was reduced under nitrogen with lithium aluminium hydride (100 mg) as described for the lower homolog. The reaction mixture was worked up in a similar manner to yield the amine (274 mg, 38%) as a pale yellow liquid, bp 189–195°C at 9 torr, identical by gas chromatography and mass spectrometry with the natural product. MS (EI) 265(M^+, 3), 264(4), 224(15), 210(4), 180(3), 168(9), 154(100), 140(6), 126(7). NMR δ_H: 1.20–1.50 (21H, m, 10 × CH_2, NH), 2.00 (4H, m, 2 × $C\underline{H}_2CH{=}CH_2$), 2.56 (4H, t, J = 7 Hz, $C\underline{H}_2NHC\underline{H}_2$), 4.70–5.00 (4H, m, 2 × $CH{=}C\underline{H}_2$), 5.78 (2H, qt, J_{cis} = 10.3 Hz, J_{trans} = 17.0 Hz, J_{CH_2} = 6.7 Hz, 2 × $CH_2C\underline{H}{=}CH_2$). δ_C: 27.35, 28.86, 29.05, 29.40, 30.15, 33.76, 50.12 (CH_2), 114.12 ($=CH_2$), 139.11 ($-CH=$).

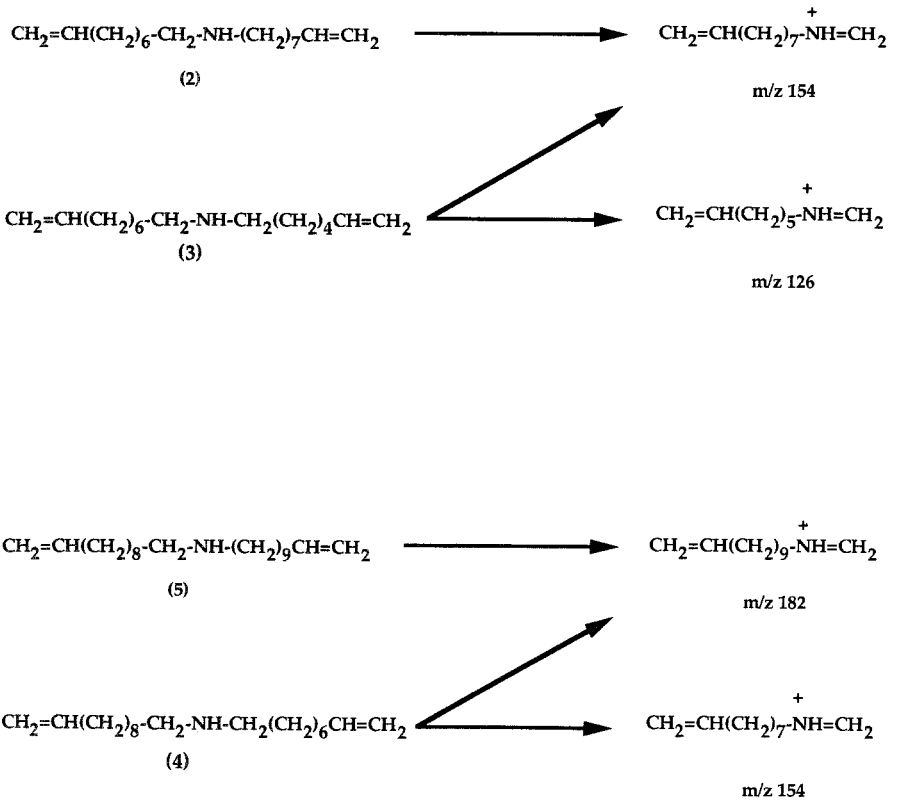
RESULTS AND DISCUSSION

A GC-MS study of the dichloromethane extract of whole ants revealed two prominent components, present in approximately equal amounts. The less volatile component furnished a small molecular ion at 265 D with its base peak at *m/z* 154; the more volatile component, which was marginally the greater compound present, displayed a weak molecular ion at 237 D and provided prominent fragment ions at *m/z* 154 and 126. Accurate mass determinations indicated molecular formulae of $C_{18}H_{35}N$ and $C_{16}H_{31}N$, respectively, for these compounds.

When a pentane solution of the crude extract was washed with dilute aqueous

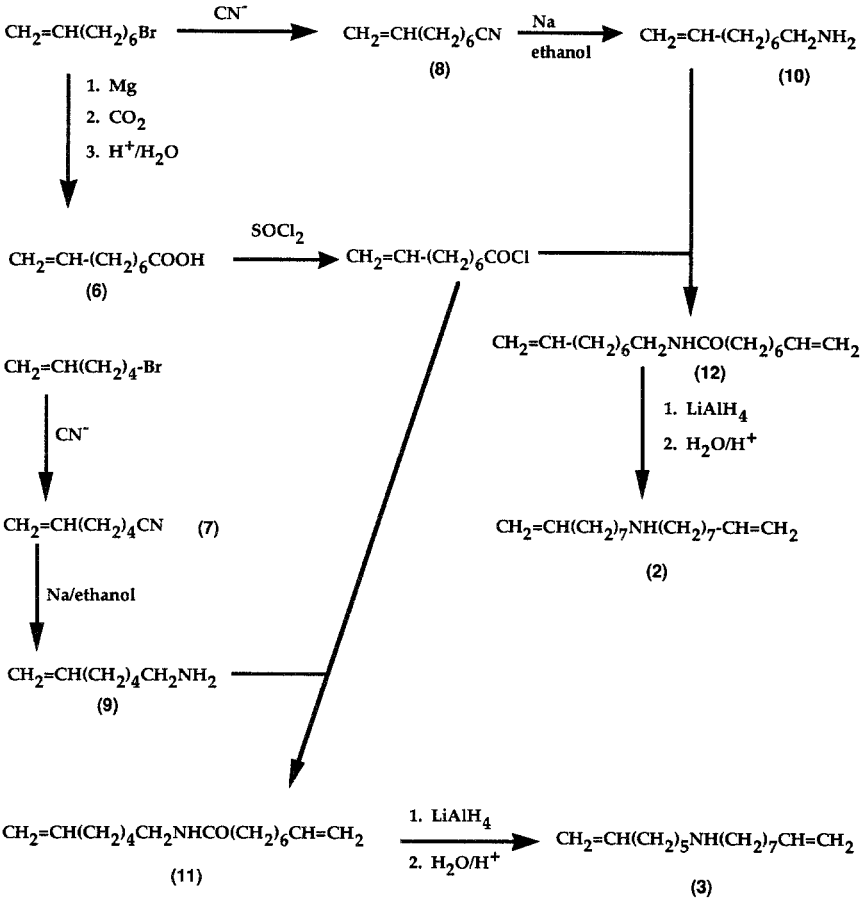
sulfuric acid, the aqueous phase then made alkaline, and the bases returned to dichloromethane, the latter extract was essentially free of any material apart from the two major nitrogenous compounds referred to above. This fraction was pure enough to give good quality NMR spectra. ^{13}C NMR spectroscopy revealed the absence of methyl groups and quaternary carbons. Methine carbons at 139 ppm and methylene carbons at 114 ppm suggested the presence of terminal double bonds, while a methylene carbon at 49 ppm indicated a carbon adjacent to nitrogen. A cluster of other methylene carbons were present with chemical shifts ranging between 26 and 33 ppm. Olefinic protons were also clearly evident from the ^1H NMR spectrum. The summation of these data suggested that the two major natural products were the unsaturated secondary amines **(2)** and **(3)** and their principal fragmentation is outlined in Scheme 1.

To confirm these formulations, the amines were synthesized. 8-Bromoct-1-ene was converted into the corresponding nitrile **(8)** by treatment with potas-



SCHEME. 1.

sium cyanide, and this derivative was reduced with sodium in ethanol to produce non-8-enamine **(10)**. Non-8-enoic acid **(6)** was obtained from the above bromide by carbonation of its Grignard derivative. Reaction of the acid chloride derivative of non-8-enoic acid with non-8-enamine furnished the amide **(12)**, which was reduced with lithium aluminium hydride in ether to give the C_{18} secondary amine **(2)**. The C_{16} secondary amine **(3)** was produced in a similar manner utilizing hept-6-enamine **(9)** to produce the intermediate amide **(11)** by reaction with the chloride derivative of non-8-enoic acid. Hept-6-enamine **(9)** was obtained by reduction of the cyanide **(7)**, derived from the commercially available 6-bromohex-1-ene. These reactions are summarized in Scheme 2.



SCHEME. 2.

Separate examination of disarticulated heads, thoraces, and gasters indicated that the amines were localized in the gaster of *Technomyrmex*.

There are a number of glandular-type structures within the gaster, any one of which might be a focus for amine production. Among them is an unusual gland in *Technomyrmex*, which must be accepted as a venom gland on morphological grounds, although it does not conform to the characteristic size reduction of this structure within the Dolichoderinae. Dichloromethane extraction of 23 of these glands and 51 glands subsequently isolated by microdissection and analyzed by GC-MS confirmed the presence within them of amines **(2)** and **(3)**, now recognized as venom constituents. In addition, this study of the gland extracts revealed the presence of two other amines at levels of approximately 1/100 to 1/1000 of the major compounds. The mass spectra of these additional components (the spectra of these two compounds shown in Figure 1 are background-subtracted spectra) indicated that they were probably the C_{11}–C_9 ($C_{20}H_{39}N$) and the bis C_{11} ($C_{22}H_{43}N$) amine analogs **(4)** and **(5)** of the major components, although there was insufficient material to carry this characterization further. No trace of the cyclopentanoid monoterpene alkaloid, actinidine **(1)**, characteristic of dolichoderine ant anal (i.e., pygidial) glands, was found in the venom gland, although its presence was confirmed in the extract of the whole gaster of the ant.

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STEREOSPECIFIC ANTENNAL RESPONSE BY RED TURPENTINE BEETLE, *Dendroctonus valens* TO CHIRAL MONOTERPENES FROM PONDEROSA PINE RESIN

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Abstract—The antennal response of *Dendroctonus valens* to host monoterpenes from the resin of ponderosa pine was studied using the electroantennogram (EAG) technique. Male and female beetles were given a single dose of each of 11 different monoterpenes. Response amplitude to the different compounds did not vary between sexes and was generally well correlated with results from field attraction studies. Response to (*S*)-(−)-β-pinene was greatest. The relative amplitude of the responses to the (*R*)-(+) and (*S*)-(−) enantiomers of α-pinene, however, were reversed from their relative attractiveness in the field. A dose-response study was conducted for the (*R*)-(+) and (*S*)-(−) enantiomers of α-pinene, plus a reciprocal differential saturation test with successive doses of first one enantiomer of α-pinene and then the other. Comparison of EAG traces suggests different receptors for the two stereoisomers of α-pinene. Differential saturation curves suggest that while one set of receptors may respond to both enantiomers, some receptors respond only to the (*S*)-(−) enantiomer.

Key Words—Coleoptera, Scolytidae, Dendroctonus valens, EAG, electroantennogram, enantiomer, kairomone, host attraction, bark beetle, α-pinene, β-pinene

INTRODUCTION

Dendroctonus valens (Leconte) is attracted to diseased and wounded conifers principally in the genus *Pinus*. Unlike its congeners and more well-known bark

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beetles, *D. valens* does not typically mass attack its host and is not generally an aggressive tree-killing species (Eaton and Lara, 1967). Instead of a powerful aggregation pheromone, *D. valens* may rely more on host odors or kairomones to locate and select its host. *D. valens* is attracted to the resin of its host ponderosa pine, *Pinus ponderosa* Lawson (Vité and Gara, 1962; Owen, 1985; Hobson, 1992). The principal attractants in the resin are the monoterpenes (*S*)-(−)-β-pinene, (*S*)-(+)-Δ-3-carene and (*R*)-(+)-α-pinene. The (*S*)-(−) enantiomer of α-pinene interrupts attraction of *D. valens* to its optical isomer, (*R*)-(+)-α-pinene (Hobson et al., 1993).

In the foothills of California's central Sierra Nevada *D. valens* feeds on two principal hosts, *P. ponderosa* and sugar pine, *Pinus lambertiana* Dougl. The resin of *P. ponderosa* is composed principally of (*S*)-(−)-β-pinene, (*S*)-(+)-Δ-3-carene and (*S*)-(−)-α-pinene with smaller amounts of myrcene and (*S*)-(−)-limonene (Smith, 1977; Hobson et al., 1993). The resin of *P. lambertiana* is composed principally of (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene, (*S*)-(+)-Δ-3-carene, and myrcene in decreasing order of percent composition (Hobson et al., 1993).

This study was conducted in tandem with the identification and field testing of the attractants for *D. valens* from ponderosa pine resin. The aim was to investigate the sensitivity and specificity of the antennal response of *D. valens* to a range of monoterpenes, for correlation with field test results, and thus to help further our understanding of host tree location mechanisms.

METHODS AND MATERIALS

D. valens adults were collected at the University of California's Blodgett Forest Research Station (El Dorado County, California) in the foothills of the central Sierra Nevada in August 1988. Adults were captured in Lindgren flight traps (Lindgren, 1983) baited with commercial gum turpentine (T&R Chemical, Clinton, Texas) and sexed by male stridulation (Pajares and Lanier, 1990).

The antennal response of *D. valens* to individual compounds was determined using the EAG technique of Schneider (1957), with methods described previously by White and Birch (1987). Responses were recorded from isolated heads of *D. valens*, mounted ventral surface uppermost on a glass stage using double-sided adhesive tape. The recording electrode (a glass micropipet filled with insect saline) was inserted into a hole punched in the terminal club segment using an etched tungsten needle, while the indifferent electrode was inserted into the back of the head. The electrodes were connected via chloridized silver wires to a Grass P-16 preamplifier, and EAG responses were displayed on a Tektronix 502 oscilloscope. A permanent record of EAG responses was made

using a Gould Brush 220 pen recorder, from which measurements of EAG amplitudes were made.

Odor stimuli were delivered from cartridges as 1.0-sec pulses into a continuous airstream (1000 ml/min) directed onto the antenna, as described previously by White and Birch (1987). Each material (10 μl) was applied to a filter paper strip and the solvent allowed to evaporate before use. A blank cartridge (solvent only) was presented before each test material, and the resulting EAG response subtracted from the subsequent test response before analysis of the results, to control for the effects of the solvent and mechanical disturbance of the airflow in the first experiment. Eleven compounds, obtained from Aldrich Chemical Company with chemical purities shown in Table 1, were tested. Nine were previously identified as the major monoterpenes from resin extracts of ponderosa pines at Blodgett Forest: (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene, β-phellandrene, (*S*)-(+)-Δ-3-carene, myrcene, (*S*)-(−)-limonene, (*R*)-(+)-limonene, and terpinolene (Hobson et al., 1993); two (longifolene, a sesquiterpene and estragole, a resin and foliage aromatic ether) were included to test the response of the antennae to terpenes from sympatric conifers and foliage of ponderosa pine. Each was tested at a single (10-μg) dose applied to a filter paper strip in 10 μl of dichloromethane, which was allowed to evaporate before the paper was inserted into the cartridge. The order of presentation of compounds was randomized between beetles. A total of 10 individuals (five male, five female) were tested.

The results from testing the 11 compounds were analyzed using a two-layered analysis of variance to investigate the effect of beetle sex, and the different chemicals on the EAG response followed by Bonferroni pairwise tests ($\alpha = 0.05$) (Crunch Interactive Software, San Francisco, California).

TABLE 1. CHEMICAL PURITIES OF TESTED COMPOUNDS

(*R*)-(+)-α-pinene	98% (optical purity 96%)[a]
(*S*)-(−)-α-pinene	98% (optical purity 91%)[a]
(*S*)-(−)-β-pinene	99%
β-Phellandrene	[b]
(*S*)-(+)-Δ-3-Carene	95%
Myrcene	85%
(*S*)-(−)-Limonene	97% (optical purity 91%)
(*R*)-(+)-Limonene	97% (optical purity 91%)
Longifolene	98%
Terpinolene	[b]
Estragole	98%

[a] Determined by chiral GC column (Hobson et al., 1993).
[b] Not recorded.

In a separate experiment, a log dilution series in dichloromethane was made up for each of the two stereoisomers of α-pinene. Each series was presented to six male *D. valens*, again randomizing the order of presentation.

To determine the specificity of the antennal receptors to the two enantiomers of α-pinene, a differential saturation test was carried out (Payne and Dickens, 1976) using methods previously described White (1987). This involved recording the EAG response (as described above), while the antennal receptors were presented first with a continuous saturating dose (1 mg) of one enantiomer, then, after a 1.5-sec delay, with a simultaneous pulse (1.0-sec duration) of the other enantiomer (1 mg). The odors were presented using a three-inlet delivery tube, keeping the total airflow over the antennae at 1000 ml/min throughout the presentation. The procedure was repeated for three male individuals. In each case the antenna was presented with first one enantiomer of α-pinene as the saturating dose and then the other. To ensure that the first presentation did saturate the EAG response, each antenna was also presented with a saturating dose of each enantiomer, followed by a test dose of the same enantiomer.

RESULTS AND DISCUSSION

EAG Responses to Individual Components. Analysis of variance showed no difference in EAG responses between the sexes ($F = 0.07$, $P = 0.8$), therefore, the data were pooled for presentation. There were clear differences, however, between the responses to the components tested ($F = 12.5$, $P < 0.001$), with (*S*)-(−)-β-pinene giving the largest EAG responses and longifolene the smallest (Table 2). Where optically active components were tested, there was evidence of chiral specificity in the response of *D. valens* antennal receptors. There was a clear difference between the responses to (*R*)-(+)-α-pinene and (*S*)-(−)-α-pinene ($P < 0.01$, Bonferroni test), although there was no specificity shown between (*R*)-(+)-limonene and (*S*)-(−)-limonene. The lack of differences in response between the sexes suggests that both male and female *D. valens* antennae possess similar numbers and types of receptor sites (Payne, 1975).

The lack of sexual differences in EAG responses agrees with the overall even ratio of sexes caught in the traps (Hobson et al., 1993). The closely related *D. terebrans* also showed very little sexual difference in EAG response to α-pinene, β-pinene, turpentine, and six scolytid pheromones (Delorme and Payne, 1990). *Dendroctonus frontalis* (Zimmermann) males and females did not differ in their EAG response to four different enantiomeric blends of frontalin (Payne et al., 1982). *Dendroctonus ponderosae* (Hopkins) males and females did not differ in their response to three terpenoid pheromones (*trans*-verbenol, *cis*-verbenol, verbenone) and differed in their response to three bicyclic ketal pheromones (*exo*-brevicomin, frontalin, *endo*-brevicomin) only at the highest

TABLE 2. RESPONSE OF *D. valens* TO HOST MONOTERPENES[a]

Compound	EAG (μV)[2b]	*D. valens* catch[3c]	*D. valens* catch[4d]
(*S*)-(−)-β-Pinene	467 ± 165[a]	27.8[a]	61.1[a]
(*S*)-(+)-Δ-3-Carene	193 ± 115[c]	2.6[b]	11.2[b]
(*R*)-(+)-α-Pinene	162 ± 92[c]	2.2[b]	0.7[d]
(*S*)-(−)α-Pinene	452 ± 155[a]	0.1[c]	
Myrcene	385 ± 262[ab]		2.5[c]
(*R*)-(+)-Limonene	146 ± 71[c]		0.8[d]
(*S*)-(−)-Limonene	208 ± 154[bc]		
β-Phellandrene	162 ± 162[bc]		
Terpinolene	125 ± 125[bc]		
Estragole	421 ± 173[ab]		
Longifolene	21 ± 28[c]		

[a] Means followed by the same superscript are not significantly different ($\alpha = 0.05$).
[b] Mean and SD for 10 beetles.
[c] Mean *D. valens* caught in test 5 (Hobson et al., 1993).
[d] Mean *D. valens* caught in test 2; α-pinene and limonene tested as natural enantiomeric mix in *P. ponderosa* resin (Hobson et al., 1993).

two concentrations (10 and 100 μg) tested (Whitehead et al., 1989). In contrast, female *D. pseudotsugae* had a higher EAG response to limonene and more receptor cells that responded to either α-pinene or limonene (unspecified enantiomers) than did males (Dickens et al., 1983, 1984). *D. pseudotsugae* females were also more attracted to host odors than were males (Rudinsky, 1966).

EAG responses to individual components of the resin correlated to some extent with their attractiveness in field tests, although not all compounds tested on the antenna were tested in the field. (*S*)-(−)-β-pinene gave the largest EAG responses at the dose tested and was the most effective in attracting beetles to traps, although (*S*)-(−)-α-pinene and myrcene gave EAG responses that were not significantly different from (*S*)-(−)-β-pinene, yet produced much lower trap catches. A notable exception to the correlation between EAG response and attraction in the field was α-pinene, where (*R*)-(+)-α-pinene produced a lower EAG response than (*S*)-(−)-α-pinene but was significantly more attractive in the field (Table 2).

This stereospecificity was confirmed in the second experiment, where the dose–response curve of the two stereoisomers of α-pinene showed a significantly greater EAG response to the (*R*)-(+) enantiomer at all doses >1 μg (Figure 1).

The presence of a large EAG response suggests the presence of many receptor sites for a given compound, although it cannot determine whether such compounds will act as attractants, repellents, or in some other way. Ecologically

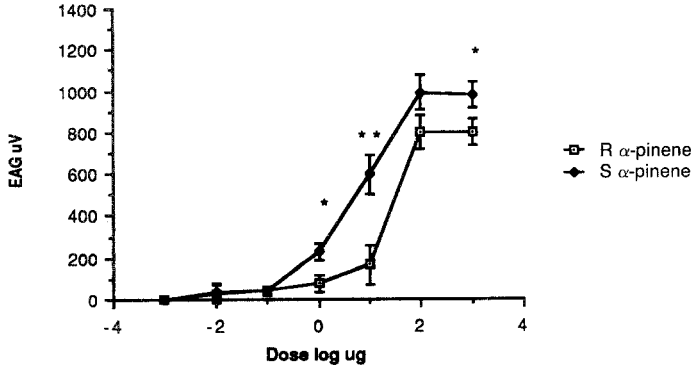

FIG. 1. Dose–response curve of EAG response (mean ± SE) of six male *D. valens* to dilutions of (*R*)-(+)- and (*S*)-(−) α-pinene. *$P < 0.05$; ** $P < 0.01$.

relevant materials with high EAG activity are likely, however, to be of behavioral significance to the insect (see Masson and Mustaparta, 1990, for a review). The greater EAG response to the *S* enantiomer of α-pinene, relative to the *R* enantiomer, suggests that the *S* enantiomer may have a role to play in the host location mechanism. Trap catch results have shown that (*S*)-α-pinene acts as an interruptant and reduces the trap catch caused by (*R*)-(+)-α-pinene (Hobson et al., 1993). This, together with the abundance of (*S*)-(−)-α-pinene in sympatric nonhost conifers, suggests that (*S*)-(−)-α-pinene may be a cue whereby *D. valens* can discriminate nonhosts (Hobson et al., 1993).

The reversal of the relative amplitudes of the antennal EAG response from the preference exhibited in the field prompted a closer examination of the shape of the EAG curves for the two enantiomers of α-pinene (Figure 2). Measurements of the time taken for the EAG response to recover to half of the maximum response amplitude showed that recovery following stimulation by (*S*)-(−)-α-pinene takes significantly longer than recovery following stimulation by (*R*)-(+)-α-pinene ($t = 5.48$; 5 *df*, $P < 0.01$ for 100-μg doses). This suggests that different receptor populations may be involved. The differential saturation curves support this conclusion (Figure 3). When 1 mg of (*R*)-(+)-α-pinene was delivered, followed by 1 mg of (*S*)-(−)-α-pinene there was an initial strong antennal response and a subsequent additional response, indicating that a separate set of (*S*)-(−) receptors were capable of responding after a saturation dose of (*R*)-(+). Saturation of the antennal receptors was demonstrated at this dose level by presenting two successive doses of 1 mg of (*R*)-(+) and obtaining only one response (Figure 3b). In contrast, a 1-mg dose of (*S*)-(−) followed by the same dose of (*R*)-(+) produced only one response (Figure 3c). Again, saturation at the 1-mg level of (*S*)-(−) was demonstrated by two successive 1-mg pulses of (*S*)-(−), which produced only one response.

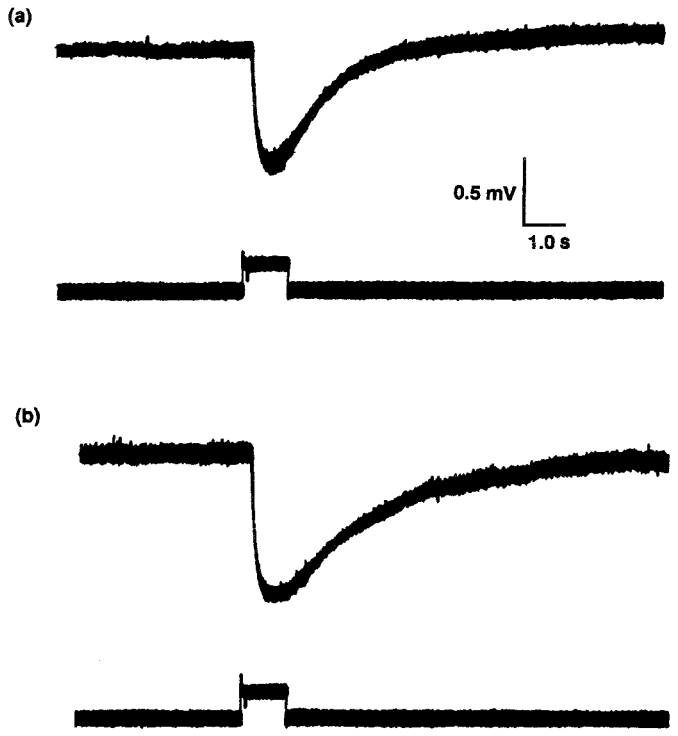

FIG. 2. EAG trace of *D. valens* to 100 μg of (*R*)-(+)-α-pinene (a) or (*S*)-(−)-α-pinene (b) Lower trace shows pulse duration of odor stimulus.

Delorme and Payne (1990) suggest from saturation curves that *D. terebrans* receptors for α-pinene and β-pinene may be triggered by other monoterpenes, but *D. terebrans* antennal receptors continued to respond to doses of α- and β-pinene beyond the saturation dose of turpentine. However, the relative proportions of (*R*)-(+) and (*S*)-(−) enantiomers were not known for the α-pinene or the turpentine.

The responses of *D. valens* antennae suggest at least two sets of receptors of α-pinene, those that may respond to either (*R*)-(+) or (*S*)-(−), (at least at high concentrations) and those that respond to (*S*)-(−) only. These results do not rule out the existence of receptors specific for the *R* enantiomer. This technique tests extremely high concentrations of the odors; specificity for the *R* enantiomer may be shown by some receptors at lower, physiologically and ecologically more relevant concentrations. Single-unit recordings would be required to confirm this. At the high concentrations presented here, such discrimination may break down and the *R* receptors may be saturated by the *S*

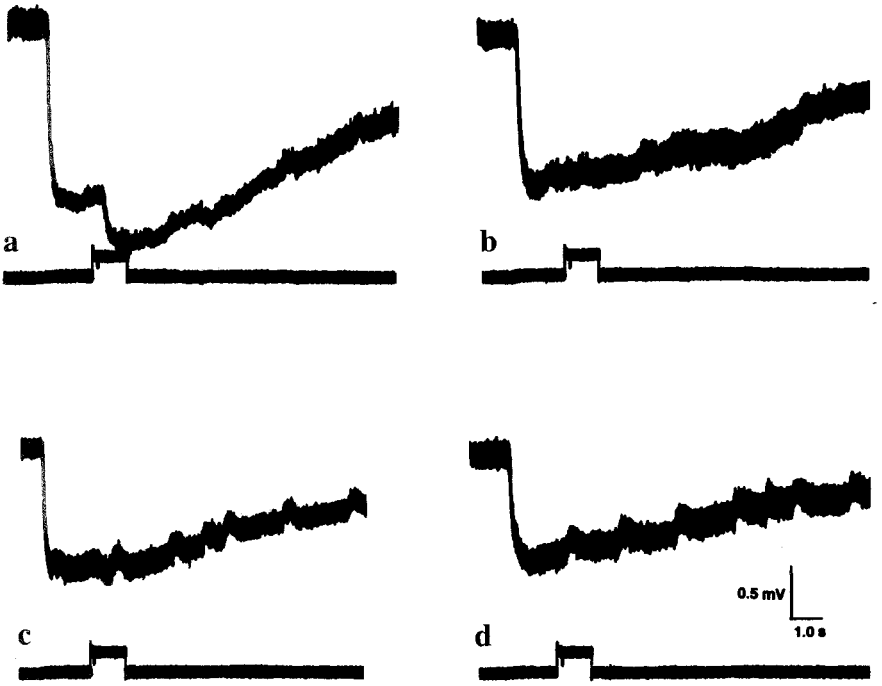

FIG. 3. Differential saturation EAG trace of *D. valens* to 1-mg doses of α-pinene enantiomers: (*R*)-(+) then (*S*)-(−) (a), (*R*)-(+) then (*R*)-(+) (b), (*S*)-(−) then (*R*)-(+) (c), and (*S*)-(−) then (*S*)-(−) (d). EAG (top trace) shows onset of first (saturating) odor stimulus, which was kept on for 5 sec. Lower trace shows pulse duration of second (test) odor stimulus.

enantiomer. These results do demonstrate, however, that there are at least two distinct populations of receptors for α-pinene and that one population responds specifically to the *S* enantiomer.

The specificity of the putative (*S*)-(−) receptors is indicated by the additional response obtained after 100 μg of (*R*)-(+) failed to exhaust the antennae's capacity to respond to (*S*)-(−). Similarly *I. pini* antennal receptors sensitive to ipsdienol maintained their specificity when tested with a 500-μg dose of other odors (Mustaparta et al., 1979). In contrast, the most specific antennal receptors of *D. terebrans* (sensitive to 3,2-MCHone and 3,2-MCHol) responded to a 1-μg dose of other odors such as α-pinene (Dickens et al., 1984). The specificity of the putative (*S*)-(−) receptor in *D. valens* antennae suggests that the ability to distinguish the two enantiomers of α-pinene that are widespread and abundant in host and nonhost conifers is important in host selection. Separate receptors that discriminate different enantiomers of pheromones are known for *Ips para-*

confusus Lanier, *Ips pini* (Say), and *Scolytus scolytus* (F.) (Mustaparta et al., 1979, Wadhams et al., 1982) although *D. pseudotsugae* and *D. frontalis* received both (+)- and (−)-frontalin on a single receptor (Dickens et al., 1985; Payne et al., 1982). Prior to the current study, however, few comparative EAG or single cell electrophysiological tests of enantiomers of chiral host monoterpenes have been done. Both enantiomers of limonene and α-pinene elicited significant EAG responses from *Anthonomus grandis* Boh., but there was no evidence of chiral specificity (Dickens, 1984).

Additional EAG or single-cell dose–response studies with other host monoterpenes that are attractive to *D. valens* in field tests (e.g., β-pinene and Δ-3-carene) are likely to further our understanding of *D. valens*' host selection. Ultimately we may answer the question of how *D. valens* can, by olfaction, identify its host species and individual hosts in a forest of mixed species and variable individuals with broadly overlapping host odor components.

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AGGREGATION PHEROMONE OF *Carpophilus antiquus* (COLEOPTERA: NITIDULIDAE) AND KAIROMONAL USE OF *C. lugubris* PHEROMONE BY *C. antiquus*

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Abstract—Males of *Carpophilus antiquus* Melsheimer (Coleoptera: Nitidulidae) emit an aggregation pheromone that was found to be a novel hydrocarbon, (3*E*,5*E*,7*E*,9*E*)-6,8-diethyl-4-methyl-3,5,7,9-dodecatetraene. A synthetic scheme and spectra (mass and proton NMR) are given for the compound. Beetles produced the pheromone when feeding on a variety of media, including the brewer's yeast-based artificial diet, fermenting whole-wheat bread dough, corn, and prunes; live baker's yeast was generally added to the food media. Males held individually produced, on average, 25× more pheromone per beetle than males held in groups of 10 or more. Pheromone was not produced until males were at least 5 days old but was still detected from the oldest beetles tested (47 days). In field tests, the pheromone was attractive to both sexes of *C. antiquus*, and it was synergized by food volatiles: A combination of pheromone and fermenting whole wheat dough attracted 2.5× more beetles than pheromone alone, but dough by itself was not significantly more attractive than the control. Semiochemical interactions were studied among *C. antiquus* and two other sympatric species for which pheromones are known, *C. lugubris* Murray and *C. freemani* Dobson. *C. antiquus* responded readily to the pheromone of *C. lugubris*, but all other interspecific responses to the pheromones were weak. In a sample of naturally infested corn ears, the presence of *C. antiquus* was strongly associated with the presence of *C. lugubris*, as would be expected if the pheromone of *C. lugubris* serves as a kairomone for *C. antiquus*.

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Key Words—*Carpophilus antiquus*, *C. lugubris*, *C. freemani*, Coleoptera, Nitidulidae, pheromone, kairomone, hydrocarbon, tetraene.

INTRODUCTION

Carpophilus antiquus Melsheimer (Coleoptera: Nitidulidae) is a small (2.4 mm length), reddish brown sap beetle that is most often found associated with corn. Williams et al. (1983) and references therein reported that its range in North America is from New Jersey south through the Carolinas and westward into Minnesota and Missouri. We have found it to be quite common in corn fields and woodlands in central Illinois.

Male-produced aggregation pheromones have been identified for a number of sympatric species including *C. lugubris* Murray (Bartelt et al., 1991) and *C. freemani* Dobson (Bartelt et al., 1990b). We had previously noticed that *C. antiquus* responds clearly to the pheromone of *C. lugubris* (unpublished), and we wished to know whether the two species simply shared a pheromone or whether a more complex semiochemical interaction existed between them. We sought to clarify the relationship by identifying the pheromone produced by *C. antiquus*, by measuring the relative attractiveness of the species' pheromones in field experiments, and by determining the degree of association between species in naturally infested corn ears. The field experiments were expanded to include the pheromone of *C. freemani* also, when it became clear that this species was common enough to provide meaningful comparisons with the others.

Irrespective of ecological interactions among species, having a pheromone for *C. antiquus* could be of practical importance because the species is a significant member of the sap beetle complex attacking corn in the Midwest. Additional control and monitoring techniques for these beetles may become more valuable because they are now known to vector mycotoxin-producing fungi (Lussenhop and Wicklow, 1990).

METHODS AND MATERIALS

Beetles. A culture of *C. antiquus* was started from beetles collected near Kilbourne, Illinois, in April 1991. The rearing method followed the concepts described by Dowd and Weber (1991) except that the pinto beans in the diet were replaced by additional brewer's yeast (the "brewer's-yeast diet"). Later on, finely chopped pitted prunes were added to the diet as well (30/liter, the "prune–brewer's-yeast diet"). We found *C. antiquus* to be difficult to rear in large numbers, primarily because of poor egg production. However, best results were with the prune–brewer's-yeast diet. The culture eventually produced enough

beetles to allow pheromone identification, but beetle numbers were never sufficient for conducting wind-tunnel bioassays.

Pheromone Identification. Because laboratory bioassays could not be used to guide pheromone purification, the approach was to seek consistent, sex-specific differences in volatile collections from male and female beetles by gas chromatography (GC). Any sex-specific compounds would be considered as pheromone candidates, based on previous experience, and identification of these through spectroscopy and synthesis would be attempted. Pheromonal activity of the synthetic compounds would then be verified in field bioassays.

Pheromone Collections. Volatile collections from feeding beetles were made as described previously for *C. hemipterus* (Bartelt et al., 1990a). Briefly, male or female beetles and food materials were placed into 50-ml flasks. Filters of Tenax or, later, Super Q porous polymer (Alltech Associates, Deerfield, Illinois, for both materials) were used to clean the incoming air and to capture the volatiles from the feeding beetles. The temperature during collections was 27°C, the humidity of incoming air was ca. 30%, and the photoperiod in the incubator was 14:10 hr (light–dark). Volatiles were eluted from filters with hexane.

Several food types were tried: brewer's yeast diet, prune–brewer's-yeast diet, water-soaked corn seeds (autoclaved to retard mold formation), prunes, and whole-wheat bread dough (whole-wheat flour, sugar, and water in a 4:1:2 blend, by volume). Dried baker's yeast (Fleischman's Yeast Inc., Oakland, California) was sprinkled over all these media. In addition, the brewer's yeast and prune–brewer's-yeast diets were tried without additional baker's yeast. This range of food materials was chosen based on previous experience with other *Carpophilus* species (Bartelt et al., 1990a,b, 1991, and other, unpublished data). Live baker's yeast was usually added because nitidulid beetles are typically attracted to fermenting media, and they presumably colonize and emit pheromone readily from such food sources. Initially, sand (ca. 1-cm layer, extracted with hexane prior to use to reduce background volatiles) was placed in the bottoms of the flasks to provide a more natural feeding site for the beetles (i.e., the food–sand interface) and to retain dampness. Damp sand led to more rapid spoilage of the food, however, and this practice was discontinued.

Beetles were always segregated by sex, and between 1 and 150 individuals were added to flasks. The initial number was 150; it was expected that the pheromone would be more prominent against the background of food volatiles if more individuals were present. However, when fewer beetles were in the flasks, pheromone production per beetle was greater (see Results). Later collections were made from smaller numbers of beetles.

One hundred thirty-one male-derived and 56 female-derived volatile collections were analyzed. (Collections from females were terminated after it was clear that a male-specific compound existed). Multiple regression analysis was

used to explore relationships between pheromone production in males and factors such as beetle age, numbers of beetles in volatile collectors, and food source.

Chromatography and Spectroscopy. Instrumentation was as described previously (Bartelt et al., 1990a). Male- and female-derived volatile collections were compared by GC on a 15-m × 0.25-mm-ID capillary column (DB-1, with 1.0-μm film thickness, J&W Scientific, Folsom, California). Oven temperature was programmed from 70 to 220°C at 10°/min.

The male-derived samples for all days for which a male–female difference was detected were combined for further analysis. A parallel sample was prepared from the female-derived volatile collections. These were subjected to column chromatography on silica gel; the elution solvents were hexane and 5%, 10%, and 50% ether in hexane. These fractions were again analyzed by GC for male–female differences.

The major male-specific compound in the hydrocarbon fraction was analyzed by GC–mass spectrometry (GC-MS) and was further purified by high-performance liquid chromatography (HPLC) on a silver-nitrate-coated silica column (Heath and Sonnet, 1980); the solvent was 10% toluene in hexane. A nuclear magnetic resonance (NMR) proton spectrum was obtained for the purified compound (300 MHz, deuterobenzene).

Synthetic Hydrocarbons. The structures of five hydrocarbons discussed in the text are shown in Figure 1, along with assigned structure numbers. These are: (3*E*,5*E*,7*E*,9*E*)-6,8-diethyl-4-methyl-3,5,7,9-dodecatetraene **(1),** (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene **(5),** (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **(6),** (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene **(7),** and (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene **(8).** The pheromone components of *C. lugubris* and *C. freemani* **(5–8)** were available from earlier research (Bartelt et al., 1990c, 1991).

The *C. antiquus* pheromone **(1)** was synthesized as outlined in Figure 1. Commercially available aldehyde **2** was used in a Wittig-Horner condensation with triethyl 2-phosphonobutyrate. The resulting ethyl ester was reduced to the corresponding alcohol with $LiAlH_4$, and the alcohol was converted to aldehyde **3** with MnO_2. These steps were repeated a second time, starting with **3** to yield aldehyde **4,** and a Wittig condensation using (propyl)triphenylphosphonium iodide completed tetraene **1.** Reaction conditions were as described previously for related syntheses (Bartelt et al., 1990c). The final product **1** and the ester intermediates were distilled (Kugelrohr). Compound **1** was passed through a silica gel column with hexane to remove polar impurities. Over the seven synthetic steps, the yield of all-*E* **1** was 8% from aldehyde **2,** and the purity of **1** in the final product was 61%. Impurities were primarily *Z* isomers of **1.** Another impurity (6%) was triene **8,** which was generated because of incomplete Wittig-Horner condensations. The product was diluted with hexane to 150 μg of **1** per microliter and was used to prepare trap baits for field tests. Chromatographic

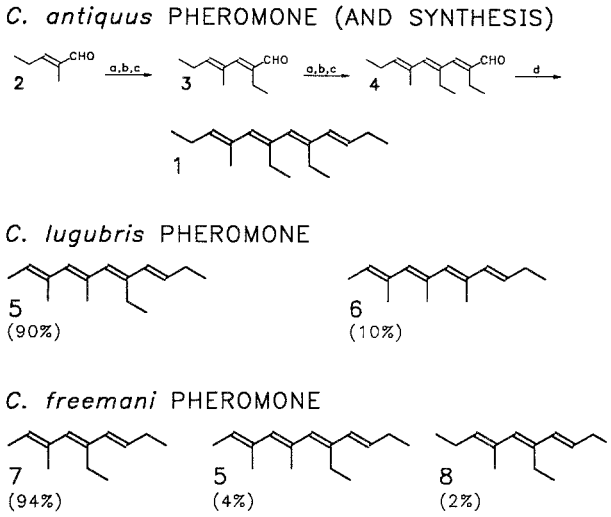

FIG. 1. Hydrocarbon pheromones of three *Carpophilus* species and assigned structure numbers. Synthetic scheme shown for *C. antiquus* pheromone **(1)**; a = Wittig-Horner condensation with triethyl 2-phosphonobutyrate, b = reduction with $LiAlH_4$, c = conversion to aldehyde with MnO_2, d = Wittig condensation with (propyl)triphenylphosphonium iodide (see text). For *C. lugubris* and *C. freemani*, proportions of components in pheromones are indicated as percentages.

removal of unwanted geometrical isomers and other impurities was not practical because of the large amount of material needed for field tests. An analytical sample of **1** was prepared by HPLC on the silver nitrate column (5% toluene in hexane) followed by HPLC on a size-exclusion column (PLgel 50A, Polymer Laboratories, Shropshire, U.K.) with hexane as solvent to remove the last traces of toluene prior to NMR analysis. As for the beetle-derived sample, a proton spectrum was obtained at 300 MHz in deuterobenzene.

Formulations for Field Use. Synthetic **1** was applied to rubber septa (20 mm × 11 mm diameter, red rubber, Aldrich Chemical Co., Milwaukee, Wisconsin) for use in field traps. The compound was loaded at the rate of 1.4 mg/septum and was immediately followed with 300 μl of methylene chloride. The release rate from the septa was 0.60 μg/hr during the first day after preparation, measured in volatile collections taken at 27°C and with an airspeed of 48 cm/min.

For *C. lugubris*, septum loads for **5** and **6** (0.44 and 0.05 mg, respectively) were chosen so that the emission rate of **5** was the same as that for **1** from the *C. antiquus* septa. (It was verified by GC that the *C. lugubris* pheromone used in the field study did not contain **1**). The load rates of **7** and **5** for the *C. freemani*

septa were 0.49 and 0.01 mg, respectively (the standard load rate in earlier studies). As discussed below, **8** was not added to the septa.

Field Studies. Experiments were conducted in 1992 near Kilbourne, Illinois, in an oak woods adjacent to a corn field, and near Washington, Illinois, in a stand of mixed hardwoods adjacent to an apple orchard. Wind-oriented funnel traps (Dowd et al., 1992) were used for these studies. The traps were hung from tree branches between 1 and 2 m above the ground, and traps were separated by at least 10 m. Within each block, traps were rerandomized weekly. Trap baits included the pheromone-treated septa described above and a source of food volatiles, which was fermenting whole wheat bread dough (ca. 15 g/trap).

One field study tested the activity of synthetic **1** and whether there was a synergistic response to synthetic **1** and food volatiles. Treatments were: **1** alone, dough alone, **1** plus dough, and empty (control) trap. All four treatments were present in each of two blocks. The beetles were collected weekly from September 15 to October 27 and were sorted according to species; captured *C. antiquus* were sexed. Both pheromone and dough baits were replaced weekly. This study was conducted only at Kilbourne.

A second study tested for cross-attraction between *C. antiquus* and *C. lugubris*. Treatments were **1** plus dough, *C. lugubris* pheromone (**5** + **6**) plus dough, and dough alone. Beetles were collected weekly and sorted according to species. Captured *C. antiquus* were sexed. This was conducted both at Kilbourne (four blocks) and at Washington (two blocks). This study ran from April 10 to May 15 and again from September 15 to October 27 at both locations. (In previous unpublished studies, *C. antiquus* never responded to traps in midsummer.) During the spring period, the pheromone baits were replaced every two weeks. Otherwise, all pheromone and dough baits were replaced weekly.

A fourth treatment, *C. freemani* pheromone (**7** + **5**) plus dough, was added to the experiment at Kilbourne between October 6 and October 27 because *C. freemani* were being captured in some of the other treatments in fairly high numbers.

The studies were analyzed as randomized complete block experiments by analysis of variance. Trap catches were transformed to the log $(X + 1)$ scale to stabilize variance.

Corn Ear Samples. As part of another study by one of us (P.F.D.), 120 milk-stage ears of corn were harvested on August 5, 1992, from a farm near Green Valley, Illinois. The corn was an open husk variety, which allowed beetles easy access to kernels. Ears were examined for *Carpophilus* beetles, and numbers of each species on each ear were recorded. A chi-square procedure was used to test for any association between the presence or absence of *C. antiquus* and *C. lugubris*.

RESULTS

Male-Specific Compound. By GC, one compound was detected in 101 of 131 male-derived volatile collections that was never detected in female-derived samples. Relative to *n*-alkanes, the compound had an equivalent chain length of 16.20 carbon units. After column chromatography on silica gel, the compound was present only in the hydrocarbon fraction. (No other sex-specific compounds were noted when the more polar silica gel fractions were compared.)

The molecular weight of the male-specific hydrocarbon was 232 by GC-MS, and the spectrum was identical to that shown in Figure 2. By analogy to the previously identified *Carpophilus* pheromones, the compound was postulated to be a 17-carbon tetraene; a molecular formula of $C_{17}H_{28}$ would account for the molecular weight of 232. When the male-derived, hydrocarbon fraction was purified further by HPLC on the silver nitrate column, the compound eluted 5.4–5.8 ml after injection (the void volume was 3.2 ml); elution after the solvent front supported the presence of double bond(s). After HPLC, a 12-μg sample of the unknown compound was available for NMR; by GC, the purity was 93%.

The NMR spectrum for the isolated compound was virtually identical to that for the synthetic tetraene (Figure 2) except for additional signals due to residual toluene from the HPLC step (δ 2.15 and >7) and unknown impurities (small singlets at δ 2.36, 1.95, 1.58, and 1.38). The spectrum indicated four ethyl groups and one methyl group attached to olefinic systems and five olefinic protons, for a total of 28 hydrogens. Two of the ethyl-group methylenes (δ 2.41 and 2.60) were split only by terminal methyls (δ 1.11 and 1.22, respectively, J = 7.5 Hz), but each of the other two (δ 2.09 and 2.11) was split by an olefinic proton (δ 5.56, J = 7.2 and δ 5.76, J = 6.6 Hz, respectively) as well as by terminal methyls (δ 0.99 and 1.02, respectively, J = 7.5 Hz). These two methylene quintets partially overlapped, giving the appearance of a four-proton sextet, and this signal was further complicated by the toluene signal in the natural sample. However, the triplet olefinic signals at δ 5.56 and 5.76 clearly indicated the attachment of two separate methylenes. The signal at δ 5.76 was further split by the proton at δ 6.13. The large coupling constant (J = 15.7 Hz) was consistent with an *E* configuration at a disubstituted double bond. A one-proton singlet (δ 6.10) overlapped the right-hand peak of the one-proton doublet centered at δ 6.13. There was another unsplit olefinic proton at δ 5.95 and an unsplit methyl signal at δ 1.78.

The shifts and couplings were matched to portions of similar structures reported previously (Bartelt et al., 1991, 1992), and compound **1** was chosen for synthesis as the probable structure of the pheromone. Synthetic **1** matched the natural compound by GC, NMR, and mass spectrometry.

Minor amounts (<5% as abundant as **1**) of other male-specific compounds were also detected when the combined, beetle-derived hydrocarbon fractions

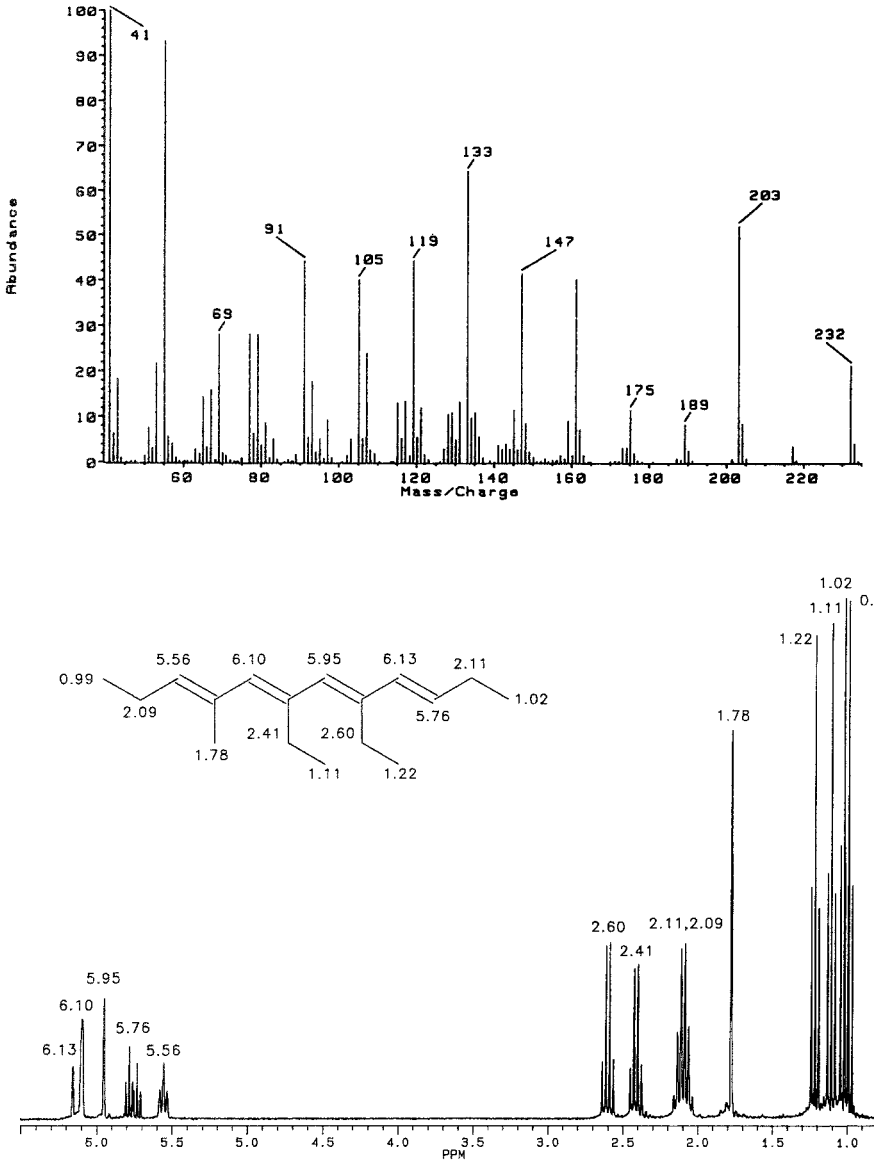

FIG. 2. Electron impact mass spectrum and proton NMR spectrum for synthetic **1.**

were carefully compared by GC. By GC retention (elution before **1**), mass spectrometry (spectra similar to **1**), and experience with other tetraenes (Bartelt et al., 1992), these other compounds were believed to be *Z* isomers of **1.** Some isomerization/degradation has invariably occurred with all of the tetraenes (Bartelt et al., 1992), and it is unknown whether the minor isomers are actually in emissions from *C. antiquus*. These were not analyzed further in this study.

Pheromone Production. Pheromone production in the laboratory was highly variable, but it was clearly dependent on beetle age and on numbers of beetles per collection flask (Table 1). By regression analysis, old beetles (≥ 5 days) produced significantly more pheromone than young beetles (1–4 days old, $P < 0.001$). Furthermore, on a per male basis, collections from single males contained significantly more pheromone than those from groups of 10 or more males ($P < 0.001$). The interaction of these factors was also significant ($P < 0.001$) because the difference between single and crowded beetles was seen only for old beetles; young beetles did not produce pheromone at any density. The highest observed production rate for a single male was 279 ng/day at 17 days of age. The largest value for a group was 31 ng/male/day for thirty 17-day-old males. Pheromone production could continue for over a month; one group of 50 males produced 3.6 ng/male/day at age 39 days and 0.5 ng/male/day at 47 days.

Pheromone production was detected for all of the food materials tested. The highest rates of pheromone production occurred for beetles feeding on the prune–brewer's-yeast diet with added baker's yeast, but differences among food media were not significant. Pheromone emission ceased whenever the collection flask became too wet (condensation on flask wall) or too dry (food material hard and brittle). Pheromone was detected in flasks containing some mold, but mold

TABLE 1. PHEROMONE PRODUCTION BY MALE *C. antiquus* (FITTED VALUES FROM REGRESSION ANALYSIS[a] OF 131 VOLATILE COLLECTIONS)

Beetles in collector		Pheromone production	Range	Number of
Age (days)	Number	(ng/male/day)	(ng/male/day)	collections
1–4	1	0.0		6
1–4	≥10	0.1	0–0.3	12
≥5	1	102	0–279	36
≥5	≥10	4.1	0–31	77

[a]For regression analysis, $R^2 = 0.46$, $P < 0.001$. Difference between age categories, difference between beetle-number categories, and the age by beetle-number interaction were all significant ($P < 0.001$).

on all food surfaces was an unhealthy situation that usually led to beetle mortality.

Activity of C. antiquus *Pheromone.* Synthetic **1** was attractive to *C. antiquus* in the field (Table 2). Fermenting dough was not significantly more attractive than the control. Dough did synergize the pheromone, however, with the combination attracting 2.5× more *C. antiquus* than the pheromone alone. Both sexes responded about equally to the pheromone treatments. *C. lugubris* was not affected by the pheromone of *C. antiquus*; both treatments containing dough attracted slightly more beetles than those that did not. *C. freemani* responded slightly but significantly to the combination of **1** and dough; the other treatments were not different from the control.

Cross-attraction. In all field tests, *C. antiquus* responded to the pheromone of *C. lugubris* plus dough dramatically better than to dough but generally not as well as to its own pheromone plus dough (Table 3). On the other hand, *C. lugubris* responded well only to its own pheromone plus dough. Combinations of the other pheromones with dough were not more effective for *C. lugubris* than dough by itself. Finally, *C. freemani* did respond significantly to the *C. antiquus* bait plus dough, but this effect was very small in comparison with the response to its own pheromone. No significant cross attraction between *C. lugubris* and *C. freemani* was observed in this study.

Corn Ear Infestation. The distributions of *C. antiquus* and *C. lugubris* were highly correlated in the corn ear sample (Table 4). Of the 27 ears on which *C. antiquus* was found, 25 also had *C. lugubris*. This was significantly higher than the 13.5 ears expected under the null hypothesis of independent distributions of the species (Pearson's chi-square statistic = 25.3, 1 *df*, $P << 0.001$).

TABLE 2. ATTRACTIVENESS OF SYNTHETIC *C. antiquus* PHEROMONE (COMPOUND **1**) TO THREE *Carpophilus* SPECIES UNDER FIELD CONDITIONS, (SEPTEMBER 15–OCTOBER 27, KILBOURNE, ILLINOIS

Treatment	Mean trap catch per week[a]		
	C. antiquus (% males)	*C. lugubris*	*C. freemani*
Compound **1** + dough	53.7 a (55%)	2.6 a	2.0 a
Compound **1**	18.8 b (55%)	0.0 b	0.3 b
Dough	0.1 c	2.9 a	0.0 b
Control (empty trap)	0.0 c	0.0 b	0.0 b

[a] In each column, means followed by the same letter are not significantly different (LSD, 0.05 level). Analysis was done in log (X + 1) scale, and means were returned to numerical scale for presentation. N = 12 for all means. Sex of captured *C. antiquus* shown for total captures over 10.

TABLE 3. ATTRACTIVENESS OF THREE SYNTHETIC *Carpophilus* PHEROMONES TO THREE SPECIES UNDER FIELD CONDITIONS[a]

Pheromone treatment	Mean trap catch per week[b]		
	C. antiquus (% males)	*C. lugubris*	*C. freemani*
April 10–May 15 (N = 30)			
C. antiquus + dough	92.4 a (46%)	1.1 b	0.5 a
C. lugubris + dough	17.3 b (47%)	88.6 a	0.1 b
Dough	0.5 c (52%)	1.5 b	0.0 b
September 15–October 27 (N = 24)			
C. antiquus + dough	17.8 a (55%)	1.3 b	2.1 a
C. lugubris + dough	4.8 b (53%)	78.0 a	0.3 b
Dough	0.1 c	1.4 b	0.0 b
October 6–27 (N = 6) (Kilbourne site only)			
C. antiquus + dough	59.1 a (52%)	0.6 b	1.4 b
C. lugubris + dough	34.4 a (51%)	47.9 a	1.3 b
C. freemani + dough	0.9 b	2.5 b	24.6 a
Dough	0.0 b	0.6 b	0.0 b

[a] Data were combined for the Kilbourne and Washington, Illinois, sites.
[b] In each experiment, means in a column followed by the same letter are not significantly different (LSD, 0.05 level). Analysis was done in log (X + 1) scale, and means were returned to numerical scale for presentation. Sex of captured *C. antiquus* shown for total captures over 10.

TABLE 4. ASSOCIATION BETWEEN *C. antiquus* AND *C. lugubris* IN A SAMPLE OF 120 CORN EARS FROM GREEN VALLEY, ILLINOIS: NUMBERS OF EARS ON WHICH JUST ONE, BOTH, OR NEITHER SPECIES WAS PRESENT

		C. lugubris		Totals for *C. antiquus*[a]
		Present	Absent	
C. antiquus	Present	25 (13.5)[b]	2 (13.5)	27
	Absent	35 (46.5)	58 (46.5)	93
Totals for *C. lugubris*[a]		60	60	120

[a] Overall, 23% of the ears were infested with *C. antiquus*, and 50% were infested with *C. lugubris*. Mean numbers of beetles per ear (standard deviation): *C. antiquus*, 0.44 (0.98); *C. lugubris*, 2.39 (3.61).
[b] Expected values are given in parentheses for numbers of ears with one, both, or neither species under the null hypothesis that the species acted independently. Null hypothesis was rejected (chi-square statistic = 25.3, 1 df, $P < 0.001$). Of the 27 ears infested with *C. antiquus*, 25 (93%) also had *C. lugubris*. This was far higher than the 13.5 (50%) expected under the null hypothesis, given the overall abundance of *C. lugubris* in the sample.

The mean number of *C. antiquus* per ear was 0.44 (±0.98 SD); the mean for *C. lugubris* was 2.39 (±3.61 SD).

DISCUSSION

Aggregation Pheromone. As with the other, previously studied *Carpophilus* species (Bartelt et al., 1990a,b, 1991), *C. antiquus* males produce an aggregation pheromone to which both sexes are attracted. As with the other species, the pheromone is synergized by host volatiles, the combination of food- and beetle-derived volatiles being more attractive than either odor source alone.

C. antiquus was unusual, however, in that the pheromone was quite attractive in the field even without a synergist. Only with *C. freemani* was there a comparably high response to the pheromone alone relative to food volatiles (Bartelt et al., 1990b). This property may correlate to an ability to colonize relatively unripe, undamaged fruits (although the beetles are certainly not restricted to these). Male *C. antiquus* or *C. freemani* at such a site could attract conspecifics even before the rich volatile bouquet associated with overripe fruit and accompanying microorganisms is present to synergize the pheromone. Smilanick (1979) found that *C. freemani* tended to accept unripe figs more readily than did *C. mutilatus* or *C. hemipterus*.

In the laboratory, the decreased pheromone-emission rates in the presence of other beetles may be evidence for an aggregation-terminating mechanism. So far we have found no evidence for antiaggregation pheromones in the nitidulids, but a mechanism for terminating aggregations under field conditions must exist.

The relatively low pheromone production per male from groups of beetles had not been noted before in our nitidulid studies, but the phenomenon has been reported in other beetle groups. For example, Burkholder and Dicke (1966) found that groups of 10 or 20 *Trogoderma inclusum* females were far less attractive than groups of two or four females. It was later learned that crowding interfered with calling behavior, resulting in reduced total pheromone emission (W.E. Burkholder, personal communication).

Chemistry. The pheromone of *C. antiquus* (compound **1**) has a novel structure. It is like several other *Carpophilus* pheromones in being an all-*E* tetraene (Bartelt et al., 1992), but it has the most carbon atoms (17) of any so far encountered. It is the first shown unequivocally to have two ethyl side chains. The mass and NMR spectral data are presented for the compound, and a synthetic method for preparing it is outlined. The compound fits the general biosynthetic scheme for the *Carpophilus* pheromones (Bartelt et al., 1992; Petroski et al., 1993); it is probably assembled from two propionate and three butyrate acyl units.

Interactions involving C. freemani *in Field Studies.* One potential compli-

cation in understanding the field studies is that triene **8** was unavoidably produced as an impurity in the synthesis of **1.** Triene **8** was discovered previously as a very minor male-specific hydrocarbon in *C. freemani* (Bartelt et al., 1991b). The compound had significant wind-tunnel activity but is not added to the pheromone for this species for field use because it did not synergize the activity of **7** (Bartelt et al., 1991b). The slight attraction of *C. freemani* to the synthetic pheromone for *C. antiquus* (Tables 2 and 3) was probably due to triene **8,** although the activity of **1** cannot be totally ruled out. In any case, the response of *C. freemani* to the synthetic *C. antiquus* pheromone was far weaker than to its own pheromone (Table 3).

There were also weak trends in Table 3 for cross-attraction between *C. freemani* and *C. lugubris*, but the effects were not significant and were very minor compared with responses to the species' own pheromones. Cross-attraction would not have been surprising because tetraene **5,** the major pheromone component of *C. lugubris*, is also a minor component for *C. freemani*, but the effect was not strong.

Interactions between C. antiquus *and* C. lugubris *in Field Studies.* Although both species responded best to their own pheromones, the responsiveness of *C. antiquus* to the pheromone of *C. lugubris* (Table 3) suggests a kairomonal role for the *C. lugubris* pheromone. *C. antiquus* could locate and cocolonize sites first established by *C. lugubris*. For example, corn ears are an acceptable food source for both species, but *C. lugubris*, being larger and stronger, could more easily penetrate the husk and gain access to and colonize developing kernels. It would be an advantage for *C. antiquus* to locate the *C. lugubris* infestations; otherwise, it could be very difficult for *C. antiquus* to use this food source. The present corn ear sample was collected from an open husk variety, which *C. antiquus* could enter fairly easily, but the association with *C. lugubris* was clear nevertheless, supporting that *C. antiquus* used the *C. lugubris* pheromone to find the ears. Competition from *C. antiquus* on a corn ear probably has little impact on *C. lugubris* because food is rarely limiting even when both species are present.

As with the bark beetles, which have been studied in far greater depth over the years (e.g., review by Birch, 1984), the nitidulid beetles seem to be involved in a variety of chemical interactions under field conditions that go beyond simple pheromonal attraction.

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National Museum collection. Mr. Dan Duval allowed the corn ear collection from his farm near Green Valley, Illinois.

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BIOLOGICALLY ACTIVE SECONDARY METABOLITES OF BARLEY. I. DEVELOPING TECHNIQUES AND ASSESSING ALLELOPATHY IN BARLEY

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Abstract—Allelopathic effects of barley (*Hordeum vulgare* L.) on white mustard (*Sinapis alba* L.) were assessed using modified bioassays that reduced other environmental influences. In a Petri dish bioassay, germination of white mustard was delayed and the radicle lengths were significantly inhibited at a density of 0.5 barley seed/cm^2. In a 'siphoning' bioassay apparatus, when the two species were sown together, radicle elongation of white mustard was not inhibited one day after sowing but became increasingly inhibited as bioassay time increased. Barley allelochemicals were released from the roots in a hydroponic system for at least 70 days after commencement of barley germination. Solutions removed from the hydroponic system of growing barley delayed germination and inhibited growth of white mustard. The allelopathic activity of barley was further confirmed at a density of 0.3 barley seed/cm^2 in a modified stairstep apparatus.

Key Words—Allelopathy, germination, bioassay, siphoning apparatus, hydroponics, stairstep assay, barley, *Hordeum vulgare*, *Sinapis alba*.

INTRODUCTION

Separation of allelopathy from other aspects of plant interference remains one of the most challenging tasks in studies of plant interference (Harper, 1977). Methods used in studying allelopathy have received more criticism than those

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for studying competition. This has resulted in some ecologists holding deep reservations concerning the significance of allelopathy.

Techniques applied to studies of allelopathy have frequently been crude, contributing to uncertainties about the significance of allelopathic phenomena. Leather and Einhellig (1986, 1988) reviewed the literature pertaining to the use of bioassays and discussed the general suitability of different assays for studying allelopathy, demonstrating that many reports of allelopathy are questionable because the bioassays were not suitable indicators. Soaking of plant parts in either water or organic solvents, for example, may lead to the release of chemicals that are not normally released into the environment (Lovett, 1982). Interpretation of "allelopathic" effects on plants or other organisms may be confounded in these circumstances. Allelopathy occurs only if the chemicals are not only produced by a plant but released into the vicinity of other plants and, ultimately, received under the influence of natural environmental conditions. Therefore, reliable investigations of allelopathy include tests of compounds released by intact living donor plants into the vicinity of receiver plants. This is a fundamental principle in investigations of allelopathy.

Barley (*Hordeum vulgare* L.) has been reported to be a smother crop, which can suppress the growth of weeds through competition for environmental resources (Overland, 1966). However, in the absence of competition, barley still inhibited germination of *Amaranthus hybridus* L. (slim amaranth) and *Chenopodium album* (Went et al., 1952), suggesting that phytotoxins might be involved (Overland, 1966). Overland (1966) further found that the inhibitory activity of barley was selective among broad-leaved plants, chickweed (*Stellaria media* L.) being more severely inhibited than shepherd's purse [*Capsella bursa-pastoris* (L.) Medic.].

The objectives of this research were to develop techniques for separating competitive influences from allelopathy and, through these techniques, to assess allelopathic activity of barley on white mustard (*Sinapis alba* L.).

METHODS AND MATERIALS

Petri Dish Bioassay. Allelopathic activity of germinating barley was bioassayed on filter paper in 9-cm Petri dishes in an incubator at 25°C in the dark using surface-sterilized seeds of white mustard (*S. alba*), used to simulate a broad-leaved weed of the same family as *C. bursa-pastoris*, but having synchronous germination. Barley (*H. vulgare*, cv. Triumph) seeds were evenly distributed on two Whatman No. 1 filter papers at rates of 0 (control), 0.13, 0.25, 0.5, 1.0, and 2.0 seeds/cm^2 with 10 white mustard seeds in each Petri dish for bioassay. The bioassay was designed with five replications.

Because germinating barley absorbs large amounts of water (Alabushev,

1977), an experiment was carried out to determine water uptake by germinating barley. Since radicle lengths of white mustard were greatest, and similar, at 5, 6, and 7 ml sterile distilled water under the bioassay conditions over the range of densities employed (Liu, 1991), 5, 6, and 7 ml sterile distilled water for controls (i.e., three controls without barley seeds) and 6.1, 6.2, 6.3, 6.6, and 7 ml for barley density of 0.13, 0.25, 0.5, 1.0, and 2.0 seeds/cm^2, respectively, were initially applied to the medium at the commencement of the experiment. Water uptake by barley was determined by weighing barley seeds after carefully removing free water from the barley seed surface. The compensating water for the uptake by barley was added 6, 18, 30, 45, and 60 hr after sowing. The criterion for determining the time and the amount of the compensating water was: 5 ml control $\leq$ water in all treatments, $\leq$7 ml control. At each observation, the water uptake by barley during the last period was added before placing in the incubator for continuation.

In the bioassay experiment, the compensating water was added at the times and in the amounts as determined in the water uptake experiment. This design should eliminate the effect of water competition by germinating barley and ensure no effect of excess water on white mustard.

The germination of white mustard was observed at 3- to 6-hr intervals and after 72 hr the radicle length of white mustard was measured. The germination data were used to calculate the germination rate, which was defined as the time to reach the germination peak in the germination distribution curves (Hughes, 1977). Range tests were applied to determine statistically significant differences for the radicle length.

Siphoning Bioassay Apparatus. The apparatus consisted of a glass container (40 cm long, 40 cm wide, and 12 cm high), four pieces of glass (45 $\times$ 9 cm), sixteen 9-cm glass Petri dishes, and 32 pieces of Whatman No. 1 filter paper (25 $\times$ 3.5 cm). Four glass Petri dishes were set on each piece of glass. Two Whatman papers were placed, parallel, over the Petri dishes and the ends of the papers were suspended in the water container. The container was filled with distilled water. The apparatus was set in an incubator at 25°C in the dark. Figure 1 shows a siphoning apparatus similar to the one used in these experiments.

The experiment was designed to observe the effects of young barley seedlings on white mustard at different times. The treatments included white mustard seeds alone and white mustard seeds with barley seeds, harvesting at each day after sowing up to six days. There were four replications. Thus, the total number of Petri dishes was 48. Since the number of Petri dishes in the apparatus was 16, which allowed only two harvests, it was not possible to conduct the experiments at one time. Therefore, the experiment was split into three runs, in each of which the 16 Petri dishes were set up in 2 $\times$ 2 factorial designs with four replications. The harvests were day 1 and day 2 in the first run, day 3 and day

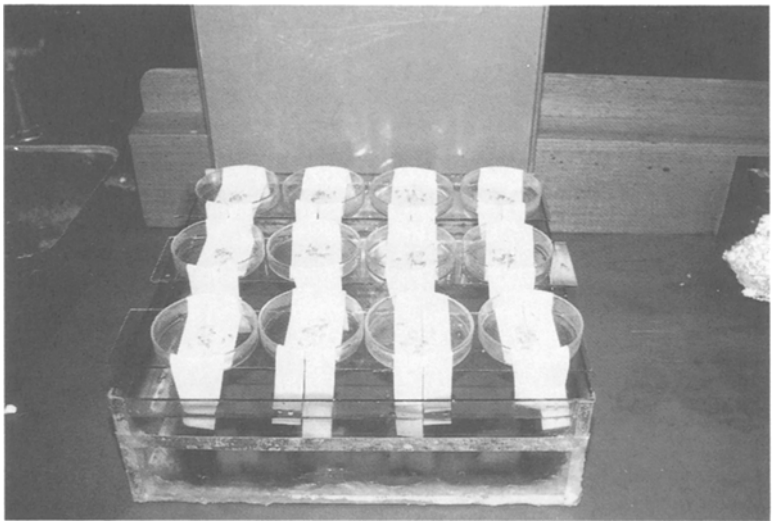

FIG. 1. Siphoning bioassay apparatus developed to eliminate competition for water while assessing the activity of allelochemicals released by germinating barley seeds.

4 in the second run, and day 5 and day 6 in the third run. Ten white mustard seeds in monoculture and 10 mustard plus 10 barley seeds in mixture were evenly sown in the Petri dishes, as appropriate. Four 6-mm filter paper disks were distributed in each dish and osmotic potentials were measured using a Wescor HR-33 dew point microvoltmeter on the harvest day.

The data for mean radicle length of white mustard collected at each harvest day were subjected to analysis of variance. The mean values of each four readings of osmotic potentials from each Petri dish at each harvest day were also subjected to analysis of variance.

Hydroponics. An 18-cm-diameter plastic saucer covered the top of a glass container (Figure 2). The volume of the glass container was periodically increased from a minimum of 0.15 liters to a maximum of 2.5-liter capacity. Hoagland's solution, varying in strength to match plant growth, was used to fill the glass containers. A glass Petri dish, 9 cm diameter and 2 cm in depth, was set on the top of the plastic saucer. There was a 3-cm hole through the glass Petri dish and plastic saucer. Two pieces of 2.5 × 4.0-cm glass were set parallel over the hole of the glass Petri dish, and roots of plant seedlings were placed through the gap between the two pieces of glass into the glass container. The epicotyls of the seedlings were surrounded by a gravel and sand mixture in the glass Petri dish. A RENA 301 air pump was used to pump air into the containers. The container was surrounded by sand in a pot, which had free drainage. Water was added to the sand around the container to avoid over heating.

FIG. 2. Hydroponic system developed to study the release of allelochemicals by intact plants over time. The tube was connected to a RENA 301 air pump, which is not shown in the picture.

The hydroponic system was set up in a glasshouse with natural light and temperature range of 16–27°C. We (Liu and Lovett, 1990) have described the experimental design and reported the response in radicle length of white mustard to growing barley in a hydroponic system. The second part of the experiment is reported here.

Stairstep Apparatus. A stairstep apparatus with four recycling nutrient units (Figure 3) was set up in a glasshouse with a mean temperature of 18°C (range 16–20°C) with natural light supply. The apparatus was developed from an earlier model (Lovett and Jokinen, 1984). Each unit comprised six steps. The nutrient solution from the top reservoir flowed, by gravity, to an incubation tray (57 × 35 × 18 cm) through three lines of pots (15 cm diameter, 14 cm height), with

FIG. 3. A modified stairstep apparatus for assessment of allelopathic effects by barley.

four replications within each set of steps, to a collector tank (50-liter capacity, on the first and sixth steps) from which it was automatically recycled by an electric pump (FP Model, Onga Pump).

Between steps 2 and 3, 3 and 4, and 4 and 5, there was an opaque tube (55 mm diameter, 800 mm long), to collect solutions from the preceding step. The topmost tube was joined to the incubation tray by a 10-mm-diameter black pipe. Connections from this tube to the next level and between lower levels were by 3-mm-diameter black tubes. A bent 3-mm-diameter black tube with a 150° angle was inserted into the opaque tube over each pot for individual solution supply. This bent tube was designed for a fine adjustment of the flow rate of solution by turning the tube clockwise or counterclockwise. The opaque tube was mounted on two steel semicircular supporters; thus, a coarse adjustment of flow rate could be obtained. In this system the flow rate of solution could be varied from 1 to 20 liters/hr.

Three centimeters of gravel (5–10 mm particle size) was sandwiched by two nylon mesh (0.8 mm) liners on the base of the incubation tray. Twelve centimeters of sand (0.7–1.5 mm particle size) covered the nylon mesh. Individual pots were filled in similar fashion and contained 500 g gravel and 2500 g sand. On day 1 the collection tank was filled with 50 liters of half-strength Hoagland's solution. Flow rates were gradually increased from 1 to 6 liters/hr during the experiment and the solution was cycled for 2 hr twice a day. Before each recycling, the electrical conductivity (EC) and pH of the solution were

monitored. A 10% Hoagland's solution was added to each unit collecting tank at day 12 and 12% Hoagland's solution was further added at day 17, in order to maintain the EC range of 1.0–1.5 mS/cm^2. The value of pH did not vary from 6.8 during the period of the experiment.

The treatments consisted of: (a) without barley (control); (b) barley at a density of 0.3 seed/cm^2 sown at the same day as white mustard; (c) barley at a density of 0.3 seed/cm^2 growing for 28 days before introduction of white mustard; and (d) 100 g oven dried plant materials of (c).

Eight seeds of white mustard were sown directly into each test pot and thinned to four seedlings in uniform positions at day 3 after sowing. The white mustard was harvested after growing for 21 days. The plants were separated into leaf, stem, and roots. The leaf area was determined using an electronic planimeter (Paton, Stepney Australia). The fresh and oven dry weights of each component were measured.

RESULTS

Petri Dish Bioassay. Total water uptake during the bioassay was up to 5.5 ml/dish (Table 1). After competition for water uptake, radicle length of white mustard was still significantly inhibited by germinating barley at a density of 0.5 seed/cm^2 or more. The critical density of barley seeds, where such inhibition may begin, was about 0.3 seed/cm^2 (determined by regression curves) under our experimental conditions. The final germination of white mustard with barley seeds was not significantly different from that without barley in general (data not shown), but the time to peak germination (germination rate) of white mustard was delayed by germinating barley. The delayed germination rate was significant, as indicated by the trends of regression equation (Figure 4).

TABLE 1. TOTAL WATER LOSSES, TOTAL AMOUNT OF COMPENSATING WATER ADDED TO SYSTEM, AND EFFECT OF GERMINATING BARLEY ON RADICLE LENGTH OF WHITE MUSTARD IN BIOASSAY

	Density (seed/cm^2)					
	0.00	0.13	0.25	0.50	1.00	2.00
H_2O losses (ml)	0.5	0.8	1.1	1.7	3.0	5.5
Compensation (ml)	0.0	0.2	0.5	1.0	2.9	6.0
Radicle length (mm)[a]	38.5 a	39.7 a	36.4 a	28.8 b	21.8 c	8.9 d

[a] Means of radicle length of white mustard that share a common letter are not significantly different at 5% level, by Student's range test.

Siphoning Bioassay Apparatus. When barley seeds and white mustard seeds were sown together, there were no significant differences between radicle length of white mustard with or without barley at day 1. However, the radicle length of white mustard was significantly ($P < 0.01$) inhibited at days 2, 3, 4, 5, and 6 (Figure 5). Although it was not possible to analyze the variance of the radicle length of white mustard between the different harvests, there was a decrease in radicle length from day 5 to day 6. Some of the mustard roots on day 5 and day 6 were decayed, possibly due to infection by microorganisms.

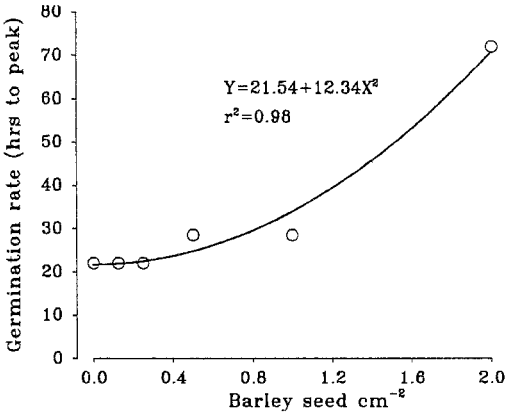

FIG. 4. Germination rates (hours to peak germination) of white mustard as affected by germinating barley seeds on filter paper at increasing densities.

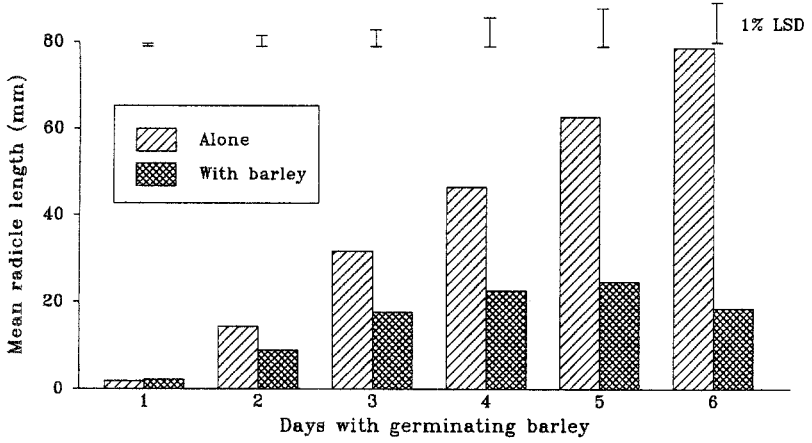

FIG. 5. Radicle lengths of white mustard as affected by germinating barley over time, where competition for water was not a factor.

There were significantly ($P < 0.01$) higher osmotic potentials on the filter paper with barley than on those without barley at all harvests (Table 2). The ranges of osmotic potentials were from −0.33 bars on day 1 to −0.95 bars on day 6, and from −0.83 bars on day 1 to −1.51 bars on day 6 for white mustard alone and white mustard with germinating barley, respectively.

Hydroponics. The inhibition of radicle length of white mustard in bioassay by the hydroponic solution in which barley roots were grown from 7 to 75 days after transplanting was previously reported (Liu and Lovett, 1990). The first-day germination was greatly reduced by hydroponic solution with barley growing in it (Figure 6). Thus, the peak germination of white mustard was delayed. The

TABLE 2. OSMOTIC POTENTIAL ON FILTER PAPER WHERE WHITE MUSTARD GERMINATED WITH OR WITHOUT GERMINATING BARLEY

	Mean osmotic potential (bars)					
	Run 1		Run 2		Run 3	
Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Without barley	−0.33	−0.44	−0.73	−0.87	−0.93	−0.95
With barley	−0.83	−1.07	−1.33	−1.41	−1.49	−1.51
P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
$P < 0.05$ LSD	−0.06	−0.08	−0.10	−0.05	−0.05	−0.07
$P < 0.01$ LSD	−0.09	−0.12	−0.15	−0.08	−0.08	−0.11

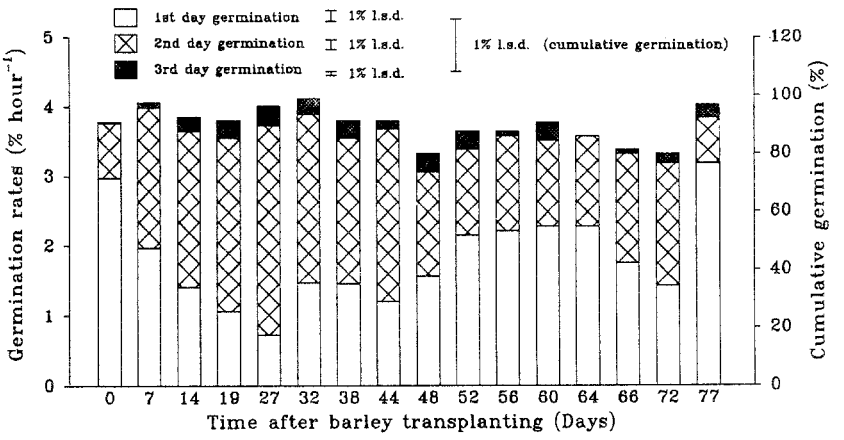

FIG. 6. Germination speed (% hour^{-1}) and cumulative germination (%) of white mustard as affected by allelochemicals from barley growing in a hydroponic system.

effect of germination, in terms of germination per hour at the first day, was not attributable to electrical conductivity, but can be attributed to the effects of allelochemicals released from barley roots (Figure 7).

Stairstep Apparatus. The barley sown simultaneously, sown 28 days before the start of the experiment, and the oven dried barley resulted in reductions of 13%, 45%, and 20% in leaf area, in reductions of 10%, 42%, and 22% in total fresh weight, and in reductions of 10%, 38%, and 24% in total dry weight, respectively (Figure 8). It was clear that barley sown 28 days before the start of the experiment exhibited the greatest inhibition of the growth of white mustard among the treatments.

The ratio of shoot to root dry weight was not significantly different ($P > 0.05$) between control and that associated with barley sown simultaneously (Figure 9). The ratio from the treatment associated with barley sown 28 days before the start of the experiment was lower, and the ratio from that associated with dead barley was higher. This result indicated that the inhibition by barley sown 28 days before introduction of white mustard inhibited shoots more than roots

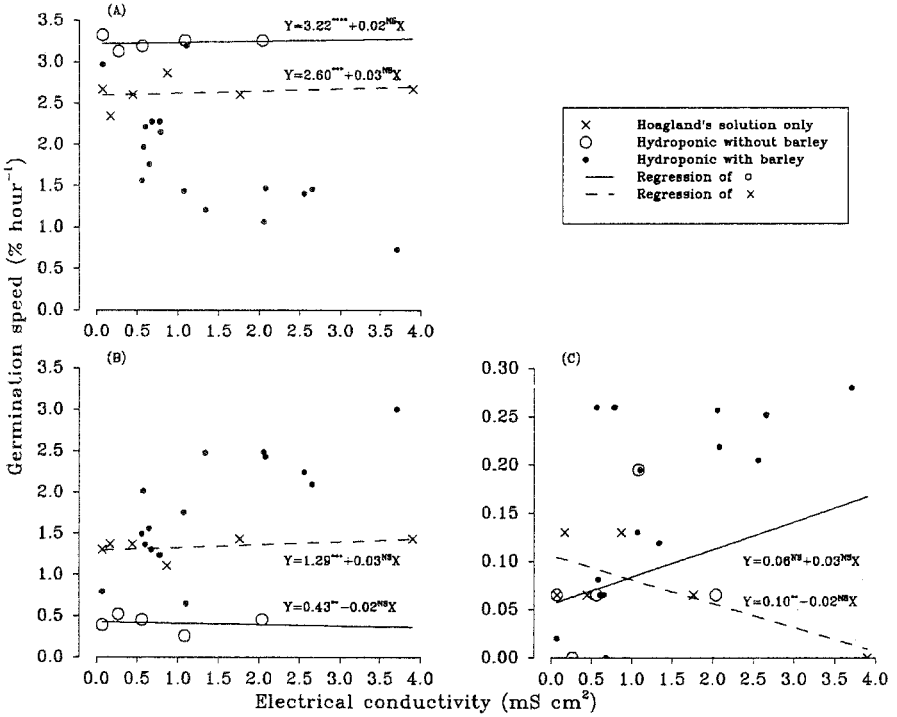

FIG. 7. Relationship between electrical conductivity and germination speed of white mustard seeds at the first day (A), the second day (B) and the third day (C).

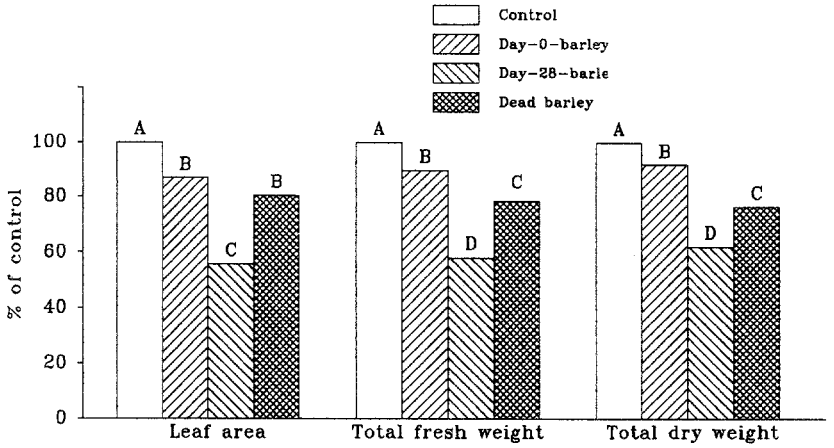

FIG. 8. Allelopathic effects of barley on leaf area, total fresh weight, and dry weight of white mustard after three weeks in a modified stairstep apparatus. The data for control are presented as 100% and the data for the treatments are presented as percent of control after analysis of variance. Any two bars within the same parameter not marked by the same letter are significantly different from each other at the 1% level, by Duncan's multiple-range test.

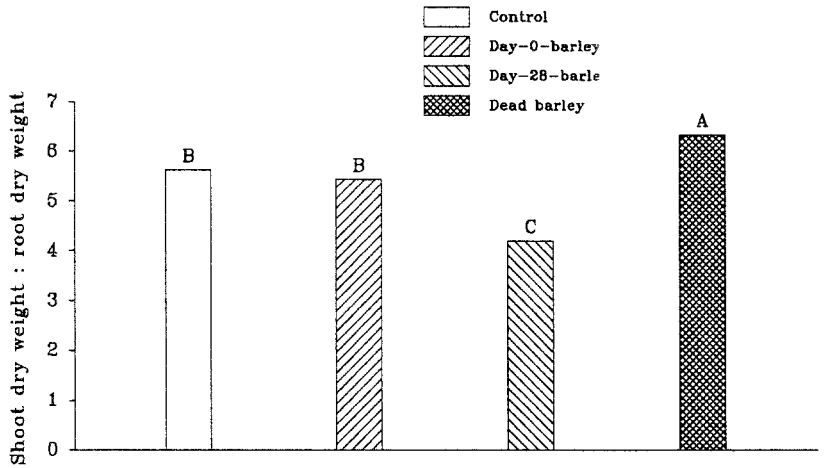

FIG. 9. Allelopathic effects of barley on the ratio of shoot dry weight to root dry weight of white mustard after three weeks in a modified stairstep apparatus. Any two bars not marked by the same letter are significantly different from each other at 1% level, by Duncan's multiple-range test.

of white mustard, while the inhibition by oven dried barley inhibited roots more than shoots. In addition, the seedlings of white mustard associated with the oven dry barley became chlorotic one week after sowing, but this symptom gradually declined two weeks after sowing and disappeared by the harvest.

DISCUSSION

Many of the problems associated with the separation of allelopathy from plant interference can be attributed to a lack of convincing methodology (Fuerst and Putnam, 1983). The common methods used in such studies were reviewed by Liu (1991). The key criteria for developing convincing methods must be that the investigations are on intact plants and that experiments should reasonably simulate the real situation of plant interference. Since numerous compounds are already present within plants (Putnam, 1988), results from, for example, testing the effects of extracted plant materials using solvents or from maceration and grinding of plant materials are certainly unreliable due to the liberation of chemicals that might not be released in nature (Lovett, 1982).

Germinating seeds are able to take up water to 88% of their dry weight for cereal crops and up to 113% of their dry weight for legumes (Alabushev, 1977). Liu (1991) showed that the average water uptake by germinating barley was 83% and 91% of their initial dry weight on filter paper and sand media, respectively. The amount of water absorbed by germinating barley at barley densities of 0.5 seeds/cm^2 or more (Table 1) could certainly alter the water available for other species. Therefore, addition of water to compensate for water depletion by barley is essential in a bioassay when an attempt is made to reduce the physical effects (competition), especially in a limited test medium such as Petri dishes or in a bioassay where the number or size of germinating seeds is large. In the siphoning bioassay apparatus (Figure 1), water was continuously supplied to the system so that the amount of water in each should, theoretically, be the same and any water uptake or evaporation should be automatically compensated. Therefore, this technique enabled conclusive separation of competition for water from allelopathic interference. In addition, since there were no physical barriers limiting the early growth of germination seeds in the open system, a bioassay could be conducted much longer than is permitted by conventional Petri dish techniques.

Allelopathic interference by germinating barley, as previously reported (Overland, 1966; Prutenskaya, 1972), was further confirmed by the modified bioassay technique. Such effect was not attributable to osmotic potentials (Table 2) as these osmotic potentials were higher than could result in an inhibition (Liu, 1991), nor to competition for water as the feature of the system (Figure 1), but

was attributable to allelochemical release by the early seedlings of barley (Liu and Lovett, 1990).

In the field situation, germinating seeds of plants are generally subject to infection by fungi, bacteria, and virus diseases. The results of allelopathic activity of crop species that delay germination (Figures 4, 6, and 7) or reduce radicle elongation of weed seedlings (Figure 5) could complement these stresses, to the advantage of establishing crop seedlings. Since the delayed germination and inhibition of root growth of weeds is a secondary expression of primary disturbance of metabolic activity by germinating seeds (Winter, 1961), the ability of seeds to provide microbial inhibitors may be important (Einhellig, 1987). Decaying roots of white mustard were noted using the siphoning bioassay apparatus suggesting that the germinating seeds were less resistant to disease, including those caused by microorganisms. Therefore, crop plants may exhibit allelopathy as indirect self-defense through interaction with other environmental stresses, and the combined effects of allelopathy and pathogenicity may help to eliminate some weed seedlings.

The aerated hydroponic system (Figure 2) provided a technique to further assess phytotoxic activity. In this system plant roots and nutrients were in the dark. Plant stands were supported by the gravel and sand mixture, thus simulating field conditions despite the roots being in the solution. Since hydroponic solution without barley did not interfere with seedling growth of white mustard, the inhibition of white mustard was attributable to the presence of barley roots. Interference by competition for plant nutrients was eliminated in this system but inhibition of white mustard was observed up to 75 days (Liu and Lovett, 1990). The results strongly suggest that inhibition of white mustard occurs as a result of the release of allelochemicals by barley and that such release is not confined to the seedling stage.

The stairstep apparatus (Figure 3) was built with opaque materials, thus keeping it free from algal growth, as observed by Lovett and Jokinen (1984). The potential for adjustment provided uniform delivery of solution over a range of flow rates. Thus, flow rate could be adapted to different experimental needs and to the changing water requirements of plants at different stages of growth. Further evidence for allelopathic effects of living barley was found using the modified stairstep apparatus. Barley, already growing for 28 days, exhibited more inhibition of the seedling growth of white mustard than barley sown simultaneously with white mustard (Figures 8 and 9).

Overall, separation between allelopathy and competition is the most difficult task in the studies of plant interference. In our studies, attempts have been made to develop convincing techniques for such separation. These techniques, at least in part, simulate plant growth in nature and have significant advantages over conventional methods.

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BIOLOGICALLY ACTIVE SECONDARY METABOLITES OF BARLEY. II. PHYTOTOXICITY OF BARLEY ALLELOCHEMICALS

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Abstract—The release of alkaloids by barley was quantified by HPLC. Hordenine was released from the roots of barley in a hydroponic system for up to 60 days. The amount reached a maximum, 2 μg/plant/day, at 36 days, then declined. Effects on white mustard by hordenine and gramine included reduction of radicle length and apparent reduction in health and vigor of radicle tips. Transmission electron microscopic examination of white mustard radicle tips exposed to hordenine and gramine showed damage to cell walls, increase in both size and number of vacuoles, autophagy, and disorganization of organelles. The evidence of the morphological and primary effects of barley allelochemicals at the levels released by living plants indicates that the biologically active secondary metabolites of barley may lead to a significant role in self-defense by the crop.

Key Words—Allelopathy, allelochemicals, phytotoxin, gramine, hordenine, HPLC, TEM, micrograph, autophagy, barley, *Hordeum vulgare*, *Sinapis alba*.

INTRODUCTION

A few well-documented examples of primary effects of alkaloids on associated plants are known. Waller and Burstrom (1969) reported that diterpenoid alkaloids of *Delphinium ajacis* L. (larkspur) inhibited internode growth in *Pisum sativum* L. (pea), possibly through interference with the synthesis of gibberellins. Olney (1968) showed that veratrum (Liliaceae) alkaloids inhibited the growth of *Avena sativa* L. (oats) and *Secale cereale* L. (rye), apparently through

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a specific effect on DNA stability. Tropane alkaloids, scopolamine and hyoscyamine, are present in the leachates of seeds and foliage of *Datura stramonium* L. (Lovett et al., 1981) and soil of *D. stramonium*-infested fields (Levitt and Lovett, 1984). These compounds inhibited the growth of *Helianthus annuus* L. seedlings, possibly by interference with starch hydrolysis (Levitt et al., 1984). Lovett and Potts (1987) tested the hypothesis that the inhibition of early seedling growth of some crop plants by scopolamine was due to interference with gibberellin-stimulated food reserve metabolism. However, in germinating barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.), they found no evidence of interference with gibberellin activity, although seedling growth of both cereals was inhibited by the alkaloids at the levels tested (Lovett and Potts, 1987). Therefore, no conclusions as to the mechanism of seedling growth of inhibition by scopolamine and hyoscamine could be drawn.

Two alkaloids, gramine and hordenine, have been suspected of contributing to allelopathy by barley (Overland, 1966). Gramine is a constituent of barley leaves and is present in several barley cultivars, reaching concentrations up to 8 mg/g dry weight (Hanson et al., 1981) at day 12 after germination, then slowly declining (Schneider and Wightman, 1974). Gramine was reported as a factor in the resistance of several barley cultivars to the aphids *Schizaphis graminum* and *Rhopalosiphum padi* (see, for example, Zuniga and Corcuera, 1986). Gramine is not present in barley seeds and roots (Schneider and Wightman, 1974). Hordenine is absent from barley seeds but appears in the roots from the first day of seed germination (Mann et al., 1963). Overland (1966) reported that both gramine and hordenine inhibited the growth of various plants including *Stellaria media* (L.) Cyr., *Capsella bursa-pastoris* (L.) Medic., and *Nicotiana tabacum* L. under controlled conditions.

Allelopathic activity of barley has been confirmed in the present work (Liu and Lovett, 1993). Liu and Lovett (1990) reported that barley seedlings released gramine and hordenine into a bioassay system, and peak concentrations of 22 ppm gramine and 48 ppm hordenine were quantified by GC-MS from washings of filter paper after barley seed germination for four days. However, the recovered concentrations declined following a peak under the bioassay system where no nutrients were supplied. Thus, it is unknown if such release could be continuous by living plants growing with adequate nutrient and other resources.

The objective of the present experiments was to identify and quantify gramine and hordenine during the growth of barley seedlings and to monitor the effects of these compounds on white mustard (*Sinapis alba* L.).

METHODS AND MATERIALS

Chemicals. All organic solvents used for high-performance liquid chromatography (HPLC) were HPLC grade. Chloroform used in the concentration procedure was purified for reuse by redistillation. Gramine and hordenine

hemisulfate were purchased from Sigma Chemical Co. Pure hordenine was obtained by dissolving the product in 5 : 1 0.1 N sulfuric acid–ammonia solution (pH 11), partitioning with chloroform, and removing the chloroform in a stream of dry nitrogen.

Quantification of Gramine and Hordenine Released by Barley at Different Stages of Growth and Development. Three 5-day-old barley (*H. vulgare* cv. Triumph) plants were transplanted to a hydroponic system as described by Liu and Lovett (1993) for up to 60 days. Samples were collected at 5- to 10-day intervals. On the sampling day, total volume of solution in each container was measured and passed through a 0.2-μm super membrane filter (Gelman Sciences). Samples (100 ml) were concentrated using the methods described by Lovett and Potts (1987). About 85 ml of the chloroform fraction was collected and evaporated to 5 ml in a water bath at 45°C. The condensed organic phase was further evaporated in a stream of dry nitrogen to dryness. The dry residue was diluted by 0.5 ml HPLC mobile phase (see later paragraph) before analysis.

HPLC was performed with a Waters Associates Liquid Chromatograph equipped with a model 680 automated gradient controller, Waters LC M-45 Solvent Delivery system, a Lambda-Maxmodel 481 LC spectrophotometer set at 219 nm, and a Waters 745 data module set at 1 mm/min. A μBondapak C_{18} reversed-phase column was used throughout the study.

Ten microliter solutions of reference compounds and samples were injected into the column using a SGE injector. The solvent was mixed by the dual pumping system in the proportion of 60% 0.05 M KH_2PO_4 pulsing triethylamine (TEA) and 40% CH_3CN at pH 7.65. Solvent flow rate was 2 ml/min. After each sample analysis, mobile phase was injected one or two times to clean up the injector.

Effect on Root Elongation of White Mustard by Gramine and Hordenine. Standard solutions of gramine and hordenine were prepared. Concentrations of each alkaloid were set at three levels, namely, 0 ppm, 15 ppm (8.61×10^{-2} mM for gramine and 9.08×10^{-2} mM for hordenine), and 50 ppm (2.87×10^{-1} mM for gramine and 3.03×10^{-1} mM for hordenine). The experimental design was a 3×3 factorial, where the 0 ppm gramine + 0 ppm hordenine were treated as controls. Seeds of white mustard were surface-sterilized with 1.25% (w/v) sodium hypochlorite solution for 2 min before sterile distilled water rinsing; 10 seeds of white mustard were placed on filter paper in 9-cm-diameter sterile Petri dishes with one Whatman No. 1 filter paper to which were added 3 ml of the appropriate solution. Seeds were incubated in the dark at 25°C for three days after which the length of the radicles was measured (Liu and Lovett, 1990).

The data were converted to percentage of reduction from the control and the synergistic or antagonistic responses of the two alkaloids calculated by the method introduced by Colby (1967):

$$E = I_g + I_h - \frac{I_g I_h}{100}$$

where I_g is the percent inhibition of growth by gramine at concentration C_g and I_h is the percent inhibition of growth by hordenine at concentration C_h. E is the expected percent inhibition of growth by gramine + hordenine at the concentration of $C_g + C_h$. When the observed response is greater than expected, the combination is synergistic; when less than expected, it is antagonistic.

Effect of Barley Alkaloids on Root Tip Ultrastructure of White Mustard. The experiment was conducted under axenic conditions. Ten surface-sterilized white mustard seeds were sown in 9-cm glass Petri dishes on filter paper moistened with 3 ml of solutions at concentrations of 0 ppm (sterilized distilled water for control), 22 ppm gramine, 48 ppm hordenine, 100 ppm gramine and 100 ppm hordenine, with three replications. A further 1 ml appropriate solution was added to each dish on day 3. The seeds were germinated in an incubator for four days at 25°C in the dark.

The techniques for processing material for transmission electron microscopy (TEM) were as outlined by Liu and Lovett (1990). More than 20 sections from each root tip were examined. The ultrastructure of the third or fourth layers of the cells of root tips from both control and treatments were recorded on micrographs.

RESULTS

Alkaloids Released by Barley in a Hydroponic System. Results of a preliminary experiment showed the presence of gramine and hordenine in samples from the hydroponic system. However, gramine was too low to be quantified. Hordenine was released into the hydroponic system up to 60 days (Figure 1). The amount reached a maximum at 36 days, then decreased. The highest amount of hordenine recovered was 2 μg/day from one barley plant.

Gross Morphological Effects of Barley Alkaloids. Gramine and hordenine inhibited radicle elongation of white mustard at concentrations of 50 ppm or more. Combinations of gramine and hordenine showed a synergistic action (Table 1), according to equation 1 defined by Colby (1967). The values of synergism were higher when the two alkaloids were combined in equal concentrations.

Effect of Barley Alkaloids on Root Tip Ultrastructure of White Mustard. Cells of white mustard root tips from sterile distilled water showed distinct nuclei with intact nuclear membranes; intact organelles, including mitochondria; small vacuoles; and uniform cell walls (Figure 2).

Cells from the treatment with 22 ppm gramine also showed distinct nuclei and normal cell walls (Figure 3). However, more vacuoles appeared in these cells, compared to control. It was noted that some of the vacuoles were tending

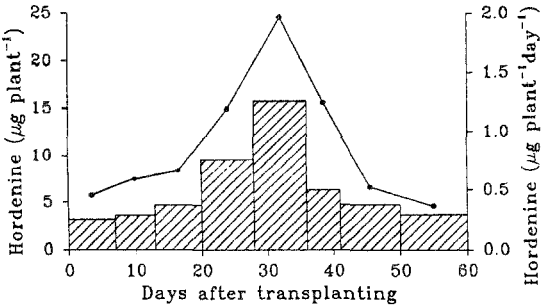

FIG. 1. Amount of hordenine released by barley in a hydroponic system. Histogram for hordenine per plant in each sample interval and line for hordenine released by one barley plant per day.

TABLE 1. EFFECT OF COMBINATIONS OF GRAMINE AND HORDENINE ON RADICLE LENGTH OF WHITE MUSTARD

Gramine (ppm)	Hordenine (ppm)	Radicle length (mm ± SE)[a]	Reduction observed (%)	Reduction expected (%)	Difference[b]
0	0	38.0 ± 1.4 a	0		
0	15	37.6 ± 0.9 ab	1.2		
0	50	35.5 ± 1.6 bc	6.6		
15	0	38.6 ± 0.8 a	−1.6[c]		
50	0	35.2 ± 1.3 bc	7.3		
15	15	34.7 ± 2.1 c	8.8	−0.4	+9.2
15	50	34.4 ± 2.1 c	9.5	0.5	+4.4
50	15	33.8 ± 1.7 c	11.1	8.4	+2.7
50	50	29.6 ± 0.7 d	22.0	13.4	+8.6

[a] Means sharing a common letter are not significantly different at 5% level, by LSR.
[b] Differences (the % reduction observed minus % reduction expected) with a plus sign indicate synergistic effect.
[c] A minus sign indicates stimulation compared to the distilled water control.

to join together and might become giant vacuoles (typically, see Figure 3a and c).

Cells from the treatment with 48 ppm hordenine showed similar results for nuclei, which appeared to be intact, but had more and larger vacuoles (Figure 4), compared to the control. There was evidence also of damaged cell walls (Figure 4a and b) and giant vacuoles, which contained some membrane fragments from other organelles (Figure 4c).

In the treatments with both 100 ppm gramine and hordenine, a further increase in vacuole size occurred, with evidence of distinct autophagy (typically,

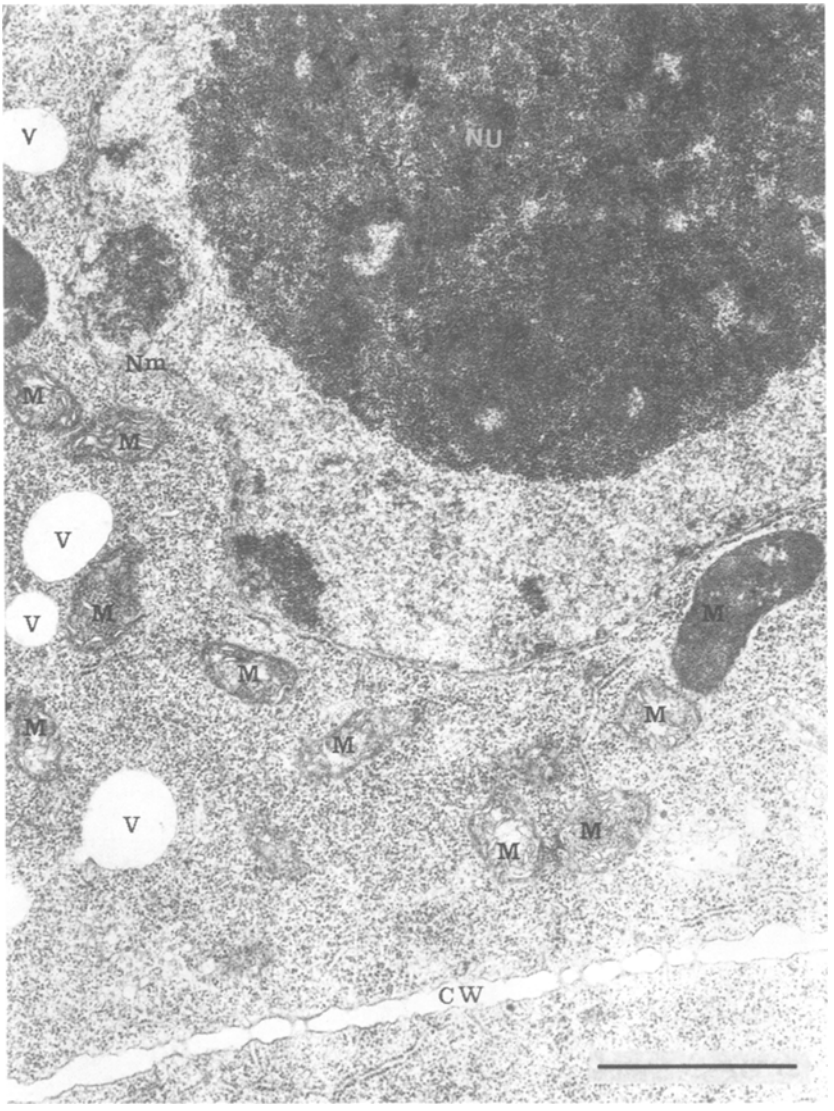

FIG. 2. Transmission electron micrograph of the cells of white mustard root tips four days after commencement of germination; sterile distilled water. Scale bar = 1 μm. CW = cell wall; V = vacuole; M = mitochondrion; N = nucleus; NU = nucleolus; Nm = nuclear membrane.

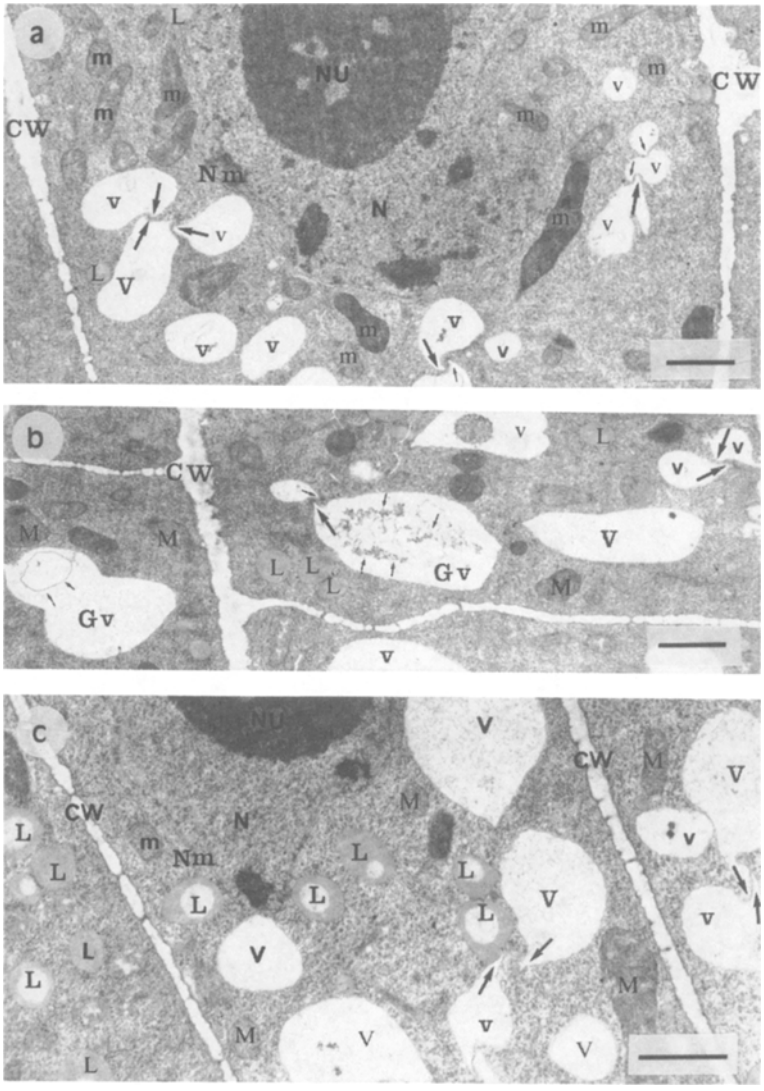

FIG. 3. Transmission electron micrographs of the cells of white mustard root tips four days after commencement of germination; 22 ppm gramine. Scale bar = 1 μm. CW = cell wall; V or v = vacuole; Gv = giant vacuole; L = lipid; M = mitochondrion; N = nucleus; NU = nucleolus; Nm = nuclear membrane; arrows indicate development of giant vacuoles.

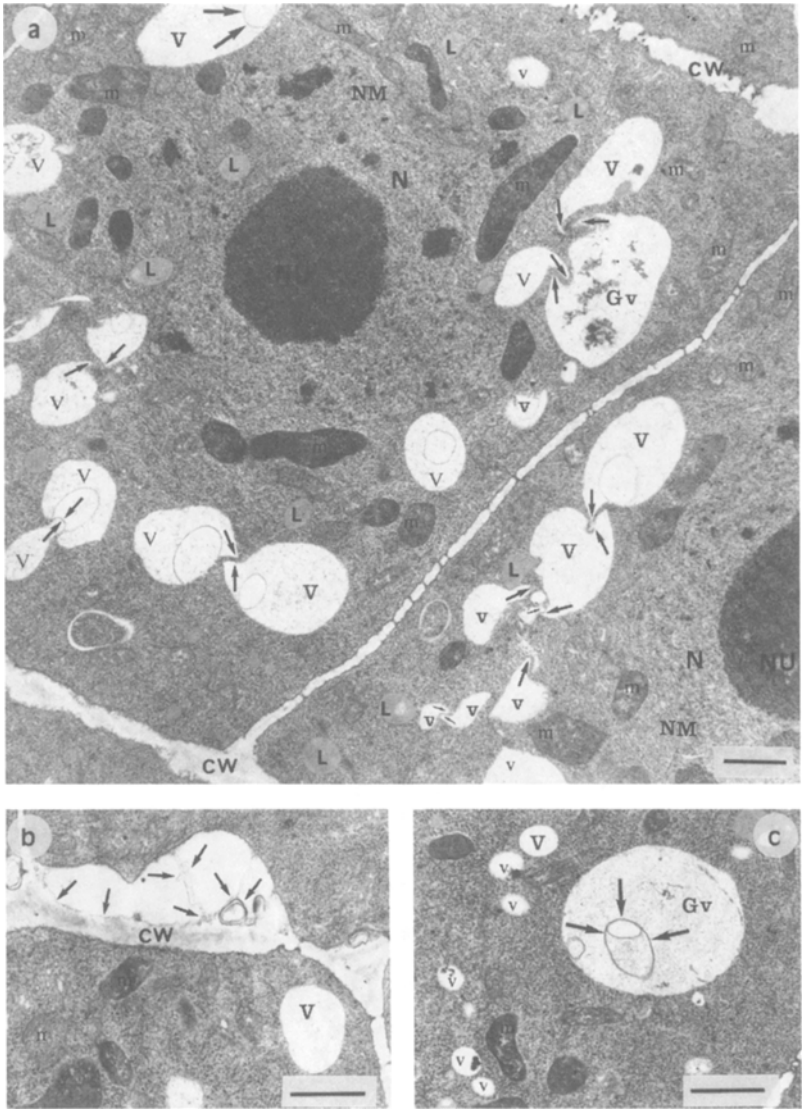

FIG. 4. Transmission electron micrographs of the cells of white mustard root tips four days after commencement of germination; 48 ppm hordenine. Scale bar = 1 μm. CW = cell wall; V or v = vacuole; Gv = giant vacuole; L = lipid; M or m = mitochondrion; N = nucleus; NU = nucleolus; Nm = nuclear membrane; arrows indicate the development of giant vacuoles and autophagy.

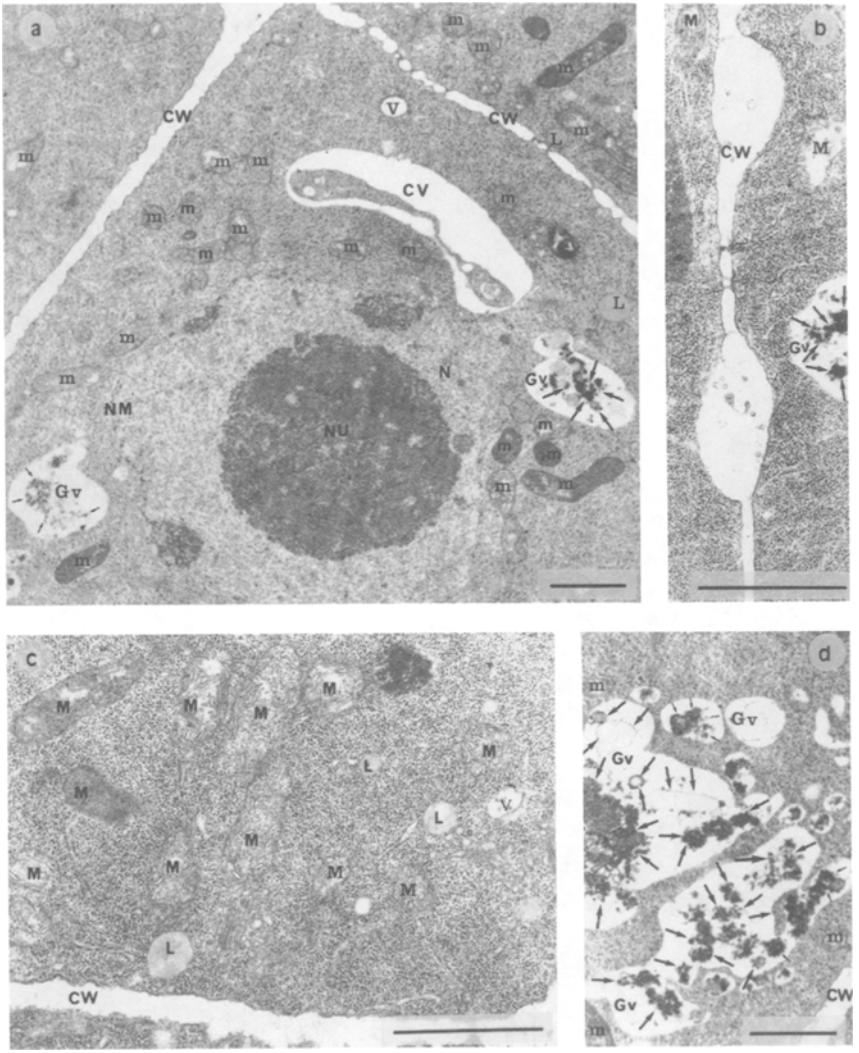

FIG. 5. Transmission electron micrographs of the cells of white mustard root tips four days after commencement of germination; 100 ppm gramine. Scale bar = 1 μm. CW = cell wall; V = vacuole; Gv = giant vacuole; L = lipid; M = mitochondrion; N = nucleus; NU = nucleolus; Nm = nuclear membrane; arrows indicate autophagy.

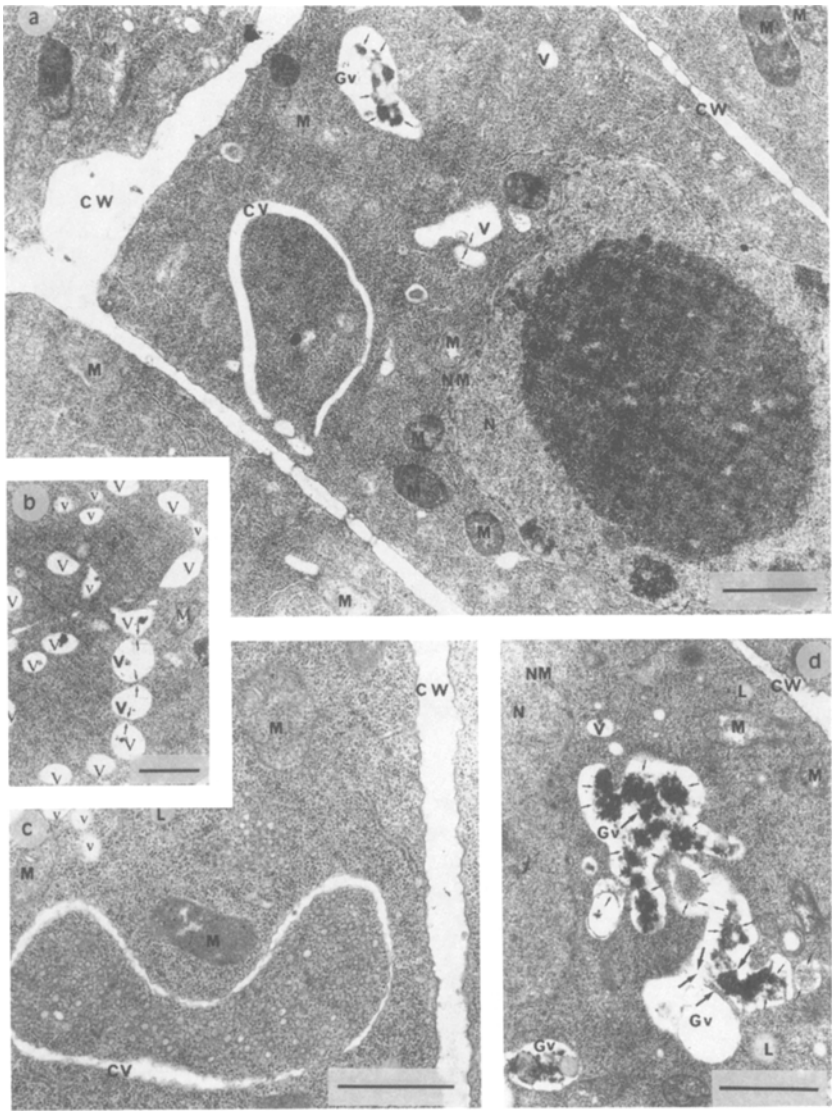

FIG. 6. Transmission electron micrographs of the cells of white mustard root tips four days after commencement of germination; 100 ppm hordenine. Scale bar = 1 μm. CW = cell wall; V or v = vacuole; Gv = giant vacuole; Cv = circular vacuole; L = lipid; M = mitochondrion; N = nucleus; NU = nucleolus; Nm = nuclear membrane; arrows indicate the development of giant vacuoles and autophagy.

see Figures 5d and 6d). At this concentration there were some circular vacuoles (Figures 5a, 6a and c), which might be created from individual small vacuoles (Figure 6b). In addition, some cell walls appeared to be damaged and some mitochondria showed evidence of disorganization (Figures 5 and 6). The nucleolus appeared normal, although there was evidence of less distinct nuclei and damaged nuclear membranes (Figure 6d).

It was noted that many lipids appeared in the treatments (Figures 3 and 4), as compared with the control (Figure 2). In general, at the concentrations of alkaloids used in these studies, there was evidence of disorganization but not of gross levels of damage.

DISCUSSION

In the bioassay of the combined effect of gramine and hordenine, a synergistic interaction occurred. This agrees with the work of Einhellig and coauthors (Rasmussen and Einhellig, 1979; Einhellig et al., 1982), who tested a number of phenolic allelochemicals and demonstrated their synergistic effects.Rasmussen and Einhellig (1979) found that an equimolar combination of 3.3 mM with any two of ferulic, *p*-coumaric, and vanillic acids showed more inhibition of sorghum seed germination than the two allelochemicals combined at different concentrations. In addition, the equimolar combination also depressed seed germination more than a combination at different concentrations. In the combination of gramine and hordenine, the synergistic effects of equal concentrations were higher than unequal concentrations (Table 1).

The evidence from electron microscopy showed the effect of allelochemicals on the ultrastructure of root tip cells of white mustard, including increases in both size and number of vacuoles (Figures 3–6). Matile (1978) reviewed the work in which the vacuoles of higher plants play fundamental roles in: (1) storage pools of intermediates such as enzymes, inorganic ions, primary and secondary metabolites; (2) regulation of turgor; and (3) detoxification. Matile (1976) discussed the detoxification of potentially toxic plant metabolites and concluded that cells protect themselves from compounds such as poisonous alkaloids by sequestering them into the vacuoles.

In our experiments, cells treated with high concentrations of alkaloids (Figures 5d and 6d) showed an autophagic phenomenon (Matile, 1975, 1976, 1978; Matile and Wiemken, 1976), which is similar to phagocytosis in animal cells. This phenomenon has been considered as either representing the autophagic elimination of damaged organelles (Villiers, 1971) or the beginning of senescence of the cells (Matile, 1975, 1976; Matile and Wiemken, 1976). The latter cannot explain the results in our experiments since cells of the same age and location were compared between the control and alkaloid treatments. In the

process of autophagy, portions of cytoplasm, including damaged mitochondria, are encircled by a number of small vacuoles that ultimately fuse together and form a giant vacuole (Figures 5a, b, d, and 6). This process may represent an active and energy-dependent response to damage (Matile and Wiemken, 1976).

It has been noted that plants take up alkaloids from soil by processes similar to those in nutrient uptake (Winter, 1961). Investigations have shown that alkaloids can be freely taken up by higher plants, including alkaloid-free plants (Winter, 1961) or lower plants (Schneider and Stermitz, 1990). Lovett et al., (1981) suggested that allelopathy and responses to allelochemicals are part of a network of plant chemical communication. Plants may produce alkaloids as defensive chemicals to microorganisms and other plants; the alkaloids in the cells of a producer can be stored, released, or detoxified. Plant vacuoles are a principal place for storage or detoxification. There are at least two advantages for alkaloids entering vacuoles. First, the alkaloids can be isolated to avoid contact with other organelles that may be damaged, as observed in the cells of root tips of white mustard treated with gramine (Figures 3 and 5) and hordenine (Figures 4 and 6). Second, alkaloids stored in vacuoles can be used as self-defense chemicals to insects or other predators.

Recent results on alkaloid uptake in isolated vacuoles contradict the classic alkaloid accumulation model (Deus-Neumannand Zenk, 1986). Vacuoles of alkaloid-containing plants take up only those alkaloids that are specific to this particular plant, while vacuoles of alkaloid-free plants do not take up alkaloids (Deus and Zenk, 1982; Deus-Neumann and Zenk, 1984). Thus it would be expected that toxic alkaloids may damage the organelles in cells of such plants and may, for example, cause dysfunction of enzyme systems (Benoit and Starkey, 1968, as cited in Rice, 1984).

The appearance of many lipids after the treatment of root tips with gramine and hordenine in this experiment may be evidence of disruption of food metabolism (Figures 3 and 4), similar to the effects of benzylamine on root tip cells of *Linum usitatissimum* L. (Lovett, 1982). The effects of the tropane alkaloids, scopolamine and hyoscamine, on root tips of *Helianthus annuus* were the accumulation of lipid droplets and the abundance of amyloplasts (Levitt et al., 1984), indicating a general slowing down of the metabolism of food reserves. During the course of germination, the reserve foods, such as starch and lipids, are enzymatically degraded to low-molecular-weight carbohydrates for further metabolic utilization (Bonner and Varner, 1976). Therefore, retarded food metabolism may result from the dysfunction of enzymes by allelochemicals, as previously discussed. Hence, although the allelochemicals present are insufficient to cause the death of the seedlings, their metabolism is adversely affected (Levitt et al., 1984), consequently, a slow germination (Liu and Lovett, 1993) and short root length (Liu and Lovett, 1990) could be observed.

For studies of primary effects of allelochemicals, transmission electron

microscopy can be used to show direct evidence of such effects. When seedlings of white mustard were treated with 22 ppm gramine, there was a substantial response in the cells, typically, increases in the number and size of vacuoles (Figure 3). The same amount of alkaloids in bioassay could be expected to produce nonsignificantly different radicle lengths of white mustard (Liu, 1991), compared to cells from control. Therefore, when measuring a secondary effect, the noneffect (0% reduction) following stimulation cannot be interpreted as a true noneffect, but may only be expressed as "no difference" in a secondary indicator.

In our work, the release of the two alkaloids, gramine and hordenine, has been established in the early seedling stage (Liu and Lovett, 1990) and in the later stage from a barley growing environment (Figure 1). The effects of these alkaloids at the levels quantified have been demonstrated at both gross morphological and ultrastructural levels. The results suggest that biologically active secondary metabolites of barley may play a significant role in self-defense by the crop.

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BIOLOGICALLY ACTIVE SECONDARY METABOLITES OF BARLEY. III. A METHOD FOR IDENTIFICATION AND QUANTIFICATION OF HORDENINE AND GRAMINE IN BARLEY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract—A method was devised for the extraction and quantification of hordenine and gramine from barley (*Hordeum vulgare*) tissue using HPLC techniques. Quantification was by peak area, the relationship between peak area and concentration of authentic standards being linear for both hordenine and gramine. Significant differences in the ability of three lines of barley to produce hordenine and gramine were detected using this method.

Key Words—Barley, *Hordeum vulgare*, allelochemicals, hordenine, gramine, quantification, HPLC, cultivars.

INTRODUCTION

Hordenine (*N,N*-dimethyltyramine) and gramine (*N,N*-dimethylindolemethylamine) have been isolated from barley (Bowden and Marion, 1951; Leete et al., 1952; Leete and Marion, 1953; Rabitzsch, 1959; Steinhart et al., 1964; Schneider et al., 1972; Hanson et al., 1981; Lovett and Liu, 1987; Liu and Lovett, 1990) and have been implicated in being, at least in part, responsible for barley's vigor in competition with other species (Overland, 1966; Liu and Lovett, 1990). However, Hanson et al. (1981) failed to detect gramine in all barley cultivars they investigated, and others (Kirkwood and Marion, 1950) found that *N*-meth-

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yltyramine (the immediate precursor of hordenine) was present in greater quantities in the roots of some cultivars than hordenine itself.

Working on the tryptamine-based alkaloids of reed canary grass (*Phalaris arundinacea*) (of which gramine is one), Marum et al. (1979) found that the inheritance of gramine was controlled by two recessive genes, and they postulated that it would be possible to breed lines capable of producing this alkaloid. In a series of papers published in the 1950s and 1960s, a Canadian group (e.g., Massicot and Marion, 1957; Gower and Leete, 1963) elucidated the pathways of gramine and hordenine synthesis in barley. From this work, and on the basis of "one gene–one enzyme," it should also be possible to breed barley lines with a high alkaloid content, a desirable end if a defensive role for such compounds is accepted (Lovett et al., 1989).

To facilitate the identification of possible parents with a high alkaloid content, a rapid, reliable method of estimating the quantity of gramine and hordenine in plant samples is necessary.

To date, high-performance liquid chromatography (HPLC) has been used extensively to identify and quantify alkaloids of many types, including hordenine and gramine (Kohl et al., 1983; Renaudin, 1984; van der Heijden et al., 1987; Liu and Lovett, 1990), both quickly and accurately. Liu and Lovett (1990) isolated and quantified hordenine and gramine from hydroponic solutions in which barley (cv. Triumph) had been grown, and sample purification and concentration was achieved by partitioning against chloroform, a relatively time-consuming, expensive, and hazardous method. Further, the HPLC quantification method used a mobile phase of relatively high molarity and pH, both of which shorten column life. Johansson and Schubert (1990) described a method for the quantification of hordenine by HPLC but expressed dissatisfaction with the detection component. We have found no published method for the quantification of gramine by HPLC and no sample preparation method using sample preparation cartridges, e.g., the Water's Sep-Pak range.

We describe in this paper methods for the extraction of hordenine and gramine from barley root and shoot tissue, extract purification and concentration, and some amendments to the methods of HPLC quantification that have been previously reported.

METHODS AND MATERIALS

Standard solutions of authentic hordenine and gramine hemisulfate (Sigma Chemicals) of 5, 10, 15, and 20 ppm and 2.5, 5.0, 7.5, and 10.0 ppm respectively, were prepared.

Barley seedlings, cvv. Skiff and Proctor and line CI400117, were grown to the stage of one fully expanded leaf in a growth cabinet (12-hr day, 8–18°C

temperature regime). Fifty grains of each variety were sown in sand in 20-cm pots replicated three times on three separate occasions (blocks A, B, and C). The seedlings were separated into roots and shoots and the remains of the grain discarded. The alkaloids were extracted from the frozen material by grinding approximately 1.5-g samples in a mortar with 30 ml 0.01% acetic acid (Renaudin, 1985).

After standing for 24 hr at room temperature, the extracts were then filtered through glass wool, the pH adjusted to 9.15 and centrifuged at 3000 rpm for 5 min. Ten-milliliter aliquots of the extracts and 1-ml aliquots of the standards were applied to prepared Sep-Pak C_{18} cartridges (Waters Associates). Two replicates of each standard, pH adjusted to 9.15, were processed. The method of purification for gramine and shoot samples was based on that of van der Heijden et al. (1987) with minor modifications, viz.

Acetonitrile (ACN) 2 ml

↓

0.001 M KH_2PO_4 pH 7 2 ml

↓

aliquot applied

↓

0.05 M KH_2PO_4 pH 9.5/isopropanol (70:30) 2 ml

↓

0.05 M KH_2PO_4 pH 9.5/isopropanol (95:5) 2 ml

↓

0.05 M KH_2PO_4 pH 2.3/isopropanol (70:30) 1.5 ml

Hordenine was not adequately retained using this method and the following procedure was developed:

ACN 2 ml

↓

0.001 M KH_2PO_4 pH 7 2 ml

↓

aliquot applied

↓

0.05 M KH_2PO_4 pH 9.5/isopropanol (70:30) 2 ml

↓

0.05 M KH_2PO_4 pH 2.3/isopropanol (70:30) 1.5 ml

The final eluates were evaporated to dryness under nitrogen at 40°C and taken up in 1 ml mobile phase. Recovery of alkaloid from each batch of root and shoot extracts purified was checked by concurrently "purifying" a 10-ml aliquot of 1 ppm hordenine and gramine, respectively.

Ten-microliter aliquots of each purified standard and plant sample were injected into a Waters HPLC system consisting of an M40 pump, flow rate 2 ml/min; U6K injector; UV/VIS spectrophotometer, wavelength 221 nm, and the results recorded as peak areas using a Waters 745 data module. The column used was a Water's μBondapak Phenyl 10 μ (3.9 mm × 30 cm) and the mobile phase was 0.025 M KH_2PO_4 + 0.1% TEA (triethylamine), pH 7.15/ACN (2:1 for hordenine and root samples) (6:4 for gramine and shoot samples). Duplicate injections were made for each sample. Identification of hordenine and gramine in plant samples was by retention time relative to authentic compound and confirmed by coelution of representative samples with authentic compound. The presence of hordenine and gramine in barley material has previously been confirmed by GC-MS (Liu and Lovett, 1990).

RESULTS AND DISCUSSION

Methanol and ethanol have commonly been used for the extraction of alkaloids from plant tissue (Massicot and Marion, 1957; Schneider et al., 1972; Renaudin, 1984); however, when compared with extraction with 0.01% acetic acid, very low recovery rates of the alkaloids were achieved by us using this method. For example, when root tissue was extracted in either methanol or 0.01% acetic acid spiked with 2 ppm hordenine, the proportion of the hordenine spike recovered from the methanol extraction was 0.5%, whereas that from acetic acid extraction was 75%. Recovery from unspiked root tissue was similarly related. Reextraction of the samples in their respective solvents failed to increase total recovery by more than 5% and extraction in double the quantity of solvent gave similar results. It is probable, therefore, that small quantities of hordenine remained adsorbed on the root tissue and that, in the case of methanolic extraction, hordenine was not retained on the Sep-Pak cartridges. Data on the recovery of gramine and hordenine standards from the sample preparation protocol are presented in Table 1. Recovery of hordenine ranged from 97.9% to 109% and of gramine from 87.7% to 96%. These figures are consistent with the literature (Renaudin, 1985; van der Heijden et al., 1987) and are considered acceptable.

TABLE 1. RECOVERY OF HORDENINE AND GRAMINE FROM SEP-PAK PROCEDURES

Hordenine			Gramine		
Concn. (ppm)	Peak area	Recovery (%)	Concn. (ppm)	Peak area	Recovery (%)
5.0	102,936	109.0	2.5	233,098	87.7
	100,948	106.9		236,722	89.1
10.0	199,672	105.8	5.0	506,664	95.3
	186,629	98.9		493,887	92.9
15.0	277,231	97.9	7.5	722,800	90.7
	382,474	100.1		728,126	91.3
20.0	374,728	98.2	10.0	1,020,395	96.0
	370,886	98.2		1,004,719	94.5
10.0 Untreated std.	188,788[a]		3.0 Untreated std.	317,926[a]	

[a] Mean of four injections.

Liu and Lovett (1990) successfully used HPLC techniques to quantify hordenine and gramine released by barley into hydroponic solution; however, a Waters μBondapak 10 μ C_{18} column and a mobile phase consisting of 0.05 M KH_2PO_4 + 0.1% TEA, pH 7.65/ACN (60:40) were used. When this method was modified by using the μBondapak Phenyl 10 μ column (3.9 mm × 30 cm) recommended by Waters for the analysis of basic compoundsand a mobile phase with the molarity and pH of the buffer reduced to 0.025 M and 7.15, respectively, resolution of peaks at a flow rate of 2 ml/min was close to the baseline (Figure 1) quantification of both hordenine and gramine and, therefore, was acceptable. An added advantage of this method was reduced retention times (e.g., 3 min as opposed to 4.5 min for hordenine) (Liu, 1991). Up to 1500 injections of these alkaloid samples have been processed through one column, which is considered in the upper range for a system using buffered mobile phases.

When standards of authentic hordenine and gramine were injected directly into the HPLC, the relationship between concentration and peak area was found to be linear over the ranges tested ($r = 0.9994$ and $r = 0.9999$, respectively) as was the relationship with peak height. Regression analysis of the data presented in Table 1 for the recovery of the alkaloids from the purification protocols, excluding the untreated standards, confirmed this relationship for both hordenine and gramine ($r = 0.9993$ and $r = 0.9989$, respectively). As the purification protocol was in each case carried out across a batch of eight car-

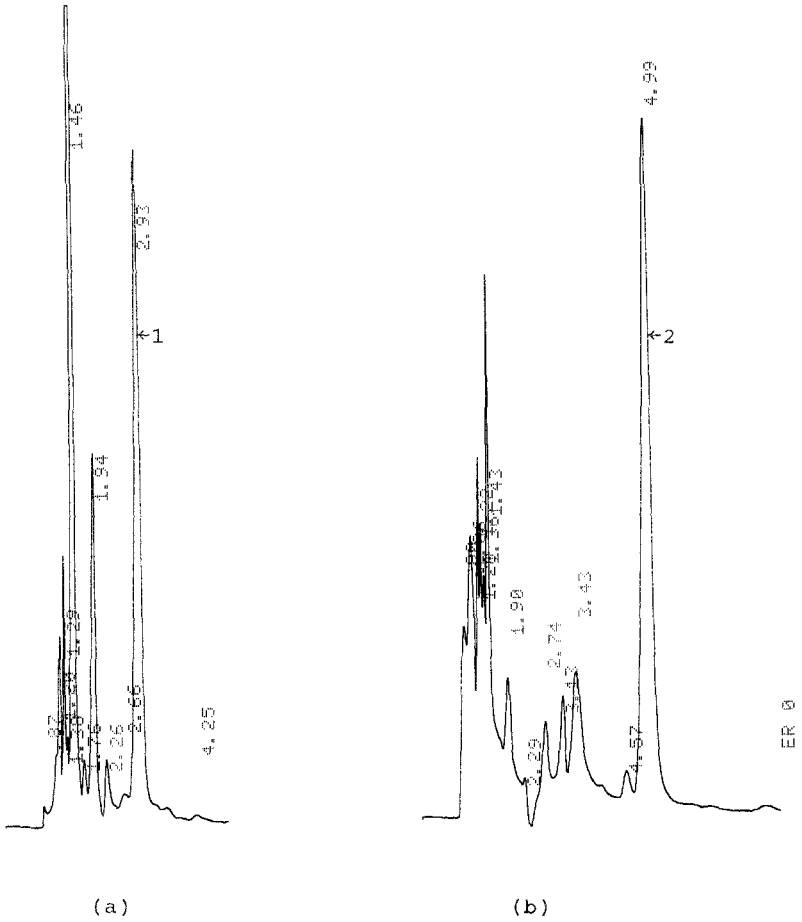

FIG. 1. Chromatographs showing separations of hordenine (a) and gramine (b) from barley root and shoot material respectively. 1 = hordenine, retention time 2.93 min; 2 = gramine, retention time 4.99 min.

tridges, the results were indicative of the homogeneity of the cartridges and the reliability of the protocol in general.

UV absorption by gramine was great enough to allow its quantification down to 1.0 μg/ml (20 μg/g dry wt. approx. of plant sample) with an accuracy of less than 5% variation between dual injections. This figure compares favorably with the lower detection limit of 30 μg/g dry wt quoted by Hanson et al. (1981).

In comparison with gramine, hordenine absorbs considerably less UV light,

resulting in a lower detection limit, in the order of 2.5 μg/ml. This figure is higher than that achieved by Johansson and Schubert (1990) using electrochemical detection and of the same order as they achieved using UV detection at 275 nm. (We found both gramine and hordenine to absorb more strongly at 221 nm than at 275 nm and, therefore, used this wavelength.) Johansson and Schubert, however, reported a run time of 35 min/injection and contamination of the electrodes used with electrochemical detection, which led to downtime. The method reported here, while not as sensitive, has a run time of 8 min and no downtime associated with electrode contamination. Further, given the concentration of hordenine found by us in barley roots (Table 2), we would argue that, for our purposes, quantification to lower levels is unnecessary.

Two major problems in quantifying hordenine in plant material were encountered. The first of these concerns the time scale of hordenine production by barley plants. A trial experiment (data not presented here) with glasshouse-grown plants of cv. Skiff indicated that hordenine content of the roots peaked and declined within the space of three days, the peak roughly coinciding with expansion of the first leaf. This is consistent with data obtained for cv. Triumph (Lovett and Liu, 1987). The apparent disparity with the data presented in Figure 1 of the preceding paper of this series (Liu and Lovett, 1993) may be explained by the sampling intervals of 5–10 days used in this work, resulting in averaging of production over a minimum of five days. The data presented here (Table 2) are for 8-day-old plants, an age chosen to accommodate the slower growth of cv. Proctor and line CI400117.

Duncan's multiple-range test was applied to the data to determine if there was a block effect; line CI400117 contained significantly (5% level) less hordenine in block B than in block C, while no differences were found for the other cultivars. Analysis of variance of the complete data showed no block effect but

TABLE 2. MEAN[a] HORDENINE AND GRAMINE CONTENTS OF THREE BARLEY LINES GROWN ON THREE OCCASIONS

	Hordenine (μg/g fresh weight)				Gramine (μg/g fresh weight)		
Block	Proctor	Skiff	CI400117	Block	Proctor	Skiff	CI400117
A	33^{cde}	14^{ab}	26^{bcd}	A	5	1	975
B	37^{de}	14^{ab}	21^{abc}	B	3	3	900
C	42^{e}	11^{a}	37^{de}	C	8	2	962
Mean	37	13	28	Mean	5	2	946

[a]Mean of three replicates. LSD (hordenine) (5%) = 6.67. Data followed by the same letters do not differ at the 5% level.

indicated that all cultivars differed in their hordenine content at the 5% level (LSD 6.67). This indicates a potential difficulty in comparing the hordenine production capacity of different cultivars and, indeed, the lower levels of hordenine production by cv. Skiff may reflect a greater physiological age and hence declining hordenine production rather than a lesser capacity to produce the compound. It may be more appropriate to analyze plants at the stage when their first leaf is fully expanded rather than at a common chronological age.

The second problem was the difficulty in retaining hordenine on the Sep-Pak cartridges during sample purification. Considerable quantities of the plant matrix remained, but sufficient was removed that adjustment of the pH of the mobile phase to 7.15 permitted the resolution of peaks close to the baseline, thus allowing satisfactory quantification (Figure 1a). Considerable upward adjustment of the pH of the mobile phase is still possible before reaching pH 7.5 where silica starts to dissolve from the column at the parts per million level (Runser, 1981).

No such problems were encountered in quantifying gramine. Subsequent analyses of further lines have confirmed that barley lines either produce large quantities of gramine or virtually none at all (Table 3). The results for cv. Proctor are consistent with those of Hanson et al. (1981), who considered that there were two possible reasons for the lack of gramine production—lack of capacity to synthesize the compound or the capacity to degrade it faster than it is made. The detection of trace amounts of the compound by us suggests a third possibility: that the capacity to produce gramine is present in all lines but that breeding for standard agronomic traits has led to increased competition within the plant from other metabolic pathways for substrate(s) required for gramine synthesis.

TABLE 3. GRAMINE CONTENT OF BARLEY LINES OF VARIOUS GENETIC BACKGROUNDS

Name/number	Background	Gramine production
H. agriocrithon	ancestral	High
H. spontaneum	ancestral	High
cv. Prior	English (old)	Medium
cv. Proctor	English (1952)	Low
cv. Triumph	English (1985)	Low
cv. Grimmett	Australian (1982)	Low
cv. Skiff	Australian (1988)	Low
CI400117[a]	Ethiopian	High
CI490224[a]	Syrian	Low

[a]These are lines obtained by the Australian Winter Cereals Collection from ICARDA and represent germplasm from closer to the crop's centre of origin. High = 1000–1500 μg/g fresh weight; medium = 300–1000 μg/g fresh weight; low = 0–25; μg/g fresh weight.

CONCLUSIONS

The alkaloids hordenine and gramine can be isolated from plant material and quantified by using Sep-Pak C_{18} cartridges to prepare the samples, followed by reversed-phase liquid chromatography. This method is faster and easier to use than methods hitherto reported and is capable of detecting differences between cultivars in their ability to produce both hordenine and gramine.

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CONSTITUENTS OF WING GLAND AND ABDOMINAL HAIR PENCIL SECRETIONS OF MALE AFRICAN SUGARCANE BORER, *Eldana saccharina* WALKER (LEPIDOPTERA: PYRALIDAE)

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Abstract—In addition to *trans*-3,7-dimethyl-6-octen-4-olide (eldanolide), vanillin, and 4-hydroxybenzaldehyde, identified by French workers in the wing gland and abdominal hair pencil secretions of the male African sugarcane borer, *Eldana saccharina*, we have, in an earlier note, reported the presence of several other terpenoid, aromatic, and unbranched-chain compounds such as, (*Z*)-3,7-dimethylocta-2,6-dienoic acid, 6,10,14-trimethyl-2-pentadecanol, 4-hydroxy-3-methoxybenzyl alcohol, 1-octadecane thiol, 16-hexadecanolide, and 18-octadecanolide in these secretions. In the present paper experimental details and spectral evidence supporting the identification of these compounds, as well as the identification of (*Z*)-9-hexadecenal and *cis*-3,7-dimethyl-6-octen-4-olide (*cis*-eldanolide), are reported. Using electroantennography it was found that male and female antennae reacted approximately equally strongly to both secretions. This result was confirmed in analyses of the secretions using coupled gas chromatography–electroantennography and it was found that male as well as female antennae responded to eldanolide. Vanillin, substituted phenols related to vanillin, and some oxygenated monoterpenes elicited weak responses in male and female antennae. In some analyses 6,10,14-trimethyl-2-pentadecanol, present in the secretions of the insect, gave a strong antennal response. The results obtained in dynamic and static

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headspace determinations showed that several of the organic compounds present in the glandular secretions are released in detectable quantities and are present in widely varying quantitative ratios in the effluvia of individual calling male moths.

Key Words—*Eldana saccharina*, Lepidoptera, Pyralidae, exocrine secretions, sex pheromone, aggregation pheromone, electroantennograms, electroantennographic detection, NMR.

INTRODUCTION

The stalk borer *Eldana saccharina* Walker is found in a number of cyperaceous natural host plants in Africa (Atkinson, 1980). Since 1970 it has become an increasingly serious pest in South African sugarcane fields (Smaill and Carnegie, 1979; Carnegie and Smaill, 1980), and it is also a recognized pest of maize and other cereal crops in West and East African countries (Carnegie, 1974). Male moths of this insect, in common with other gallerines, have a gland at the base of each forewing with a duct opening towards the wing tip, as well as a pair of prominent hair pencils on the eighth segment of the abdomen (Atkinson, 1982; Farine, 1983). These disseminating structures are assumed to be involved in pheromone communication in this insect. The possible use of a synthetic pheromone for controlling and/or monitoring the insect has led to considerable interest in the identification and synthesis of the pheromone secreted by the male moth. The γ-lactone, *trans*-3,7-dimethyl-6-octen-4-olide (eldanolide), was identified as a component of the wing gland secretion (Kunesch et al., 1981, 1982), and several syntheses have been elaborated for the natural (3*S*,4*R*) isomer (e.g., Vigneron et al., 1982, 1984; Uematsu et al., 1983; Davies et al., 1985; Suzuki et al., 1985; Ortuño et al., 1987; Butt et al., 1987; Bloch and Seck, 1989; Ebata et al., 1990). It has been suggested (Zagatti, 1981; Kunesch et al., 1982) that eldanolide acts as an "attractant at a distance" of the female. Vanillin and *p*-hydroxybenzaldehyde have been found in the abdominal hair-pencil secretion and were reported to act as a courtship pheromone (Zagatti, 1981; Zagatti et al., 1981). In these studies extensive use was made of laboratory methods for the evaluation of the biological activity of the compounds identified in the exocrine secretions of the male moth.

In field tests carried out concurrently with the present investigation of the chemical composition of the exocrine secretions of the male insect, it was, however, found that neither the synthetic compounds nor extracts of the glandular secretions showed any activity (Bennett et al., 1991), whereas live males in traps attracted more males than females. The male moths normally call in groups in the sugarcane canopy (Atkinson, 1981), so the male exocrine secretions may contain one or more components constituting an aggregation pheromone for males. Finally, Bennett et al. (1991) have recently shown that the

male moth produces a sound with a frequency of approximately 50 kHz, which is created by the buckling of the tymbal on the tegula to produce a pulse train. The lack of pheromone activity observed when extracts of the exocrine glands or synthetic constituents were used in field tests can therefore most likely be ascribed to the absence of this sound, but what then is the function of the two glandular structures and the pheromones they produce? These results show that the intraspecific communication in *E. saccharina* is still far from being clearly understood.

In a preliminary communication (Burger et al., 1985), we have reported the identification of a number of compounds in addition to those found in the exocrine secretions of the male moth by the French workers. We now wish to give experimental details and spectral evidence supporting the identification of these and some additional compounds and to present the results of experiments carried out to determine which of these compounds are present in the effluvium of the calling male insect and elicit electroantennographic responses in male and female antennae.

METHODS AND MATERIALS

General. Analytical gas chromatographic (GC) separations were carried out on a Carlo Erba Fractovap 4160 gas chromatograph equipped with a flame ionization detector (FID) and using 40-m glass capillary columns coated with the apolar phase PS-255 at film thicknesses of 0.5, 0.75, and 1.0 μm. Details of GC parameters are given in the figure legends. Headspace determinations were done with a Siemens Sichromat 2 dual-oven gas chromatograph equipped with flame ionization detectors and a glass capillary column coated with OV-31-OH at a film thickness of 0.5 μm as analytical column in one of the two ovens of the instrument. Quantification was done with a Hewlett-Packard 3385A Lab Automation System or with a Hewlett-Packard XTRA CHROM II Data System using a 192-k Nelson Analytical interface. Preparative GC separation of components of wing gland and hair pencil secretions, as well as the purification of certain synthetic products were carried out according to procedures described by Burger et al. (1985) using a wide-bore glass column (40 m $\times$ 0.5 mm), coated with immobilized PS-255 at a film thickness of 2.0 μm, as well as a glass column (14 m $\times$ 3.0 mm), packed with 3% SE-30 on 60–80 mesh Chromosorb WAW-DMCS.

Electron impact mass spectra (EI-MS) were recorded at 70 eV on Varian MAT 311A, Finnigan MAT 4500, and Carlo Erba QMD 1000 GC-MS systems, using the capillary columns described above. Chemical ionization mass spectra (CI-MS) were obtained with methane and ammonia as reactant gases. High-resolution mass spectral data were obtained with the Varian MAT 311A instru-

ment and a Kratos DS90 data system. ^1H and ^{13}C NMR spectra were recorded at, respectively, 79.54 MHz and 20.00 MHz (34°C) on a Varian FT 80 NMR spectrometer as well as at, respectively, 299.905 MHz and 75.42 MHz (25°C) on a Varian VXR 300 NMR spectrometer.

Synthetic samples of some of the compounds identified in male exocrine secretions and starting materials for syntheses of compounds not commercially available, were obtained from Merck (Darmstadt), Fluka (Buchs), Aldrich (Milwaukee), Sigma (St. Louis), and BASF (Ludwigshafen). Boiling points are uncorrected.

(Z)-3,7-Dimethylocta-2,6-dienoic Acid. This was prepared by the oxidation of a commercially available mixture of (*E*)- and (*Z*)-3,7-dimethylocta-2,6-dienal (12.0 g, 71.4 mmol). A suspension of Ag_2O (27 g, 117 mmol) in water (70 ml) and ethanol (10 ml) was added to the aldehyde. After the addition of a solution of NaOH (12 g, 300 mmol) in water (24 ml) to this mixture over a period of 2 hr, the reaction mixture was stirred for 60 hr at room temperature. The precipitated silver and other solid material were filtered off and were washed thoroughly with hot water. Unchanged aldehyde was extracted from the filtrate with ether, whereafter the water layer was acidified with 1.5 M H_2SO_4 solution and the mixture of geranic acid isomers extracted with ether. The combined ether extracts were dried on anhydrous Na_2SO_4, the drying agent removed by filtration and the solvent removed under reduced pressure. Distillation of the resulting oil gave a mixture of (*E*)- and (*Z*)-3,7-dimethylocta-2,6-dienoic acid in a ratio of 2:1 (9.1 g, 69%), bp 162–163°C/20 torr. The *E* and *Z* isomers were separated gas chromatographically on a wide-bore column and characterized by NMR spectrometry.

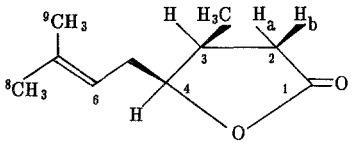
(*E*)-3,7-Dimethylocta-2,6-dienoic acid: ^1H NMR ($CDCl_3$): δ 5.694 (m, J = 1.2 Hz, H-2); 2.186 (m, H-4); 2.176 (m, H-5); 5.076 (m, J = 8.1, 6.7, 1.4 Hz, H-6); 1.690 (q, J = 1.2 Hz, H-8); 1.610 (d, J = 1.5 Hz, H-9); 2.174 (d, J = 1.3 Hz, H-10). ^{13}C NMR ($CDCl_3$): δ 172.19 (s, C-1); 115.21 (d, C-2); 163.00 (s, C-3); 41.23 (t, C-4); 26.05 (t, C-5); 122.83 (d, C-6); 132.69 (s, C-7); 25.67-q, C-8); 17.70 (q, C-9); 19.15 (q, C-10).

(*Z*)-3,7-Dimethylocta-2,6-dienoic acid: ^1H NMR ($CDCl_3$): δ 5.685 (q, J = 1.4 Hz, H-2); 2.644 (t, J = 7.9 Hz, H-4); 2.156 (m, H-5); 5.144 (m, J = 7.3, 1.4 Hz, H-6); 1.686 (q, J = 1.4 Hz, H-8); 1.618 (d, J = 1.3 Hz, H-9); 1.926 (d, J = 1.4 Hz, H-10). ^{13}C NMR ($CDCl_3$): δ 171.70 (s, C-1); 115.74 (d, C-2); 163.49 (s, C-3); 33.72 (t, C-4); 26.85 (t, C-5); 123.51 (d, C-6); 132.41 (s, C-7); 25.67 (q, C-8); 17.56-q, C-9); 25.70 (q, C-10).

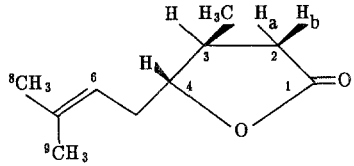
6,10,14-Trimethyl-2-pentadecanol. This was obtained by the hydrogenation of a mixture of farnesyl acetone isomers (1.33 g, 5 mmol) with 5% Pt on activated charcoal in methanol (20 ml). The reaction product was diluted with ether and filtered through a thin layer of anhydrous $MgSO_4$ to remove the catalyst, whereafter the filtrate was concentrated in a slow stream of purified

N_2. Bulb-to-bulb distillation of the resulting viscous oil yielded the required compound, 1.02 g, bp 170°C (air bath temperature)/10^{-5} torr; ^{1}H NMR ($CDCl_3$): δ 3.799 (m, J = 6.1 Hz, H-2); 1.525 (m, J = 6.6 Hz, H-14); 1.0–1.47 (bm, 20 H); 1.189 (d, J = 6.1 Hz, 3 H, H-1); 0.867 (d, J = 6.5 Hz, 6 H, H-15 and H-16); 0.856 (d, J = 6.5 Hz, 3 H, H-17 or H-18); 0.844 (d, 6.5 Hz, 3 H, H-18 or H-17). ^{13}C NMR ($CDCl_3$): δ 23.5 (q, C-1); 68.2 (d, C-2); 39.8 (t, C-3); 23.3 (t, C-4); 37.4 (t, C-5); 32.8 (d, C-6); 37.0 (t, C-7); 24.4 (t, C-8); 37.4 (t, C-9); 32.8 (d, C-10); 37.4 (t, C-11); 24.8 (t, C-12); 39.4 (t, C-13); 28.0 (d, C-14); 22.6 (q, Me on C-14); 19.7 (q, Me on C-17); 19.7 (q, Me on C-4).

*(±)-*cis*-Eldanolide and (±)-*trans*-Eldanolide.* These were isolated by preparative gas chromatography from a mixture of the diastereomeric eldanolide isomers prepared from ethyl 3-methyl-4-pentenoate according to the procedure elaborated by Uematsu et al. (1983). NMR spectral assignments are given with reference to the following formulae:



cis-Eldanolide



trans-Eldanolide

cis-Eldanolide: ^{1}H NMR ($CDCl_3$): δ 2.690 (dd, J = −16.7, 7.8 Hz, H-2a); 2.211 (dd, J = −16.7, 3.6 Hz, H-2b); 2.604 (m, H-3); 4.442 (ddd, $J_{4,5a}$ = 7.8, $J_{4,5b}$ = 6.4, $J_{4,3}$ = 5.6 Hz, H-4); 2.416 (m, J = −14.9, H-5a); 2.268 (m, J = −14.9, H-5b); 5.126 (m, $J_{6,5}$ = 7.1, 1.4 Hz, H-6); 1.722 (m, J = 1.4 Hz, 3 H, H-8); 1.645 (m, J = 1.1 Hz, 3 H, H-9); 1.040 (d, J = 7.0 Hz, 3 H, H-10). ^{13}C NMR ($CDCl_3$): δ 176.79 (s, C-1); 37.59 (t, C-2); 32.87 (d, C-3); 83.39 (d, C-4); 28.77 (t, C-5); 118.13 (d, C-6); 135.16 (s, C-7); 25.80 (q, C-8); 18.01 (q, C-9); 13.89 (q, C-10).

trans-Eldanolide: ^{1}H NMR ($CDCl_3$): δ 2.674 (dd, J = −16.5, 7.4 Hz, H-2a); 2.170 (dd, J = −16.5, 9.1 Hz, H-2b); 2.26 (m, H-3); 4.055 (ddd, $J_{4,5a}$ = 6.3, $J_{4,5b}$ = 7.1, $J_{4,3}$ = 5.4 Hz, H-4); 2.425 (m, J = −15.6 Hz, H-5a); 2.350 (m, J = −15.6 Hz, H-5b); 5.173 (m, $J_{6,5}$ = 7.3, 1.5 Hz, H-6); 1.728 (d, J = 1.1 Hz, 3 H, H-8); 1.638 (bd, 3 H, H-9); 1.134 (d, J = 6.5 Hz, 3 H, H-10). ^{13}C NMR ($CDCl_3$): δ 176.34 (s, C-1); 36.93 (t, C-2); 34.98 (d, C-3); 86.97 (d, C-4); 32.08 (t, C-5); 117.91 (d, C-6); 135.25 (s, C-7); 25.65 (q, C-8); 17.60 or 17.80 (q, C-9); 17.80 or 17.60 (q, C-10).

Insects. Adults of *E. saccharina* were obtained from a culture that was started at the South African Sugar Association Experiment Station (Mount Edgecombe, Natal) from larvae collected in the field and at sugarcane mills from cut

sugarcane. The larvae were fed on a chick pea medium (Atkinson, 1978) and kept at 25°C on a 12-hr light and 12-hr dark photoperiod schedule. The pupae were sexed, air freighted to Stellenbosch, and maintained in environmental chambers using a 12-hr at 26°C and 12-hr at 18°C temperature cycle. For analytical work, emerged moths were exposed to constant light regime to prevent extrusion of the hair pencils.

Collection of Material. About 24 hr after the moths had emerged, they were anesthetized with chloroform vapor for the collection of the wing gland and hair pencil secretions. The application of slight pressure to the abdomen of the males resulted in a gradual extrusion of the hair pencils, which were removed with a pair of ophthalmic scissors before they were fully everted and allowed to fall into a Reacti-Vial with dichloromethane (Merck, Residue Analysis Grade). The wing glands were excised with a sharp blade, likewise collected in dichloromethane, and ground with a pointed glass rod to extract the organic material. Both extracts were filtered through a small sintered glass filter at 3000 rpm in a centrifuge and used for quantitative work without further concentration. For qualitative analyses some of the extracts were concentrated in a purified (activated charcoal) nitrogen atmosphere.

Electrophysiological Measurements. Measurements of the electrophysiological response of male and female antennae to glandular secretions and synthetic compounds were carried out using glass capillary/Ag–AgCl microelectrodes filled with insect saline solution (Roelofs, 1984) to which polyvinylpyrrolidone K90 (Fluka) was added to increase the viscosity of the solution (Van der Pers, 1980). Experiments with intact moths were carried out by immobilizing the intact insects in a glass tube as follows: One end of a short glass tube was flame polished to form a constriction allowing only the head of a moth to protrude from this end of the tube. Using a small glass blower's torch, a thin glass rod of about the length of the insects' antennae was attached sideways to the constriction. A moth was inserted from the other end of the tube and kept in place with a plug of cotton wool. In the process the insect's antennae were pinned to its sides in the tube. One of the antennae was carefully withdrawn from the flame-polished end of the tube and fastened to the glass rod using a sliver of glue peeled off from carpet tape. The recording electrode was inserted into the distal end of the antenna and the indifferent electrode into the cranial cavity of the moth. Due to the attempted movements of the moth, this preparation produced a very noisy baseline in EAD recordings, and it was therefore used only for some EAG measurements.

GC analyses with flame ionization and electroantennographic detection (GC-FID/EAD) were performed with severed antennae inserted lengthwise into the air duct as shown in Figure 1. As the GC separations took more than 2 hr, antennal preparations were replaced two or three times during analyses. The column effluent was diluted with 15 ml/min of He and split 1 : 1 between the

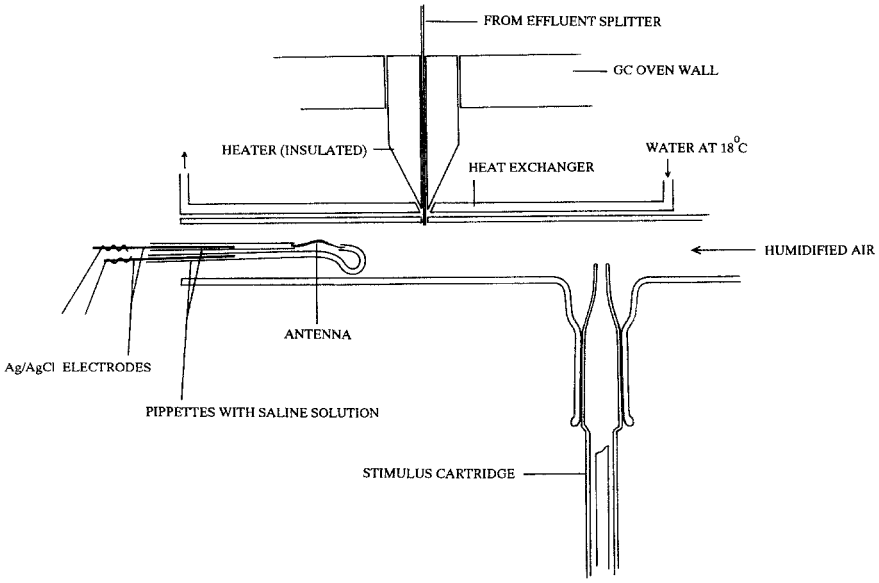


FIG. 1. Antennal preparation inserted lengthwise into the air duct for EAG measurements or FID/EAD analyses.

two detection systems, using instrumentation recently described by Burger et al. (1991a).

Some EAG measurements were made with the same instrumentation and with stimulus cartridges (90 mm × 6 mm ID) fitted with drawn out B-10 drip cones allowing the delivery of the test volatiles directly into the stream of purified (activated charcoal) and humidified air (Figure 1). Samples dissolved in dichloromethane and solvent blanks were pipetted onto filter paper strips (80 mm × 6 mm), the solvent was allowed to evaporate, and the strips were inserted into the stimulus cartridges, which were closed with glass stoppers before and between measurements. Each stimulus cartridge was inserted into a B-10 socket on the air duct and 5 ml of air was puffed through the cartridge by depressing the plunger of a glass syringe by hand.

To compare the response of male and female antennae to wing gland and hair pencil extracts, paper strips were impregnated with one to five moth equivalents of the extracts. Measurements were repeated with one antenna each from 11 male and 11 female moths. In this experiment the EAG amplitudes were standardized by dividing the amplitude produced by the glandular extract by the amplitude produced by air puffed over solvent blanks prepared by evaporating the solvent, dichloromethane, from a paper strip. In a comparison of the sensitivity of antennae to individual constituents of the secretions of the insect,

amplitudes were standardized by dividing the amplitude produced by these materials by those of 1-hexadecanol, which was used as a standard reference compound. EAG amplitudes were stored on an analog storage oscilloscope (Hewlett-Packard 1201 B) or recorded on a strip chart recorder (Houston OmniScribe D 5000). Two recorders with matched chart speeds were used in FID/EAD analyses.

Headspace Determinations. The fluidum of a single calling male moth was trapped on a film/organic-polymer open-tubular trap (FOPOTT) (Burger and Munro, 1986). The smallest possible container in which typical calling behavior could be induced by light intensity and temperature manipulation was constructed by partly flattening a cylindrical glass vessel (45 ml) on one side and fusing a few glass fragments into the inside flattened surface to provide the moth with purchase. To eliminate irreversible adsorption as far as possible, the inside surface of the vessel was silylated with a monofunctional silylation agent according to procedures described by Grob (1986). Before use, the glass vessel was rinsed with dichloromethane and heated overnight at 200°C in a well-ventilated oven. A virgin male moth was placed in the vessel and calling behavior induced by switching to red light and decreasing the room temperature to 18°C. As soon as the moth started calling, purified air was sucked over the calling male and through the FOPOTT and a similar guard trap at a flow rate of 30 ml/min. Trapping of the volatiles was interrupted after 20 min, and the moth was quickly removed from the headspace vessel. The FOPOTTs were replaced by two fresh traps and trapping was continued. The guard trap used in the first trapping cycle was installed in the first, and the analytical OV-31-OH capillary column in the second oven of the Sichromat 2 gas chromatograph. Using helium as a carrier gas at a linear flow velocity of ca. 40 cm/sec, the volatiles were desorbed from the FOPOTT by programming the first oven from 40°C to 230°C at 10°C/min, the second oven being held isothermally at 10°C to trap the volatiles on the analytical column. The first oven was then cooled to room temperature and the guard FOPOTT (second in line) replaced by a similar length of uncoated capillary tubing, whereafter the volatiles that had been trapped on the analytical column during the previous desorption step were analyzed using helium at a linear flow velocity of 28.6 cm/sec and a programming rate of 2°C/min from 40°C to 260°C. Having determined that no volatiles had broken through to the guard FOPOTT, the volatiles trapped on the other FOPOTT (first in line) were similarly desorbed and quantitatively analyzed, using eldanolide and vanillin as external standards. Further cycles of trapping, desorption, and analysis were carried out until all the volatile material secreted by the moth had been removed from the vessel. To avoid overloading the capillary column, the volatiles were initially trapped for periods of only 20 min, but as the material in the vessel became depleted the volatiles could be trapped for longer periods.

In other experiments the removal of the volatile material from the vessel

was quantitatively monitored by sucking purified air through the vessel and an uncoated 1-m fused silica capillary at a constant flow of 30 ml/min and replacing the 1-m uncoated section of the fused silica tubing with a 1-m FOPOTT for a fixed time at certain intervals, after which the FOPOTT was subjected to the analytical procedure described above.

Experiments were also done in which the volatiles secreted by a calling male were rinsed from the headspace vessel with a suitable solvent (Baker et al., 1980). A virgin male was allowed to call in the vessel for periods of up to 2.5 hr, after which the vessel was cooled to 5°C for 5 min. The moth was removed, the vessel rinsed three times with 0.4-ml quantities of either purified (Al_2O_3) diethyl ether or dichloromethane, and the washings combined for quantitative gas chromatographic determination of the volatiles released by the insect. Decane and 2-phenylethanol were used as internal standards.

RESULTS AND DISCUSSION

Gas chromatographic (GC) and gas chromatographic–mass spectrometric (GC-MS) analyses of extracts of the hair pencils and wing glands of *Eldana saccharina* males on an efficient capillary column showed that, in addition to eldanolide, *p*-hydroxybenzaldehyde, and vanillin, identified by French workers (Kunesch et al., 1981, 1982; Zagatti et al., 1981), the secretions contain several hitherto unidentified constituents that could conceivably also play a role in the semiochemical communication of the insect. GC-MS analyses of these secretions of the male insects were repeated at least 40 times at irregular intervals over a period of seven years. Care was taken to avoid using glassware and syringes that had been used for other analyses or for the handling of extracts. The presence of some minor constituents that appeared irreproducibly in some of the analyses and were not solvent contaminants, can therefore not be ascribed to contamination with substances handled in our laboratory. The only explanation that can be offered is that some of these constituents could have been introduced in the diet of the larvae. Typical total-ion chromatograms of the hair pencil and wing gland secretions are shown in Figures 2 and 3, respectively. The compounds identified in the wing gland and hair pencil secretions of the male insect are listed in Table 1. Compounds such as, for example, constituents **1** and **2** that were not present in more than a few analyses, are listed as possible contaminants.

4-Hydroxy-4-methyl-2-pentanone (**1**) was identified as a constituent in some wing gland extracts by comparison of its mass spectrum and gas chromatographic properties with those of the authentic synthetic material. Constituent **2**, present in both tail brush and wing gland extracts, has a mass spectrum practically identical to those of α-phellandrene and α-terpinene, with a base peak

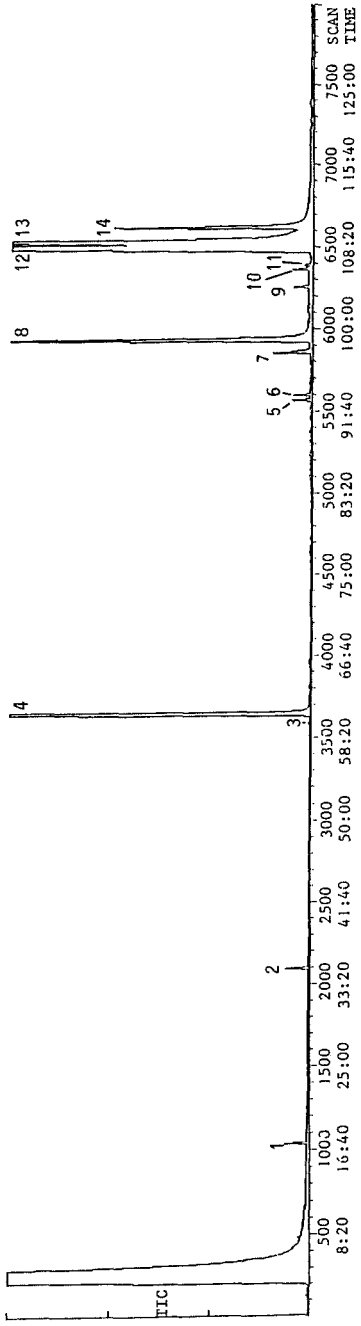


FIG. 2. Total-ion chromatogram of the wing gland secretion of the male African sugarcane borer *Eldana saccharina*. Glass capillary column coated with PS 255 (40 m × 0.3 mm, film thickness 1.0 μm); 40–240°C at 2°C/min.

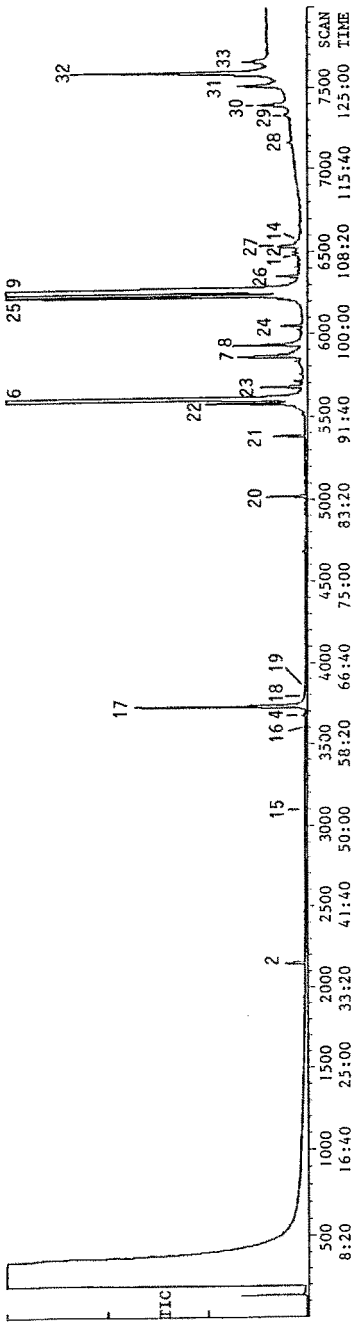


FIG. 3. Total-ion chromatogram of the abdominal hair pencil secretion of the male African sugarcane borer *Eldana saccharina*. GC parameters as in Figure 2.

TABLE 1. COMPOSITION OF VOLATILE ORGANIC FRACTION OF WING GLAND SECRETION AND ABDOMINAL HAIR PENCIL SECRETION OF MALE AFRICAN SUGARCANE BORER

Wing gland secretion[a]			Hair pencil secretion[b]		
Peak in Figure 2	Abundance (μg/male)	Component	Peak in Figure 3	Abundance (μg/male)	Component
1		4-Hydroxy-4-methyl-2-pentanone[c,d,i]	**2**		Unidentified[c]
2		Unidentified[c]	**15**		Unidentified[c]
3	<0.0001	(Z)-3,7-Dimethylocta-2,6-dienoic acid[d,i]	**16**	0.012	4-Hydroxybenzaldehyde[d,i]
4	0.45	*trans*-3,7-Dimethyl-6-octen-4-olide (*trans*-eldanolide)[d,e,f,g,h,i]	**4**	0.033	*trans*-3,7-dimethyl-6-octen-4-olide (*trans*-eldanolide)[d,f,i]
5		Unidentified[c]	**17**	0.58	4-Hydroxy-3-methoxybenzaldehyde (vanillin)[d,e,f,g,h,i]
6	0.007	6,10,14-trimethyl-2-pentadecanol[d,i]	**18**	0.0025	*cis*-3,7-Dimethyl-6-octen-4-olide (*cis*-eldanolide)[d,i]
7	0.019	16-Hexadecanolide[d,i]	**19**	0.00075	4-Hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol)[d,f,i]
8	0.29	Hexadecanoic acid[d,i]	**20**	0.016	(Z)-9-Hexadecenal[d,i]
9	0.016	(Z)-9-Octadecenol[d,i]	**21**		Unidentified[c]
10		Unidentified[c]	**22**		Unidentified[c]
11		Unidentified[c]	**6**	2.88	6,10,14-Trimethyl-2-pentadecanol[d,e,f,g,h,i]
12	0.64	(Z, Z)-9,12-Octadecadienoic acid[d,i]	**23**	0.081	1-Hexadecanol[d,i]
13	0.47	(Z)-9-Octadecenoic acid[d,i]	**7**	0.81	16-Hexadecanolide[d,f,i]
14	0.21	Octadecanoic acid[d,i]	**8**	0.79	Hexadecanoic acid[d,i]

24	0.0053	(*Z*)-9-Octadecenal[d,i]
25	0.45	(*Z*, *Z*)-9,12-Octadecadienol[d,e,f,g,i]
9	6.42	(*Z*)-9-Octadecenol[d,f,i]
26	0.061	1-Octadecanethiol[d,i]
12	0.19	(*Z*, *Z*)-9,12-Octadecadienoic acid[d,i]
27	0.68	18-Octadecanolide[d,i]
14	0.059	Octadecanoic acid[d,i]
28		Unidentified[c]
29	0.56	*cis*-2-[(*Z*)-8-pentadecenyl]-5-hydroxy-1,3-dioxane[d,e,f,g,h,i]
30	1.61	*cis*-2-[(*Z*)-8-pentadecenyl]-4-hydroxymethyl-1,3-dioxolane[d,e,f,g]
31	2.12	*trans*-2-[(*Z*)-8-pentadecenyl]-4-hydroxymethyl-1,3-dioxolane[d,e,f,g,h,i]
32		Unidentified[c]
33	0.3	*trans*-2-[(*Z*)-8-pentadecenyl]-5-hydroxy-1,3-dioxane[d,e,f,g,h,i]

[a] Secretion extracted from the wing glands of 10 male moths.
[b] Secretion extracted from the hair pencils of 8 male moths.
[c] Not present in all extracts. In some cases probably a contaminant.
[d] Low-resolution EI-MS.
[e] High-resolution EI-MS.
[f] Low-resolution CI(CH_4)-MS.
[g] ^{1}H NMR.
[h] ^{13}C NMR.
[i] Retention time comparison with synthetic material.

and molecular ion at *m/z* 93 and 136, respectively. Gas chromatographic retention time and mass spectral data indicate that this component is a terpene, but it remained unidentified, as no supporting evidence could be obtained by GC and GC-MS comparison with available terpenes having similar mass spectra, such as, among others, the ocimene and allo-ocimene isomers. Constituent **3** was present in the wing gland extract in a very low concentration and its mass spectrum contains very little structural information. However, it has the same molecular mass as eldanolide and, as it eluted just before this compound, it was assumed to have a structure similar or related to that of eldanolide. 3,7-Dimethylocta-2,6-dienoic acid is one of the structures meeting these requirements that comes to mind. A mixture of the *E* and *Z* isomers of this acid was prepared by Ag_2O oxidation of citral and, by retention time comparison with the two preparatively separated isomers, the natural compound was found to be the *Z* isomer.

Eldanolide **(4)** was identified in the wing gland extract by comparison of its mass spectrum with published mass spectral data and by gas chromatographic retention time comparison with the synthetic compound. 4-Hydroxybenzaldehyde **(16),** vanillin **(17),** and 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol) **(19)** were similarly identified in the hair pencil extract.

Eldanolide was found also in hair pencil extracts from males that had called, and it is reasonable to assume that this compound is filtered from the air that is fanned over the hair pencils by the calling insects' wings. However, eldanolide and some of the other constituents of the wing gland secretion also appear in material extracted from the hair pencils of moths that had not called. It is therefore possible that these constituents are either normally present in different proportions in both secretions or are translocated by mutual contact between the males or by some other mechanism. In the present study this problem was not further investigated. It was recently found that a minor constituent **(18)** of the hair pencil secretion has a mass spectrum (Figure 4) identical in every detail to that of eldanolide. Retention time comparison with synthetic *cis-* and *trans*-3,7-dimethyl-6-octen-4-olide revealed this compound to be the *cis* isomer (*cis*-eldanolide). This isomer has not been handled in our laboratory for at least five years, and its presence in the hair pencil extract can therefore not be ascribed to contamination with the synthetic material. The absolute configuration of this eldanolide isomer has not been established in the present investigation and further work will have to be devoted to this aspect.

Constituent **6,** having ions at *m/z* 255.2698 ($C_{17}H_{35}O$) and 252.2811 ($C_{18}H_{36}$) in its mass spectrum, was identified as the terpenoid alcohol 6,10,14-trimethyl-2-pentadecanol. Further proof of its identity was obtained by ^{13}C NMR spectral analysis and retention time comparison with authentic synthetic material. This alcohol has been identified as a courtship pheromone in the female rice moth, *Corcyra cephalonica* (Hall et al., 1987), and an enan-

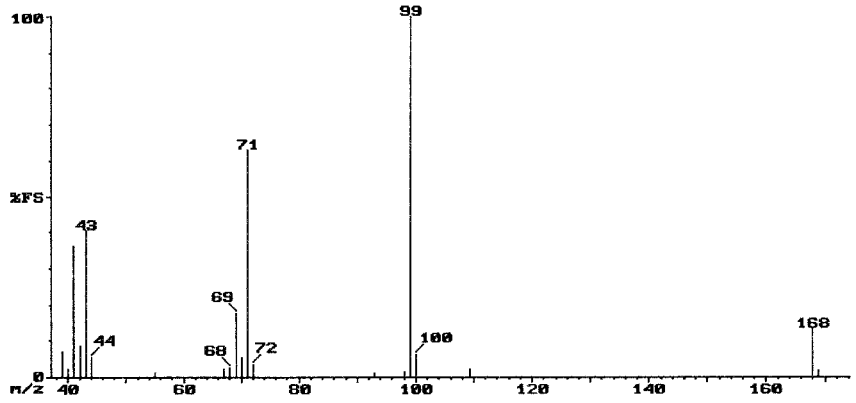


FIG. 4. Mass spectrum of *cis*-3,7-dimethyl-6-octen-4-olide (*cis*-eldanolide).

tiomer of this terpenoid alcohol showing high activity in behavioral and electrophysiological bioassays was subsequently synthesized by Mori et al. (1991).

The mass spectra of constituents **7** and **27** contain relatively little structural information and insufficient material was available for NMR analysis. The tentative identification of these constituents as hexadecanolide (dihydroambrettolide) and octadecanolide was therefore based mainly on data published for compounds isolated from the solitary bee *Colletes cunicularius* by Bergström (1974). The presence of the two cyclic lactones was confirmed by mass spectral and retention time comparison with the synthetic compounds. Hexadecanolide is another example of a compound that is present in both glandular extracts. Constituent **26,** having a molecular ion at *m/z* 286 and a weak but nevertheless significant M − 34 ion in its mass spectrum, was identified as 1-octadecanethiol, which was commercially available for comparison. Constituents **8, 9, 12, 13, 14, 20, 23, 24,** and **25** were identified as long-chain saturated and unsaturated fatty alcohols, aldehydes, and acids by, among others, mass spectral and retention time comparison with the commercially available synthetic compounds.

The hair pencil secretion contains a group of five components **(29–33)** with similar mass spectra, each having a molecular ion at *m/z* 312 and a very prominent base peak at *m/z* 103. Four of these compounds were identified as the cyclic glycerol acetals of (*Z*)-9-hexadecenal, i.e., *cis*- and *trans*-2-[(*Z*)-8-pentadecenyl]-5-hydroxy-1,3-dioxane, **29** and **33** respectively, and *cis*- and *trans*-2-[(*Z*)-8-pentadecenyl]-4-hydroxymethyl-1,3-dioxolane, **30** and **31,** respectively (Burger et al., 1991b). The mass spectra of the dioxane **29** and the dioxolane **30** are given in Figures 5 and 6 as representative examples of the spectra of these compounds. The base peak at *m/z* 103 in the EI mass spectrum of the remaining member **(32)** of this group of constituents has a much lower relative abundance than that of the other four constituents. It is, furthermore,

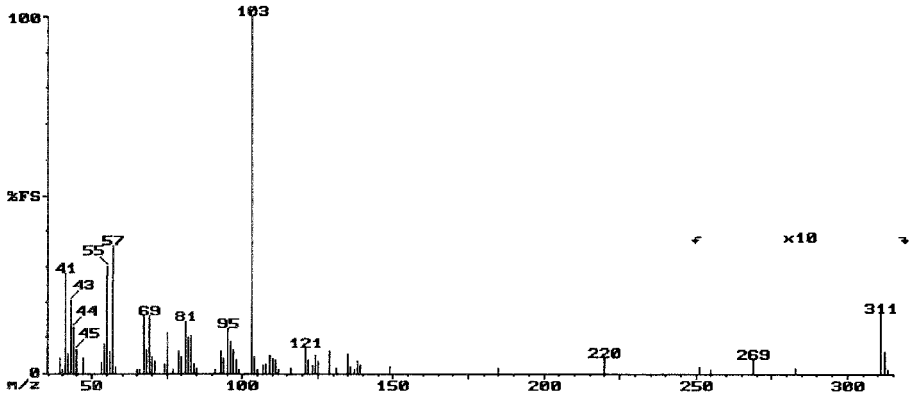


FIG. 5. Mass spectrum of *cis*-2-[(*Z*)-8-pentadecenyl]-5-hydroxy-1,3-dioxane.

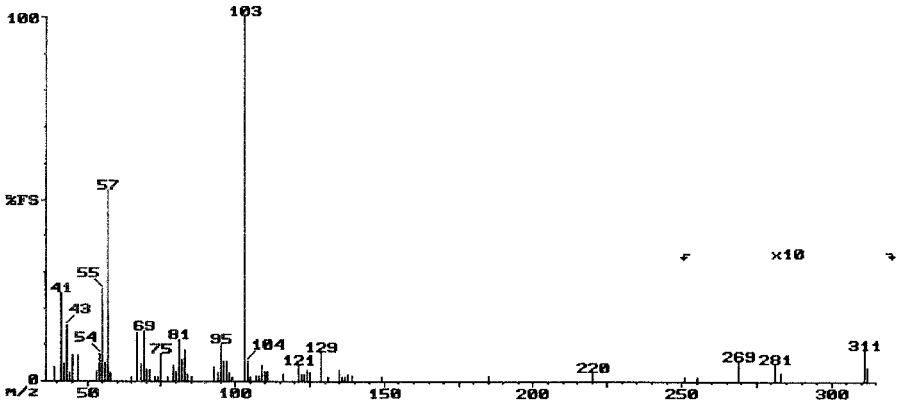


FIG. 6. Mass spectrum of *cis*-2-[(*Z*)-8-pentadecenyl]-4-hydroxymethyl-1,3-dioxolane.

only observed in the first two or three GC or GC-MS analyses with a new capillary column. This compound is apparently either sensitive to contaminants remaining in older columns or it elutes as such a broad peak from these columns as to be indistinguishable from the baseline hump in this part of the chromatogram. It remained unidentified as its mass spectrum contains very little structural information and it could not be isolated by preparative gas chromatography. It has been shown that the smooth muscle contracting activity of Darmstoff (Vogt, 1949) resides exclusively in 2-[(*Z*)-8-heptadecenyl]-4-hydroxymethyl-1,3-dioxolanedihydrogen phosphate (Wiley et al., 1970), and in our recent paper on the identification of the four cyclic glycerol acetal derivatives in the tail brush secretion of *Eldana* males, we have mentioned the possibility that these compounds

or their dihydrogen phosphate esters could play a part in the eversion or retraction of the tail brushes of the insect.

The results of Bennett et al. (1991) were not fully reconcilable with eldanolide being an "attractant at a distance" of the female and vanillin an aphrodisiac as proposed by French workers (Zagatti, 1981; Kunesch et al., 1982). The French workers were unaware of the ultrasound component, and it seems possible that all three components, wing gland pheromone, hair pencil pheromone, and ultrasound, perhaps acting synergistically, may be necessary to attract females. For example, extracts of wing glands and of hair pencils, either singly or together, were totally unattractive to moths of either sex in field-trapping experiments, while in olfactometer experiments hair pencil extract seemed the more attractive. In the work reported here, the antennae of *Eldana* moths gave only relatively weak EAG responses in laboratory experiments. It was found that two moth equivalents of a hair pencil extract elicited standardized EAG responses of 1.67 ($\pm$0.34 SD; $N = 11$) and 1.73 ($\pm$0.40 SD; $N = 11$) in male and female antennae, respectively, and that the same quantity of a wing gland extract produced responses of 1.22 ($\pm$0.54 SD; $N = 11$) and 1.40 ($\pm$0.40 SD; $N = 11$) in male and female antennae, respectively. These results support the results obtained by Bennett et al. (1991) in field trapping experiments in which the attractivity towards males and females of intact males was compared to that of males having either their wing glands blocked or their hair pencils removed and are in agreement with the conclusions reached by these authors that the communication system as a whole of the male moths is directed towards other males as well as females.

Since lures impregnated with gland extracts proved to be ineffective attractants in the absence of the sound produced by calling males, testing individual compounds in the field was not considered to be a viable method to obtain information on the semiochemical activity of the constituents of the glandular secretions of the insect. GC separations of the extracts with FID and EAD in parallel were therefore done with one to three male moth equivalents to determine which of the constituents of these extracts produce EAG responses in male and female antennae. The antennae were normally replaced two or three times during an analysis with fresh preparations. Large differences were observed in the baseline noise levels and EAD response amplitudes produced by individual male and female antennae. *trans*-Eldanolide gave the strongest response in all analyses. The response evoked by vanillin was so weak in some analyses as to be almost indistinguishable from the background noise of the system. In some analyses compounds with retention times shorter than those of eldanolide and vanillin evoked responses that were reproducible for the extract under investigation but were absent in analyses of other extracts. These compounds eluted in the retention time range expected for certain monoterpenoid compounds and simple substituted phenols. As eldanolide is a terpenoid compound, FID/EAD

analyses were done with a few monoterpenoid compounds. It was found that the oxygenated monoterpenoids geraniol, nerol, and (*E*)- and (*Z*)-citral gave strong EAD responses, whereas antennae did not respond to the monoterpenes limonene and (*E*)- and (*Z*)-ocimene. Substituted phenols related to vanillin were also found to be EAD active. Guaiacol, producing a particularly strong response in the antennae of male moths, eluted with a retention time corresponding to the EAD response found in some analyses. However, it was not possible to confirm the presence of this compound in any of the glandular secretion extracts. 6,10,14-Trimethyl-2-pentadecanol evoked a very strong antennal response in male antennae in a few analyses, while no response was recorded in others. Using a synthetic mixture of the enantiomers of this compound, male and female antennae, however, produced only relatively weak EAD responses. This result could be due to the fact that possibly only one of the eight stereoisomers present in the synthetic material evokes antennal responses, while the others might be inactive or could even have an inhibitory effect. Mori et al. (1991) have, for example, found one of the enantiomers of this compound to be eight times more active in a behavioral bioassay than a diastereomeric mixture of equal amounts of eight of its stereoisomers.

The four cyclic acetals **29, 30, 31,** and **33** did not produce antennal responses in FID/EAD analyses. It was suggested (W. Francke, personal communication) that the acetals could possibly be precursors for the release of (*Z*)-9-hexadecenal, which could then act as a pheromone. Using 1-hexadecanol as reference compound, two male moth equivalents of the hair pencil extract, (*Z*)-9-hexadecenal, and vanillin evoked standardized EAG responses of, respectively, 2.22 ($\pm$0.61 SD; $N = 15$), 1.22 ($\pm$0.10 SD; $N = 15$), and 1.23 ($\pm$0.15 SD; $N = 15$) in male antennae. In female antennae responses of, respectively, 3.50 ($\pm$1.46 SD; $N = 15$), 1.06 ($\pm$0.061 SD; $N = 15$), and 1.045 ($\pm$0.17 SD; $N = 15$) were obtained. Similar results were obtained when the responses evoked by solvent blanks were used in calculations. Standardized responses of 1.02 ($\pm$0.083 SD; $N = 15$) and 0.95 ($\pm$0.21 SD; $N = 15$), respectively, in male and female antennae were found for 1-hexadecanol if calculations were done on this basis. (*Z*)-9-Hexadecenal therefore does not appear to evoke a particularly strong EAG response in male or female antennae, in agreement with the absence of antennal responses corresponding to the elution of this compound in FID/EAD analyses. However, it must be taken into consideration that vanillin, which is claimed to have a semiochemical function in this insect, also did not evoke appreciable antennal responses in these measurements and analyses. Examples of these FID/EAD analyses appeared in a recent paper on the EAD analyses of semiochemicals concentrated on thick film capillary traps (Burger et al., 1991a).

Although the main thrust of the present investigation was the determination of the qualitative and quantitative composition of the glandular secretions of the male moth and of the effluvia of individual insects, a series of EAG measure-

ments was carried out in an attempt to compare the possible semiochemical activity of individual constituents of the secretions. This work was, however, discontinued as it is doubtful whether any valid conclusions can be drawn from results obtained with compounds that are released from the paper strips at widely different rates due to their different vapor pressures. The constituents of the glandular secretions of the male insect also have widely different polarities and therefore elute from a capillary column with different peak shapes. It is therefore also doubtful whether FID/EAD analyses can be used for the quantitative comparison of the semiochemical activity of, for example, 6,10,14-trimethyl-2-pentadecanol, the fatty acids found in the secretions, and (*Z*)-9-hexadecenal. The development of methods whereby air containing a known concentration of a compound can be puffed over the antennae is therefore being investigated.

To determine which of the constituents of the male glandular extracts are present in the effluvium of calling males, purified air was drawn over a calling male for 20 min and the organic volatiles released by the insect trapped on a 1-m fused silica trap (Burger and Munro, 1986). Large quantities of eldanolide were trapped and transferred to the analytical column, but no indication could be found that any of the compounds identified in the glandular extracts other than eldanolide and vanillin were present in the effluvium of calling males. According to the results of eight determinations summarized in Table 2, calling males released between 0.80 and 2.25 μg of *trans*-eldanolide, and between 0.009 and 0.85 μg of vanillin in 20 min. Although the smallest vessel in which a male could be induced to exhibit calling behavior was used and the glass surface was deactivated by silylation (Grob, 1986), between 55 and 65 liters of purified air had to be passed through the vessel at a flow rate of 30 ml/min to achieve

TABLE 2. DYNAMIC HEADSPACE DETERMINATION OF *trans*-ELDANOLIDE AND VANILLIN PRODUCED BY CALLING *Eldana saccharina* MALES[a]

	Quantity released in 20 min(μg)	
Experiment	*trans*-Eldanolide	Vanillin
1	1.09	0.009
2	1.21	0.012
3	1.87	0.029
4	0.99	0.049
5	0.88	0.33
6	1.85	0.048
7	0.80	0.056
8	2.25	0.85

[a]Volatile compounds, produced by one male in each of the experiments, were trapped on a 1-m fused silica capillary trap and thermally desorbed for GC determination.

TABLE 3. STATIC HEADSPACE DETERMINATION OF EFFLUVIUM OF ONE CALLING *Eldana saccharina* MALE[a]

Peak[b]	Compound	Quantities released in 20 min (μg)[c]				
		Exp. 1, 150 min	Exp. 2, 75 min	Exp. 3, 35 min	Exp. 4, 30 min	Exp. 5 20 min
4	*trans*-Eldanolide	1.65	2.95	2.11	1.87	5.76
17	Vanillin	0.05	0.68	0.17	0.11	0.0045
18	*cis*-Eldanolide			0.00021	0.0011	
6	6,10,14-Trimethyl-2-pentadecanol	0.46	39.28	0.099	0.12	0.056
23	1-Hexadecanol		2.38	0.0084	0.0022	
7	16-Hexadecanolide				0.0025	0.056
8	Hexadecanoic acid	0.13	25.96	0.013	0.0094	0.038
9	(Z)-9-Octadecanol	0.004	0.042	0.18	0.29	0.037
27	18-Octadecanolide		0.094	0.0056	0.0033	
29	*cis*-2-[(Z)-8-pentadecenyl]-5-hydroxy-1,3-dioxane			0.0012	0.0012	0.0014
30	*cis*-2-[(Z)-8-pentadecenyl]-4-hydroxymethyl-1,3-dioxolane			[d]	0.011	0.0037
31	*trans*-2-[(Z)-8-pentadecenyl]-4-hydroxymethyl-1,3-dioxolane			0.0035	0.011	0.0036
33	*trans*-2-[(Z)-8-pentadecenyl]-5-hydroxy-1,3-dioxane			0.0012	0.0011	0.0026

[a] Volatile compounds adsorbed on the wall of a 45-ml glass vessel were washed out with solvent for GC determination.
[b] Constituent numbers refer to Figures 2 and 3, and Table 1.
[c] Blank spaces indicate the absence of a compound in a particular sample.
[d] Contaminated peak.

complete transfer of these compounds to the trap. As far as the adsorption of polar compounds on glass surfaces is concerned, this result once again underlines the importance of approaching with due caution the results of headspace gas determinations in which the gas has been transferred with glass syringes. The possibility therefore exists that other compounds are present in the effluvia of calling males but are either too involatile or too strongly adsorbed on the glass surface to be transported to the trap in quantities detectable in this experiment. This was confirmed by experiments in which the volatiles produced by a calling male were rinsed from the vessel with a suitable solvent and determined gas chromatographically (Baker et al., 1980). The results of these experiments are given in Table 3. Several of the compounds previously found in glandular extracts of the male insect were found to be present in the material washed from the vessel and are apparently present in the effluvium of the insect. Guaiacol and other compounds with retention times shorter than that of *trans*-eldanolide and evoking antennal responses in previous analyses were not present in detectable amounts in the material recovered from the vessel, whereas 6,10,14-trimethyl-2-pentadecanol was one of the major volatile constituents of this material.

As far as the constituents of the hair pencil secretion are concerned, it must be taken into account that the everted abdominal brushes of the calling males can come into contact with the inside glass surface of the vessel used in these experiments and that the heavy compounds present in the material rinsed from the vessel could have been transported directly from the brushes to the glass surface. Possibly this mode of transfer is responsible for the presence of small quantities of the cyclic acetals **29, 30, 31,** and **32** in several of the analyses. However, it is unlikely that the hair pencil secretion could have been transported to the glass surface in this manner in those cases where the analyses showed the presence of large quantities of, for example, 6,10,14-trimethyl-2-pentadecanol and hexadecanoic acid in the absence of the cyclic acetals.

The results of all the quantitative determinations indicate large variations in the quantitative composition of the glandular secretions of virgin males and the effluvia of calling males. It is possible that these variations have no semiochemical significance, but these differences could also be the basis on which females select a specific male from groups of males aggregating in the leaf canopy. This possibility will have to be investigated by analyzing the effluvia of males that are accepted by females.

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HERBICIDAL ACTIVITY OF SULFORAPHENE FROM STOCK (*Matthiola incana*)[1]

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Abstract—A herbicidal compound was isolated from extracts of *Matthiola incana* and identified as sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate). The ED_{50} of this compound against velvetleaf seedlings was approximately 2×10^{-4} M. Glucoraphenin, the glucosinolate that is the natural precursor of sulforaphene, was less phytotoxic, with an ED_{50} of near 6×10^{-3} M.

Key Words—Stock, *Matthiola incana*, Brassicaceae, glucosinolate, sulforaphene, glucoraphenin, phytotoxicity, allelopathy.

INTRODUCTION

Anecdotal reports (Rice, 1984) indicate that greenhouse soil in which stock (*Matthiola* and *Malcolmia* spp.) has been grown cannot be reused, suggesting that these species might be allelopathic. Both genera are members of the Brassicaceae, which includes other species reported to have phytotoxic activity (Bialy et al., 1990; Brown et al., 1991). Our interest in naturally occurring compounds with herbicidal activity prompted us to determine whether stock produces herbicidal substances.

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[1]The mention of firm name or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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METHODS AND MATERIALS

Spectroscopy. ^1H and ^{13}C NMR spectra relative to tetramethylsilane were measured in $CDCl_3$ or D_2O at 300 and 75.5 MHz, respectively, with a Bruker WM-300-WB spectrometer. Mass spectra (70 eV) were produced by a Hewlett Packard 5790 Mass Selective Detector.

Preparation of Extracts. Seeds of cv. Happi-stok [*Matthiola incana* (L.) R.Br.] were purchased from Thompson and Morgan, Inc. (Jackson, New Jersey). Two-month-old, greenhouse-grown plants (not flowering) were cut off at soil level, rinsed in water, blotted dry, and weighed. The above ground plant parts (about 300 g fresh wt) were ground in a Waring blender with 300 ml chloroform, and the extract was filtered through cheesecloth. The grinding and filtration steps were repeated twice. The filtrates were combined, filtered through Whatman 2 paper, and washed three times with water in a separatory funnel. The chloroform layer was concentrated under vacuum to a thick liquid and then diluted with a few milliliters of chloroform; the aqueous layer was freeze-dried and redissolved in water. The remaining solid plant material was ground again three times with methanol (300 ml each time); these extracts were combined and filtered as above, and concentrated until almost dry.

Roots (about 20 g) were washed with distilled water to remove dirt, then extracted as above.

Chromatography. The chloroform extract of the leaves and stems (about 3.5 g) was chromatographed on a 6 × 62-cm column of Sephadex LH-20; the eluent was dichloromethane–methanol, 1 : 1 (v/v). Seven fractions corresponding to colored bands were collected. The band found to be active by bioassay (see below) was further chromatographed on 1-mm-thick silica gel 60 plates (Merck) in chloroform–acetone, 2 : 1 (v/v). Zones (identified by fluorescent bands and the areas between them) were eluted with acetone followed by chloroform. The active zone yielded sulforaphene. ^1H NMR ($CDCl_3$): δ 2.6–2.7 (2H, m, $C\underline{H}_2$); 2.65 (3H, s, $C\underline{H}_3$); 3.64 (2H, t, J = 6 Hz, $C\underline{H}_2$—NCS; 6.35–6.5 (2H, m, $C\underline{H}$=$C\underline{H}$). ^{13}C ($CDCl_3$): δ 32.61 ($\underline{C}H_2$); 40.50 ($\underline{C}H_3$); 43.59 ($\underline{C}H_2$—NCS); 133.20 and 137.93 ($\underline{C}H$=$\underline{C}H$); 177.5 (N$\underline{C}$S).

Isolation of Glucoraphenin (4-Methylsulphinyl-3-butenyl Glucosinolate). Radish (*Raphanus sativus* L.) seeds purchased commercially were ground in a Wiley mill and extracted overnight with hexane in a Soxhlet extractor to remove the lipids. The defatted meal (about 70 g) was added to 300 ml of boiling methanol and heated on a steam bath for 3 min. The mixture was filtered through Whatman 2 paper; the solid was boiled again for 3 min in 300 ml of 75% aqueous methanol, then filtered. The filtrates were combined, filtered through Whatman 1, and concentrated under vacuum. Fifty milliliters of lead acetate–barium acetate (0.5 M each) was added and then enough water to bring the total volume to 500 ml. The mixture was centrifuged (2300 rpm, 10 min) and the

pellet discarded. The supernatant was chromatographed on a column of Sephadex LH-20 with water; the effluent was monitored by a UV detector (226 nm). The eluate corresponding to a large peak due to glucoraphenin was collected and freeze-dried. The solid residue was dissolved in ethanol, filtered, and concentrated to dryness under a stream of nitrogen. ^{1}H NMR (D_2O): δ 2.65–2.8 (2H, m, $C\underline{H}_2$); 2.73 (3H, s, $C\underline{H}_3$); 2.94 (2H, t, J = 7 Hz, $C\underline{H}_2$); 3.4–4.0 (glucose), 5.05 (1H, d, J = 10 Hz, H-1 of glucose); 6.53–6.6 (2H, m, $C\underline{H}$=$C\underline{H}$). ^{13}C NMR (D_2O): δ 29.68, 31.53 ($\underline{C}H_2$); 39.80 ($\underline{C}H_3$); 61.71 (glucose C-6); 70.22 (glucose C-4); 73.00 (glucose C-2); 78.10 (glucose C-3); 81.16 (glucose C-5); 82.73 (glucose C-1); 133.99, 142.40 ($\underline{C}H$=$\underline{C}H$); 163.28 (quaternary C). These resonances were assigned by comparison with the data of Linscheid et al. (1980) and Cox et al. (1984).

Bioassay. Velvetleaf (*Abutilon theophrasti* Medic.) seeds (Valley Seed Service, Fresno, California) were soaked in 10% Clorox for 15 min, then in water for about 4 hr. They were incubated in darkness overnight in Petri dishes lined with wet filter paper. Test solutions were added to 2.5-ml agar solutions (6 g dry agar per liter) in 50 × 9-mm plastic Petri dishes. Each experiment included control dishes with solvent alone. All crude extracts were assayed at 1 mg extract/ml agar, except for the chloroform and aqueous extracts of the aboveground plant parts; they were assayed at 2 mg/ml. Column fractions were also assayed at 1 mg of each fraction per milliliter agar. After the agar had cooled and the solvent had evaporated from the agar, four to five seedlings were placed in each dish. The dishes were covered and incubated in darkness overnight, then evaluated visually for inhibition of seedling and root growth.

Solutions of glucoraphenin (dissolved in water) and sulforaphene (in acetone) were assayed as above, but with 2 ml agar per Petri dish (two dishes at each concentration). Radical lengths (10 per concentration) were measured after 48 hr of incubation. ED_{50} values were estimated from the graphed results (see below).

RESULTS AND DISCUSSION

Identification of Phytotoxic Component. The crude chloroform extract of aboveground plant parts caused visible inhibition of velvetleaf seedling growth; the aqueous wash following chloroform extraction and the chloroform extract of roots were slightly active. Other extracts were not inhibitory. Fractionation of the chloroform extract of aboveground parts on Sephadex LH-20 gave one fraction that completely inhibited the growth of velvetleaf seedlings. When this fraction was chromatographed on thin-layer plates and the chromatograms bioassayed, the activity corresponded to a zone (R_f = 0.4–0.5) in which no spot was evident under either visible or UV light. The remainder of this fraction was

chromatographed on a preparative TLC plate, and the zone of activity was eluted with $CHCl_3$. Comparison of IR, EI-MS, and ^1H NMR data for the eluate with literature data (Balenovic et al., 1966; Spencer and Daxenbichler, 1980; Kawabata et al., 1989) indicated that the active compound was sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate), a hydrolysis product of the glucosinolate glucoraphenin (Figure 1). This compound has been reported as the major glucosinolate in seed and green parts of *Matthiola fruticulosa* (L). Maire [= *M. tristis* (L.) R. Br.; Matas, 1960; Gmelin and Kjaer, 1970; Daxenbichler et al., 1991] and in seed of *M. parviflora* (Schousboe) R. Br., *M. bicornis* (Sibth and Sm.) DC., and *M. sinuata* (L.) R. Br. (Daxenbichler et al., 1991).

Toxicity of Sulforaphene and Glucoraphenin. The sulforaphene sample from stock and the glucoraphenin isolated from radish seeds were assayed against velvetleaf seedlings to determine the phytotoxicity of these compounds. The ED_{50} of sulforaphene was approximately 2×10^{-4} M (Figure 2). A similar value was obtained when the compound was assayed with wheat seedlings (data not shown). This value is comparable to, or lower than, values obtained for several phenolic compounds implicated in allelopathy of red clover (Chang et al., 1969) and yellow fieldcress (Yamane et al., 1992). Glucoraphenin was considerably less active against velvetleaf; its ED_{50} was about 6×10^{-3} M (Figure 2).

Sulforaphene has not previously been reported to be biologically active, although the structurally similar compound 4-methylthio-3-butenyl isothiocyanate is antimicrobial (Esaki and Onozaki, 1982), and 8-methylsulfinyloctyl, 9-methylsulfinylnonyl, and 10-methylsulfinyldecyl isothiocyanates are herbicidal (Kawabata et al., 1989; Yamane et al., 1992). Sulforaphene would appear to be more active than other herbicidal isothiocyanates (Bialy et al., 1990). Furthermore, 4-methylthio-3-butenyl isothiocyanate degrades quickly in water (Esaki and Onozaki, 1982); if this is also true for sulforaphene, our assays may have underestimated its toxicity. That glucoraphenin is less active than sulfor-

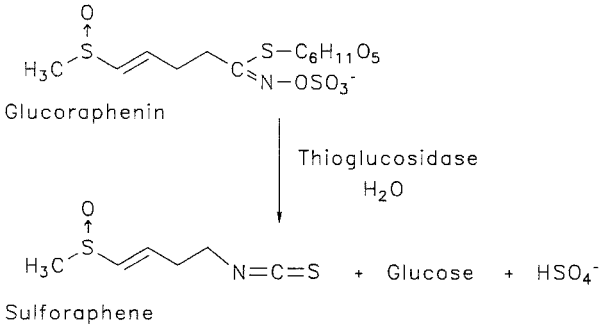

FIG. 1. Enzymatic hydrolysis of glucoraphenin to sulforaphene.

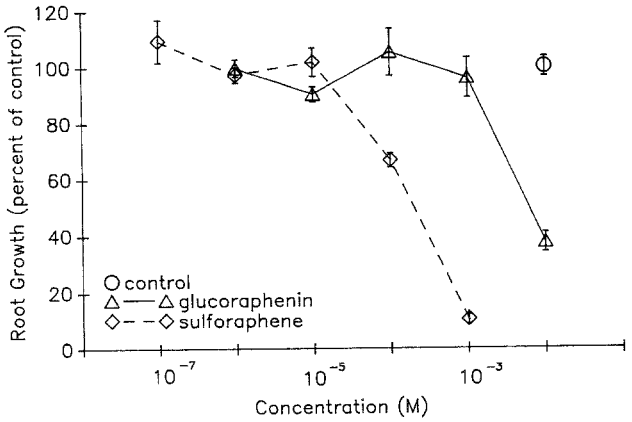

FIG. 2. Inhibition of velvetleaf seedling root growth by glucoraphenin and sulforaphene. Vertical bars represent ± 1 SE.

aphene is not surprising; isothiocyanates are generally more phytotoxic than intact glucosinolates (Bialy et al., 1990). It would appear that the isothiocyanate moiety is important for activity. Thus, any phytotoxicity due to *M. incana* or other *Matthiola* species is probably caused by sulforaphene resulting from the breakdown of glucoraphenin in soil, rather than by glucoraphenin acting directly. Measures were not taken during extraction to prevent hydrolysis of glucoraphenin, and it is assumed that sulforaphene is present in vivo at very low levels, if at all. These particular compounds are comparatively uncommon in nature and thus have not been much studied for biological activity even though glucoraphenin is the dominant glucosinolate in radish seed (Sang et al., 1984; Daxenbichler et al., 1991).

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VARIABILITY OF TERPENE CONTENT IN THE SOFT CORAL *Sinularia flexibilis* (COELENTERATA: OCTOCORALLIA), AND ITS ECOLOGICAL IMPLICATIONS

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Abstract—Colonies of the soft coral *Sinularia flexibilis* (Quoy & Gaimard) (Coelenterata, Octocorallia) were collected at Lizard Island (14°40′S and 145°28′E) Research Station. Extraction of the corals and quantitative chemical analysis for the three major diterpene components, flexibilide, dihydroflexibilide, and sinulariolide, afforded average ratios of 4:3:1 respectively. Colonies, sized on the basis of the sterile stalk circumference, were analyzed for possible correlations between size and chemical composition. The major metabolite, flexibilide, was inversely correlated with colony size, while sinulariolide concentration showed a direct correlation. The concentration of dihydroflexibilide was independent of colony size. Samples were further analyzed with respect to site of collection. Colonies were collected at three distinct reefal sites. One was characterized by large monospecific stands of *Porites cylindrica*, a second was a sandy bottom site with a mixed community of soft corals and occasional scleractinians, while the third site was a very diverse reef community with many species of scleractinian corals. *Sinularia flexibilis* was well represented at each site, and the concentration of flexibilide and sinulariolide varied significantly among sites. The concentration of flexibilide was significantly higher at the third, highly competitive site, while the concentration of sinulariolide was highest at the *Porites*-dominated site. Dihydroflexibilide levels were independent of site. It seems likely that concentrations of flexibilide, a highly cytotoxic molecule involved in interference competition, and sinulariolide, a known algicide probably responsible for colony maintenance, may be influenced by their environments.

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Key Words—Allelopathy, diterpenes, soft corals, *Sinularia flexibilis*, Coelenterata, Octocorallia, variability, competition.

INTRODUCTION

Sinularia flexibilis is a common alcyonacean soft coral that makes up a substantial proportion of living cover on some Indo-Pacific reefs (Dinesen, 1983). Like many soft corals, *Sinularia flexibilis* produces a range of secondary metabolites that participate in a variety of chemical strategies (Sammarco and Coll, 1988). These secondary metabolites, mainly diterpenes, function in defense against predation (Alino, 1989), serve as antifouling agents (see Coll, 1992), and mediate allelopathic interactions (Sammarco et al., 1983).

In terrestrial chemical ecology, the role of secondary metabolites in plant ecology has been the subject of interest for a long time (Rice, 1984). Secondary metabolites in plants are also thought to have several ecological functions, serving as defense against herbivores and pathogens, as attractants for pollinators and fruit-dispersing animals, and as allelopathic agents (Gershenzon and Croteau, 1991).

Another aspect that has been extensively studied in terrestrial chemical ecology is the intraspecific variability in the production and composition of secondary metabolites. Intraspecific variability of secondary metabolites occurs at several levels. It is observed between populations of specific plants, between specimens of a given population, and also among organs of individual plants (Gershenzon and Croteau, 1992). This has been explained on the basis of genetic differences between populations and between individual plants (Lincoln and Langenhein, 1981), the physiological condition of the plant (Rice, 1984), plant age (Bowers and Stamp, 1992), environmental characteristics (Mihaliak et al., 1989) and ecological interactions (Lincoln and Langenhein, 1979; Louda and Rodman, 1983).

In the marine environment, the chemical composition of the soft coral *Sinularia flexibilis* has been extensively studied (see Coll, 1992 for review). Little is known, however, about the variability in composition and concentration of secondary metabolites between individual colonies within this species.

For an organism such as *Sinularia flexibilis*, for which chemical defenses play a major role in its survival and colonization success, individual variability in the composition and concentration of secondary metabolites would have significant implications for the efficiency of these defensive mechanisms.

In this paper, we report the results of a survey of the chemical composition of a number of colonies of *Sinularia flexibilis* collected from three distinct reefs at Lizard Island, Great Barrier Reef, in which we measured the concentration of three major diterpenes—flexibilide, dihydroflexibilide, and sinulariolide—by

^{1}H NMR spectroscopy. The results allowed an assessment of the variability in the production of secondary metabolites by *Sinularia flexibilis*. A discussion of the likely ecological functions of these metabolites is also offered.

METHODS AND MATERIALS

Collection Sites. Colonies of *Sinularia flexibilis* were sampled from three reefs in the Blue Lagoon, Lizard Island, Great Barrier Reef (14°40′S and 145°28′E). The first site (site 1) was the lagoonal reef in front of One Coconut Tree Beach, which faces the eastern entrance of the lagoon. This reef is characterized by extensive monospecific stands of the coral *Porites cylindrica* and a few species of both scleractinian and alcyonarian corals. Site 2 was Loomis reef, which is located towards the western entrance of the lagoon and is characterized by an intermediate abundance of scleractinian and alcyonacean corals. Site 3 was the eastern side of Vicki's reef, which is immediately at the western end of the Blue Lagoon. This reef was sampled because of its very diverse coral community, represented by a large number of scleractinian coral species.

Most of the *Sinularia flexibilis* colonies that could be found at depths of 2–5 m were sampled. *S. flexibilis* normally presents a patchy distribution on the reefs, mainly because of their mode of asexual reproduction by colony division. Our collection was restricted to only one colony per patch, in order to avoid the collection of genetic clones. When a colony was selected, the circumference of the colony sterile stalk about 5 cm from its base was measured. After measurement, sampling involved the cutting with scissors in situ of three branches of the polyp-rich tissues (approximately 2–4 g dry weight of tissue) from each colony, and storing these portions in separate plastic bags. Samples were then frozen at −20°C until they were freeze-dried.

In addition to the sampling of the *Sinularia* colonies, small portions of the scleractinian corals that were in contact or in close proximity (5 cm) to each of the soft corals, were collected. The small scleractinian samples where then bleached in sodium hypochlorite solution for subsequent taxonomic identification, based on the taxonomic guides by Veron and Pichon (1976, 1979, 1982), Veron and Wijsman-Best (1977), Veron and Wallace (1984), and Veron (1986).

Sample Extraction and Preanalysis Fractionation. The freeze-dried coral tissue was extracted with dichloromethane (DCM, 10 ml/g dry weight of tissue) by soaking the ground colonies in sealed vials for two 24-hr periods and decanting the solvent from the samples after each extraction. A portion of the combined extract (1 ml) from each sample was chromatographed on a small silica gel column (Merck Si gel, type 60 for TLC; 4-cm bed packed on a cotton wool plug in a Pasteur pipet). The solvent was forced through the column using compressed air until the column was free of DCM. Second and third fractions

were obtained by elution with a mixture of acetonitrile–DCM (3:2, 1 ml each). The third fraction obtained in this way from each chromatography contained a mixture of the three major diterpenes present in each extract. This fraction was compared between samples by ^{1}H NMR spectroscopy.

^{1}H NMR Analysis. The total diterpene fraction was evaporated to dryness, an accurately known amount of 2,4-dinitrobenzene (~5 mg) added as internal standard, and deuterated chloroform (~0.5 ml) used as solvent. The ^{1}H NMR spectrum of each sample was recorded on a Bruker AM300 NMR spectrometer using a pulse delay of ~5 sec to ensure complete relaxation of the 2,4-dinitrobenzene; 64 scans of each sample were recorded. Spectra were Fourier transformed using zero line broadening and appropriate signals integrated. Signals measured for 2,4-dinitrobenzene resonated at δ7.81, 8.57, and 9.08; signals used for flexibilide resonated at δ6.46 and 3.97; sinulariolide was estimated on the basis of its lactonic methine proton at δ4.07; dihydroflexibilide was estimated on the basis of its lactonic methine signal (δ4.01), which overlapped with the same signal from flexibilide (δ3.97). The quantity of flexibilide was derived from the δ6.46 signal, and the quantity of dihydroflexibilide was derived from the two-proton signal complex near δ4.0. Integration of each spectrum was carried out in triplicate and the means used to estimate the absolute amount of each compound (in milligrams).

Statistical Analyses. Data were analyzed with standard parametric procedures. One-way analysis of variance including post-hoc tests were used for the comparison of the absolute concentrations of the compounds. Linear regression models were used to describe the relationship between the compounds and between compounds and colony size. As colony division in *Sinularia flexibilis* occurs when the basal size of the colony (as measured by its circumference) reaches about 40–50 cm, the analysis of the correlation between basal size and diterpene concentration was therefore restricted to sizes below 45 cm.

For the comparison of the diterpenoid profiles of colonies from the different reefs, we converted the concentration of each compound to a proportion of the total terpene for each colony and applied arcsin transformation to accommodate the ANOVA assumptions.

The Shannon-Wienner diversity index was calculated for the species of scleractinian corals in vicinity of the soft corals from the three reefs sampled. Diversity indexes from these reefs were then pairwise compared using Hutcheson t test. Statistical tests followed routines described by Sokal and Rohlf (1981) and Zar (1984).

RESULTS

Terpene Concentrations. Major differences were found between the concentrations of each of the three diterpenes in the soft coral tissues (one-way ANOVA, $P < 0.0001$). Flexibilide occurred at the highest concentration, with

an average of 8.25 mg/g (dry weight). Dihydroflexibilide appeared at an average concentration of 6.25 mg/g (dry weight) and sinulariolide at an average of 2.00 mg/g (dry weight) (Figure 1).

For the 73 colonies analyzed, the concentration of flexibilide was found to be negatively correlated with the concentration of sinulariolide ($r = 0.524$, $P < 0.0001$) and positively correlated with the concentration of dihydroflexibilide ($r = 0.43$, $P < 0.0001$). There was no correlation between the concentration of sinulariolide and dihydroflexibilide ($r = 0.125$, $P > 0.1$).

Variability between individuals for both the total terpene concentration and the concentration of each of the three terpenes was high. Total terpene concentrations ranged from 7.6 mg/g to 31.6 mg/g (dry weight).

The concentration of flexibilide was found to be negatively correlated with basal size ($r = 0.441$, $P < 0.001$) and, as expected, concentrations of sinulariolide were positively correlated with basal size ($r = 0.268$, $P < 0.05$). That is, as the soft coral increases in size, the concentration of sinulariolide in its tissues increases, while the concentration of flexibilide decreases. No significant correlation was found between dihydroflexibilide and basal size ($r = 0.035$, $P > 0.5$) (Table 1).

Variability Among Reefs. To reduce the size-related variability of diterpene concentrations and to include a size range well represented in all reefs, only soft corals with basal sizes between 25 and 45 cm were included in these analyses.

The average of the total terpene concentration was consistent among soft corals from the different sites (one-way ANOVA, $P > 0.1$), being 17.9 for site 1, 15.8 for site 2, and 16.3 for site 3 (mg/g dry weight).

Although total terpene content was similar at all three reefs, the proportions of the three compounds were different at each reef. Colonies of *Sinularia flex-*

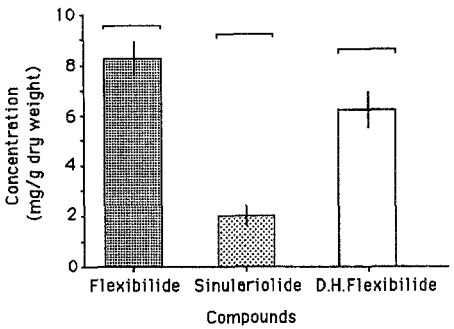

FIG. 1. Average concentration of the three major diterpene compounds from *Sinularia flexibilis*, ANOVA, $P < 0.001$, $N = 73$ for each compound. Error bars show 95% confidence intervals. Horizontal bars show similarities between groups (Tukey test, $P < 0.05$).

ibilis from site 3 contained an average of 54% of flexibilide, significantly higher than the soft corals from site 1 (average 39%). No significant difference was found between the soft corals from site 2 and site 3 (Tukey test, $P > 0.05$) or site 1 and site 2 (Tukey test, $P > 0.05$) (Figure 2).

The proportions of sinulariolide were also significantly different between reefs. This result was expected, because the concentrations of flexibilide were negatively correlated with the concentration of sinulariolide. Soft corals from site 3 contained the lowest proportion of sinulariolide, averaging 5.9% (one-way ANOVA, $P < 0.01$), significantly different from the soft corals from sites

TABLE I. REGRESSION ANALYSIS BETWEEN BASAL SIZE OF *Sinularia flexibilis* AND CONCENTRATION OF ITS THREE MAJOR DITERPENE COMPOUNDS.

	N	*r*	*F*	*P*	Significance[a]
Flexibilide	55	0.441	12.77	<0.001	**
Sinulariolide	55	0.268	4.09	<0.05	*
Dihydroflexibilide	55	0.036	0.06	>0.5	NS

[a] **, highly significant; *, significant; NS, not significant.

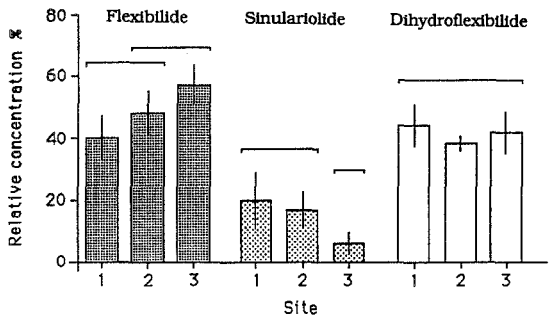

FIG. 2. Relative concentration of the three major diterpenes compounds from *Sinularia flexibilis* according to the site of collection at Lizard Island, showing a significant difference in proportion of flexibilide among sites (ANOVA, $P < 0.01$), a significant difference in proportion of sinulariolide among sites (ANOVA, $P < 0.01$), and no difference in proportion of dihydroflexibilide among sites (ANOVA, $P > 0.1$). Site 1: One Tree Coconut Reef, $N = 14$; Site 2: Loomis Reef, $N = 11$; Site 3: Vicki's Reef, $N = 16$. Proportion data were arcsine transformed to comply with test assumptions. Error bars show 95% confidence intervals. Horizontal bars show similarity between groups (Tukey-Kramer test, $P < 0.05$). Horizontal bars for dihydroflexibilide presented for comparative purposes only.

2 and 3 (Tukey test, $P < 0.05$). There was no significant variation of dihydroflexibilide between sites (one-way ANOVA, $P > 0.1$) (Figure 2).

Taxonomic identification of the scleractinian corals around the soft corals sampled indicated that *Sinularia flexibilis* colonies at site 1 were in interaction with 10 different species of scleractinian corals; at site 2 with 13 species, and at site 3 with 28 species (Table 2). The diversity of scleractinian corals species in interaction with *Sinularia flexibilis* calculated by the Shannon-Wienner index were significantly different between reefs. Site 1 presented the lowest diversity ($H' = 0.82$, $J' = 0.51$); site 2 an intermediate diversity ($H' = 1.06$, $J' = 0.66$), and site 3 presented the highest diversity scleractinian corals interacting with *Sinularia* colonies ($H' = 1.360$, $J' = 0.85$), $P < 0.001$ for all pairs (Hutcheson test) (Table 3).

DISCUSSION

Sinularia flexibilis may well be one of the most successful soft corals on tropical Indo-Pacific reefs. It certainly is the most studied in relation to its chemical composition and to the range of bioactivities associated with this chemistry. Figure 3 gives an indication of some of the chemical constituents reported from this species and suggests structural relationships between key compounds, particularly flexibilide, dihydroflexibilide, and sinulariolide. In structural terms, dihydroflexibilide appears to be something of a "dead-end" compound, in that the reactivity of the α, β-unsaturated lactone system present in sinulariolide and flexibilide has been lost by reduction. As such, it may serve as a storage compound with lower activity and greater stability than flexibilide. This is not inconsistent with the finding that levels of dihydroflexibilide are directly correlated with flexibilide concentrations.

Ecological functions have been attributed to a number of compounds shown in Figure 3, based largely on laboratory bioassays. Thus 11,12-deoxyflexibilide has been shown to be ichthyotoxic towards killifish at <1 ppm, whereas sinulariolide and flexibilide were relatively innocuous at >20 ppm in similar assays using *Gambusia affinis* (see Coll, 1992 for review). Flexibilide and, to a lesser extent, dihydroflexibilide (sinularin and dihydrosinularin) are cytotoxic to cancer cell lines (Weinheimer et al., 1977). They interfere with coral photosynthesis and respiration at <10 ppm (Webb and Coll, 1983), and eventually cause death in scleractinian corals (Coll and Sammarao, 1983). Sublethal levels (1–5 ppm) also cause zooxanthellae expulsion, nematocyst loss, and decrease in polypal activity in scleractinian corals prior to demise (Aceret et al., 1991). Flexibilide and dihydroflexibilide have been detected in the seawater around *S. flexibilis* colonies in the field at concentrations of 1–5 ppm (Coll et al., 1982). This paper reports the concentrations at which the diterpenes are present in the tissues of

TABLE 2. FREQUENCY OF SCLERACTINIAN CORAL SPECIES IN CONTACT WITH *Sinularia flexibilis* AT THREE SAMPLED REEFS

Coral species	Site 1	Site 2	Site 3
Acanthastrea hillae	—	—	1
Acropora cytherea	—	—	1
Acropora divaricata	—	—	1
Acropora humilis	—	—	1
Acropora longicyanthus	—	1	1
Acropora microphthalma	—	2	5
Acropora nasuta	—	—	1
Acropora nobilis	—	—	2
Acropora palifera	—	—	1
Acropora secale	—	2	—
Acropora selago	2	—	2
Acropora tenuis	—	3	—
Acropora valida	—	1	2
Agaricidae	—	—	1
Echinopora horrida	—	1	1
Echinopora lamellosa	2	—	2
Favidae	—	—	1
Galaxea astreata	1	—	—
Goniopora columna	—	1	—
Goniopora pandoraensis	—	1	—
Merulina ampliata	—	—	1
Montastrea curta	—	1	3
Montipora grisea	—	—	4
Montipora tuberculosa	—	—	1
Montipora verrucosa	—	—	1
Pachyseris rugosa	—	—	1
Pavona cactus	1	—	—
Pavona venosa	1	—	—
Platygyra daedalea	—	—	1
Platygyra pini	—	—	1
Pocillopora damicornis	—	1	2
Porites annae	2	—	—
Porites cylindrica	9	—	1
Porites lutea	—	1	4
Porites lychen	1	—	—
Porites sp.	—	—	1
Psammocora contigua	1	—	—
Seriatopora hystrix	—	4	—
Stylophora pistillata	1	—	1
Tubinaria raniformis	—	1	—

TABLE 3. PAIRWISE COMPARISON OF SHANNON-WIENNER DIVERSITY INDEX FOR SCLERACTINIAN CORAL SPECIES AROUND COLONIES OF *Sinularia flexibilis* SAMPLED AT THREE COLLECTION SITES (HUTCHENSON TEST)

Comparison (diversity index)	*df*	*t*	*P*	Significance
Site 1 (H' = 0.82) × site 2	112	4.33	<0.001	**
Site 2 (H' = 1.06) × site 3	123	7.56	<0.001	**
Site 3 (H' = 1.36) × site 1	100	10.55	<0.001	**

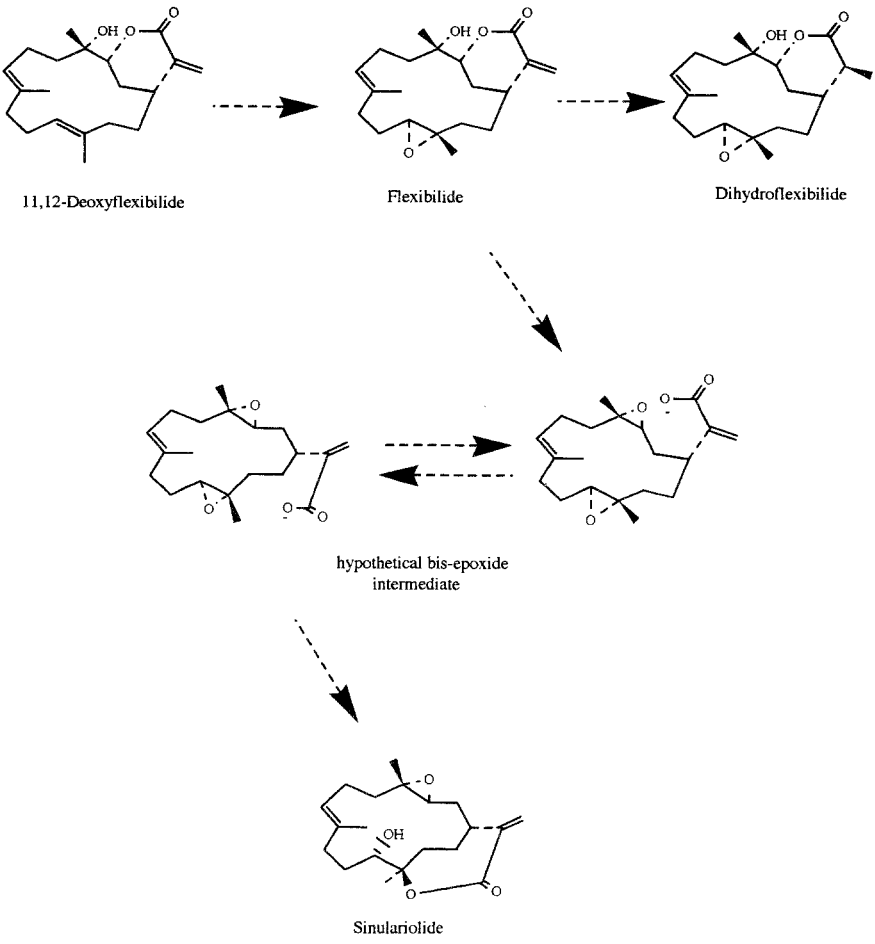

FIG. 3. Cembranolide diterpene metabolites derived from *Sinularia flexibilis* and possible biosynthetic pathways linking them.

S. flexibilis. Flexibilide kills scleractinian corals at 5–10 ppm and is strongly implicated in allelopathic effects on neighboring organisms (Sammarco et al., 1983). The two diterpenes, and particularly flexibilide, have thus been shown to be the major vectors of interference competition for *S. flexibilis*. By contrast, sinulariolide does not possess any of these properties when assayed in similar tests, although it is reported to be an effective algicide (Tursch et al., 1978; Maida, 1993). It thus seems likely that sinulariolide makes a major contribution to the prevention of fouling by algae (Coll, 1992).

Sinularia flexibilis releases secondary metabolites into the surrounding water that are capable of acting as allelopathic agents in competitive interactions (Coll et al., 1982). In a field experiment, Sammarco et al. (1983) observed that all scleractinian corals that were near or in contact with allelopathic soft corals suffered tissue necrosis and growth inhibition. In subsequent field experiments, Sammarco et al. (1985) observed that the effects of *Sinularia flexibilis* on specific neighboring scleractinian corals are variable. That is, while some colonies of a given species of scleractinian suffer deleterious effects when interacting with *Sinularia flexibilis*, other colonies in the same situation might not be affected. Although this variability of effects can be explained by an individual resistance of the scleractinian coral to allelochemicals, it may also be due to the allelopathic potential of a given *Sinularia* colony, i.e., the allelochemical content of the soft coral involved in the interaction.

Sinularia flexibilis is a long-lived soft coral, and the concretion of packed spicules in the lower, older parts of the colonies may guarantee survival, even in the face of severe competition or predation. As colonies grow older, the need for antipredator or anticompetitor compounds might thus decrease. If indeed flexibilide functions as the major vector for *Sinularia flexibilis* in competitive interactions, it might be expected that smaller colonies should have a greater need for this compound than larger colonies. This assumption is supported by the fact that the level of flexibilide was inversely proportional to colony size. The fact that the concentration of sinulariolide increased with colony size may be an indication of the need for antifouling, rather than anticompetitor compounds as colonies age, because larger size offers larger areas for fouling.

Because of the structural relationships outlined in Figure 3, it is possible that sinulariolide and flexibilide are not entirely independent products of biosynthesis. Indeed, the results show a negative correlation between the two compounds. The question then arises as to whether environmental factors play any role in the relative amounts of these metabolites in *Sinularia flexibilis* colonies.

Results from the three different reefs at Lizard Island provide an opportunity to consider this question. Two of the reefs provided the extremes in a spectrum of competitive complexity. Site 1 was dominated by *Porites cylindrica*; interactions there were mainly between *S. flexibilis* and *P. cylindrica* (9/21) and to a lesser extent with nine other species of scleractinian corals. Site 3 was a more

complex community; 45 interactions were studied between *S. flexibilis* and 28 scleractinian coral species. No single scleractinian species dominated these interactions.

Comparisons between the levels of flexibilide and sinulariolide at each of the sites is most revealing. Levels of flexibilide in colonies at the most diverse site 3 were significantly higher than those at site 1. By contrast, levels of sinulariolide at the less diverse site 1 were almost double those at site 3. The total terpene content of the three metabolites (in milligrams per gram of tissue) was independent of site. The variation occurred in the proportions of metabolites at each site. It appears that at the site with the greatest species diversity, colonies produce higher levels of flexibilide (and dihydroflexibilide) at the expense of sinulariolide biosynthesis.

The question then remains as to whether the variability in the concentration of metabolites in *Sinularia flexibilis* is due to an active biosynthetic switch that can trigger the production of one or other compound according to the ecological needs or to the selection of populations that possess specific secondary metabolite profiles that guarantee survival in a particular environment. In the case of sinulariolide and flexibilide, the former protocol is certainly a possibility.

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ORIENTATION OF BARK BEETLES *Pityogenes chalcographus* AND *Ips typographus* TO PHEROMONE-BAITED PUDDLE TRAPS PLACED IN GRIDS: A NEW TRAP FOR CONTROL OF SCOLYTIDS

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Abstract—A puddle trap was designed that is simple to build and efficient in catching bark beetles (Coleoptera: Scolytidae). The trap is insensitive to wind and should be much easier to manufacture than the more complicated perforated pipe and barrier traps commercially available. A 7 × 7 grid of 49 puddle traps baited with aggregation pheromone components of *Pityogenes chalcographus* (chalcogran and methyl decadienoate) was placed at either 1.5-, 3-, 6-, or 12-m spacing between traps in the field for two or more replicates of one day length (June 1989, Torsby, Sweden). The resulting catches showed that beetles were trapped as they flew into the grid since the inner square-ring of 24 traps caught less beetles per trap than the outer square-ring trap average (36 traps) in most experiments. *Ips typographus* also landed in puddle traps primarily on the periphery of the grid (6-m spacing only) when traps were baited with its pheromone components, (*S*)-*cis*-verbenol and methyl butenol. Computer simulation of flying bark beetles in grids of traps of various spacings and catch radii estimated that the experimental pheromone traps had an effective catch radius of 1.3 m or less for *P. chalcographus*, depending on the spacing between traps. An effective catch radius of 2 m for *I. typographus* was found for the 6-m grid spacing. *P. chalcographus* beetles were increasingly disrupted in their orientation to pheromone at the closer trap spacings since the effective catch radius declined linearly with closer trap spacing. However, landing was still precise since unbaited puddle traps within the grid did not catch any bark beetles.

Key Words—Semiochemical, pheromone, pest control, insect trap, Scolytidae, Coleoptera, mass trapping, computer simulation, disruption, effective catch radius.

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INTRODUCTION

The practical use of semiochemicals that disrupt the natural behavior and physiology of pest insects provides the economic foundation for studies of insect chemical ecology. Mass trapping using pheromone-baited traps is one of the primary strategies for control of pest insects (Silverstein, 1981). Bark beetle populations and their effects on tree mortality have been reduced by mass trapping. In 130-ha plots in California, tree mortality caused by the western pine beetle, *Dendroctonus brevicomis* LeC., was reduced to 10% that of former levels for several years following treatment (Wood and Bedard, 1977; Bedard et al., 1979; DeMars et al., 1980).

An epidemic of the European spruce engraver, *Ips typographus* L., occurred in the late 1970s in Norway and Sweden (Austarå et al., 1984). A control program using pheromone-baited traps was initiated in 1979, and in 1980 up to 5 billion *I. typographus* were trapped over extensive areas (140,000 km^2) (Bakke, 1985, 1988, 1989). The epidemic declined in 1981 and by 1982 in some areas it was hard to find Norway spruce, *Picea abies* (L.), killed by bark beetles. However, it is not known with certainty whether the mass trapping or other climatic and biological factors caused the decline. The trap used (N79 pipe trap with funnel) was relatively complex: consisting of a 1.35-m × 12-cm-diam. plastic tube with about 900 2-mm-diam. holes distributed over the surface, a 33-cm-diam. outer plastic funnel, a 12-cm-diam. inner plastic funnel and collection bottle, pheromone dispenser and holder, and a wooden stake for mounting the trap (Bakke et al., 1983; Regnander and Solbreck, 1981).

Like other pest bark beetles (Byers, 1989), *I. typographus* aggregates on host trees in response to an aggregation pheromone consisting of 2-methyl-3-buten-2-ol and (1*S*,4*S*,5*S*)-*cis*-verbenol (Bakke et al., 1977). Host-tree compounds are not effective in enhancing the attraction to pheromone components, and uninfested logs in the field are unattractive when beetles are known to be flying (Schlyter et al., 1987c). The smaller European spruce engraver, *Pityogenes chalcographus* L., is also attracted to a synergistic blend of the pheromone components, chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane) and methyl (*E*,*Z*)-2,4-decadienoate (Francke et al., 1977; Byers et al., 1988, 1989, 1990b). Monoterpenes such as α-pinene from the host increase the attraction response to the pheromone components (Byers et al., 1988).

No studies have determined the effects of spacing many pheromone traps at different distances in a grid on the orientation of these two beetles. Computer simulation of bark beetle flight through a grid of traps of various diameters also has not been attempted. The objectives of the present study were: (1) to investigate orientation of flying *P. chalcographus* and *I. typographus* in a grid of 49 pheromone-baited traps at different spacings, (2) to simulate reductions of catch as beetles fly through a grid of traps and compare these results to catches in the

field traps in order to determine effective catch radii for the pheromone-baited traps under different conditions, and (3) to construct a simple and, therefore, inexpensive trap that would be easy to set up in the field and be about as effective as the pipe trap above in catching bark beetles.

METHODS AND MATERIALS

Pheromone-Baited Puddle Traps for Control of Bark Beetles. The puddle trap is constructed from 1.5-mm wire looped into a 28-cm-diam. ring to form the rim of the "swimming pool" (Figure 1). After joining the wire to form the loop, the wire is arched to the opposite side of the ring (with a 1-cm loop at the top for attachment of dispensers and rain/sun shield) and twisted several times to strengthen the wire skeleton. A 0.5 × 0.5-m white polyethylene trash bag is cut to obtain a plastic sheet that is stretched over the ring, around the arching wires, and then under itself, whereupon the trap is placed in a scooped-out depression in the soil. Water is poured over the stretched plastic which sags slightly forming a pool with sloping plastic sides. The weight of the water and the low profile keep the trap from blowing away even under windy conditions. The final step is to attach a plastic cup or aluminum rain and sun shield that contains pheromone dispensers.

Dispensers can be taped inside plastic cups (Figure 1) that are covered with aluminum foil to shield chemicals from the sun and rain. In the present experiments, however, aluminum foil formed into a 5 × 5-cm-diam. cup was used

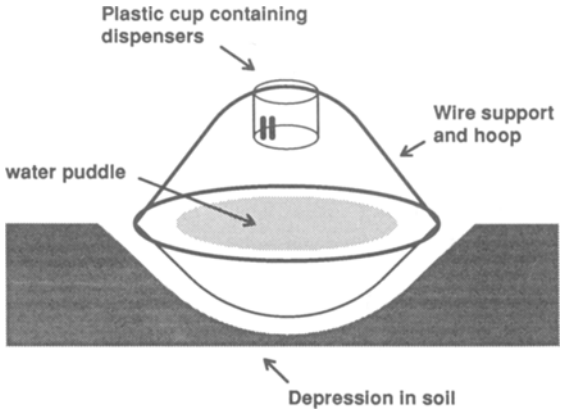

FIG. 1. Puddle trap constructed of wire hoop for support of plastic sheet containing water pool and wire arch for holding a plastic cup (covered with aluminum foil) containing pheromone dispensers. A depression, as indicated, is dug in the forest duff to form and support the water pool held by the plastic sheet (see text for details).

to shield a glass vial containing a mixture of pheromone components. For *P. chalcographus*, open 3.2 × 1.1-cm-diam. glass vials (0.525-cm-diam. opening) were used, one per trap, containing 50 μl of a stock mixture of 200 μl chalcogran (46:54 *E*:*Z*, 98% pure from W. Francke, University of Hamburg, Germany), 200 μl methyl (*E*,*Z*)-2,4-decadienoate (99.5% pure, Shell Agrar), and 2.6 ml (−)-α-pinene ($[\alpha]_D^{22} = -42°$).

The diffusion–dilution equation for obtaining predicted semiochemical release rates (Byers, 1988a) by dilution with solvent:

$$ml_s = fw_s * (g_{sem}/fw_{sem} - f_{sem} * g_{sem}/fw_{sem})/f_{sem}/g_s \quad (1)$$

can be solved for the mole fraction of the chemical (equal to the fraction of the release rate when neat):

$$f_{sem} = 1/(fw_{sem} * ml_s * g_s/(fw_s * g_{sem}) + 1) \quad (2)$$

where f_{sem} = mole fraction of semiochemical or the proportion of the release rate when neat; fw_{sem} = formula weight of semiochemical; ml_s = milliliters of solvent; g_s = grams solvent per milliliter (density); fw_s = formula weight (or molecular weight) of solvent; and g_{sem} = grams of semiochemical. Weighted averages can be used for a mixture of solvents.

Based on the release rates for a similar length tube and neat chemicals (Byers et al., 1988), ratio of the areas for dispenser openings (2.53), and the rearranged diffusion–dilution equation 2 above, the expected release (milligrams per day per dispenser) was about 0.15 mg for chalcogran, 0.003 mg for methyl (*E*,*Z*)-2,4-decadienoate, and 31 mg for (−)-α-pinene. For attraction of *I. typographus*, (1*S*,4*S*,5*S*)-*cis*-verbenol was released at 1 mg/day/trap and 2-methyl-3-buten-2-ol at 50 mg/day/trap from dispensers described previously (Schlyter et al., 1987c).

Trapping of Bark Beetles with a 7 × 7 Grid of Pheromone-Baited Puddle Traps. Grids of 49 puddle traps, as described above, were placed in two relatively flat clear-cut areas of Norway spruce (plots 1 and 2) about 1 km apart and 7 km south of Torsby, Sweden. Plot 1 was approximately 120 × 90 m and the grid (6 m between trap lines) was placed at least 15 m from the forest edge. Experiments were conducted with *P. chalcographus* on June 7, 10, and 11, and with *I. typographus* on June 15–17, 1989. Plot 2 was larger (150 × 200 m), and the 49 traps in the grid, baited with *P. chalcographus* pheromone components, were placed at 12-m spacings on June 10–12. The traps were at least 20 m from the forest edge. The spacing was changed to 3 m for experiments on June 13 and 14, and then to 1.5 m on June 15–17, 1989. Beetles that had been caught the previous day were counted the following morning before the flight period as they floated in the puddle traps. The beetles and other debris were removed by straining the water with a fine screen.

Data from the catches of puddle traps in the 7 × 7 grids were presented

graphically with a personal computer program (QuickBASIC 4.5 and Adobe PostScript command language). Contouring of the catch data was achieved with the algorithm presented by Dixon and Chapman (1980). However, a three-dimensional view was effected by plotting the x coordinate after adding to the x coordinate the corresponding y coordinate multiplied by a fixed scaling value (0.6) and plotting the y coordinate by multiplying the y coordinate by a fixed scaling value (0.45).

Computer Simulation of Trapping as Bark Beetles Immigrate into a 7 × 7 Grid of Traps. The trap grids probably would catch flying beetles as they immigrated (or were attracted) into the area. The catch per trap on traps in the outer ring (24 traps on the periphery of the grid) should thus be higher than on the 16 traps in the inner ring (traps just within the outer ring). Catches of each species per trap for the outer ring of traps, the inner ring traps, and the center-ring traps (8 traps surrounding the center trap) were averaged for each grid spacing on several dates. Ratios were calculated for the catch per trap for traps in the outer-ring trap to the inner-ring. These ratios served as a comparison to the ratios found in a computer simulation model, modified from a mass trapping simulation model (Byers, 1993) that is based on a mate-finding model (Byers, 1991). The program code is available upon request.

In the simulation model, the trap and pheromone plume radius, analogous to the effective attraction radius (Byers et al., 1989), can be independently varied as well as the x and y axes, the number of beetles, their step size and turning angles, and the number of traps and their spacing in the grid. Beetles were "released" at random only on the periphery of the area. Since they are not allowed to move outside the rectangular boundaries, the beetles rebound at random angles back toward the grid of traps (i.e., they immigrate into the grid as in nature). The simulation area enclosed grids of 49 traps of different spacing, and the simulation ended when all beetles were caught. A record of which traps (outer, inner, or central rings) caught beetles was kept so that ratios could be compared to the catch ratios from the field in order to calculate theoretical, effective catch radii.

RESULTS

Pheromone-Baited Puddle Traps for Control of Bark Beetles. Both *Ips typographus* and *Pityogenes chalcographus* were caught readily by the puddle traps (Figures 2–6). A comparison of several trap types (June 11, 1989), each baited with methyl butenol and *cis*-verbenol at the rates above from different experiments within 200 m of each other indicated that the puddle trap (Figure 1) is efficient in catching bark beetles. This trap (with plastic cup) caught 110 *I. typographus*, while four tubular sticky-screen traps at 1.5-m height (30 cm

long × 30 cm diam.; Byers et al., 1990a) averaged 81 ± 63 (±SD). Two pipe traps with funnel (Bakke et al., 1983) caught 32 ± 13, and three cross-pane window traps (Schlyter et al., 1987b) averaged 28 ± 8. It was found that detergent (for lowering the water surface tension) was not necessary to drown the beetles. In pure water, beetles would continue to move on the surface for many hours but none could leave due to their inability to climb out of the water and up the plastic sheet. Eventually beetles would sink and drown; rain caused relatively more to sink.

Observations indicated that beetles of both species oriented with a casting and/or circling flight to the trap and then either struck the plastic cup that shielded the dispensers and fell into the water or landed directly in the water or on surrounding plastic. Although white plastic traps were used in the present experiments, black plastic traps also caught the beetles. The black color worked even without water when it was sunny as beetles that landed could not find a perch to initiate flight and died within seconds from extreme heat.

Trapping of Bark Beetles with a 7 × 7 Grid of Pheromone-Baited Puddle Traps. The catch of *Pityogenes chalcographus* in puddle traps spaced 6 m apart in plot 1 on June 7 totaled 4086 and was highest along one edge of the grid nearest the forest (Figure 2A). A few days later, on June 10, a similar pattern was evident (Figure 2B) with a total catch of 2367. The next day, however, the catch of 11,974 was more uniformly spread throughout the grid of traps (Figure 2C). A second grid of traps at 12 m spacing was set up in plot 2 on June 10 (Figure 3A) and the total catch of 132 was much less than in plot 1. The next day 1136 beetles were caught (Figure 3B), and the distribution was more uniform than on the previous day. On June 12, 4794 beetles were caught in a distribution similar to June 10 in the same plot (Figures 3A and 3B).

The 12-m spacing was reduced to 3 m so that the new grid was well inside the former area. This grid caught 1216 *P. chalcographus* (Figure 4A) but the next day only 224 (Figure 4B), although the patterns were similar. The grid size was further reduced in plot 2 to a 1.5-m spacing. The catch totaled 238 on June 15 (Figure 5A), and 643 from 0900 to 1500 hr, and 323 from 1500 to 2100 hr on June 16 (Figure 5B and 5C). In all experiments, two additional traps without pheromone were placed in each grid equidistant between the diagonal corners of the inner-ring traps and the center-ring traps. None of these controls caught any bark beetles during the tests.

Since the high catches of *P. chalcographus* in plot 1 on June 11 were taxing my ability to count them, the baits were removed and replaced with those for *I. typographus*. On June 13 and 14, the pattern of catch was quite similar (total catches of only 77 and 58, respectively, Figure 6A and 6B). On June 15, a small shift in catch took place, but most still were caught closest to the forest (along the bottom edge of the figures, total of 47, Figure 6C); on June 16 a

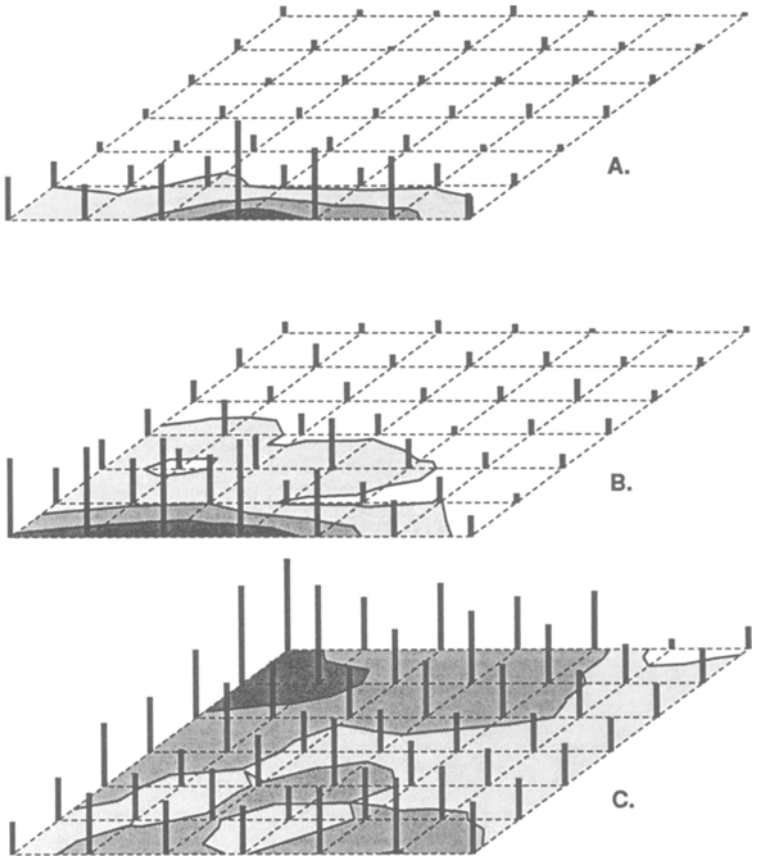

FIG. 2. (A) Catches of bark beetle *Pityogenes chalcographus* (June 7, 1989) in 49 puddle traps baited with synthetic pheromone components (see text) and placed in a grid of 6-m spacing (plot 1, Torsby, Sweden). Contour lines represent increments of 25% of the maximum trap catch (largest bar = 476). (B) Same experiment on June 10, 1989 (largest bar = 178). (C) Same experiment on June 11, 1989 (largest bar = 465).

significant proportion was caught on the side farthest from the forest (total catch of 53, Figure 6D).

Computer Simulation of Trapping as Bark Beetles Immigrate into a 7 × 7 Grid of Traps. The simulation model is represented pictorially in Figure 7, where "traps" of radius 1.3 m (for example) are shaded circles placed at a 6-m spacing. The tracks of 60 simulated beetles are shown entering the periphery of the area at random and flying in the area until caught (Figure 7). The fact that "beetles" were not allowed to leave the simulation area is equivalent to intro-

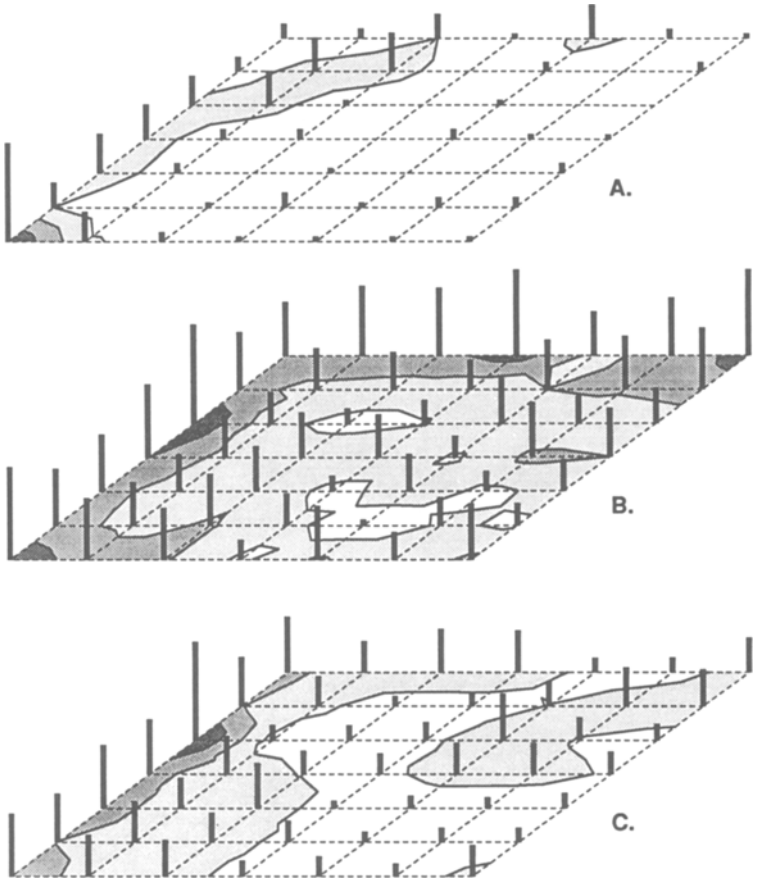

FIG. 3. (A) Catches of bark beetle *Pityogenes chalcographus* (June 10, 1989) in 49 puddle traps baited with synthetic pheromone components (see text) and placed in a grid of 12-m spacing (plot 2, Torsby, Sweden). Contour lines represent increments of 25% of the maximum trap catch (largest bar = 20). (B) Same experiment on June 11, 1989 (largest bar = 50). (C) Same experiment on June 12, 1989 (largest bar = 319).

ducing a new beetle when one leaves, and thus all catch ratios of inner to outer traps are based on the same number of beetles. In the actual simulations, the distribution of trap catches of 4000 beetles at each trap radius (Figure 8) was used to obtain ratios of inner to outer trap catches.

For a grid of 49 traps at 6-m spacing, as the simulated trap radius (effective catch radius) is changed from nearly 0 to a maximum of 3 m, the ratio of catch for the trap catch average on the outer ring of 24 traps to that on the inner ring of 16 traps increases from one to nearly infinite (Figure 8). The curve could not

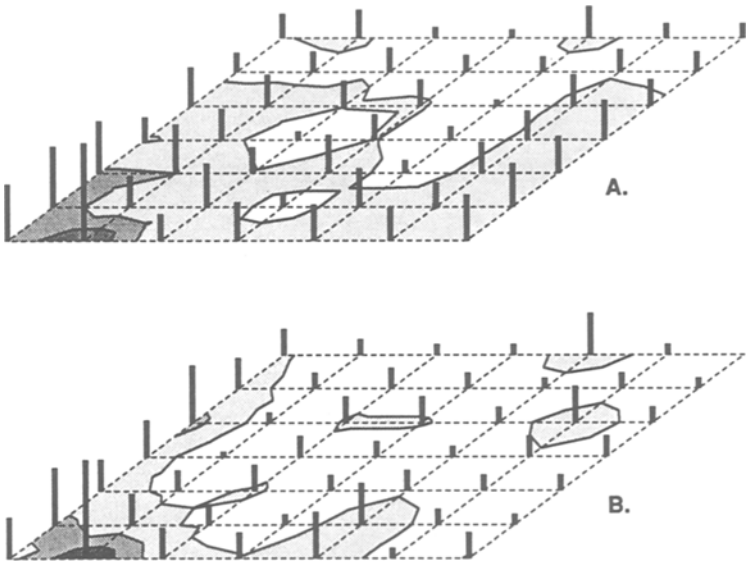

FIG. 4. (A) Catches of bark beetle *Pityogenes chalcographus* (June 13, 1989) in 49 puddle traps baited with synthetic pheromone components (see text) in a grid of 3-m spacing (plot 2, Torsby, Sweden). Contour lines represent increments of 25% of the maximum trap catch (largest bar = 82). (B) Same experiment on June 14, 1989 (largest bar = 19).

be fit by standard curvilinear equations (exponential, logarithmic, geometric, or quadratic). However, the reciprocal of the ratio (i.e., catch per trap on the inner ring divided by the catch per trap on the outer ring) gave a relationship that was fit perfectly by the quadratic equation $Y = aX^2 + bX + c$ ($r^2 = 0.999$). The best fitting equation used $a = 0.0694$, $b = -0.56$, and $c = 1.015$. Thus the equation for the relation between the trap radius and the outer/inner catch ratio is then the reciprocal of the quadratic equation. To solve for X given Y, the equations are solved in terms of Y. Therefore, a catch ratio (inner/outer) of $Y = 0.3$ yields an effective catch radius of $X = 1.59$ m for a trap as found from the equation:

$$X = \frac{-b - \sqrt{b^2 - 4a(c - Y)}}{2a} \tag{3}$$

Similarly, a catch ratio (outer/inner) of $Y = 9$ yields an effective catch radius of $X = 2.23$ m as found from the equation:

$$X = \frac{-b - \sqrt{b^2 - 4a[c - (1/Y)]}}{2a} \tag{4}$$

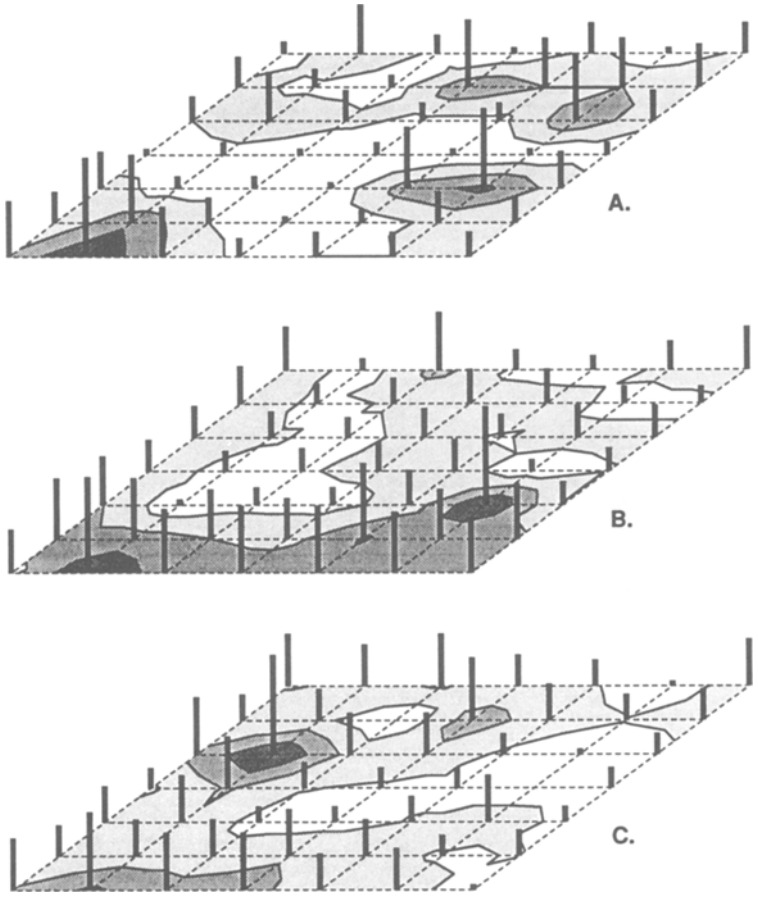

FIG. 5. (A) Catches of bark beetle *Pityogenes chalcographus* (June 15, 1989) in 49 puddle traps baited with synthetic pheromone components (see text) in a grid of 1.5-m spacing (plot 2, Torsby, Sweden). Contour lines represent increments of 25% of the maximum trap catch (largest bar = 16). (B) Same experiment on June 16, 1989, between 0700 and 1500 hr (largest bar = 34). (C) Same experiment on June 16, 1989, between 1500 and 2100 hr (largest bar = 19).

For comparison to the field catches either equation 3 or 4 can be used to solve for the effective catch radius in the field, assuming the field conditions are simulated appropriately by the model. Simulations at spacings of 1.5 m and different trap radii gave best fitting quadratic coefficients of $a = 0.8788$, $b = -2.042$, and $c = 0.97$; similarly, for 3-m spacings: $a = 0.2067$, $b = -1.026$,

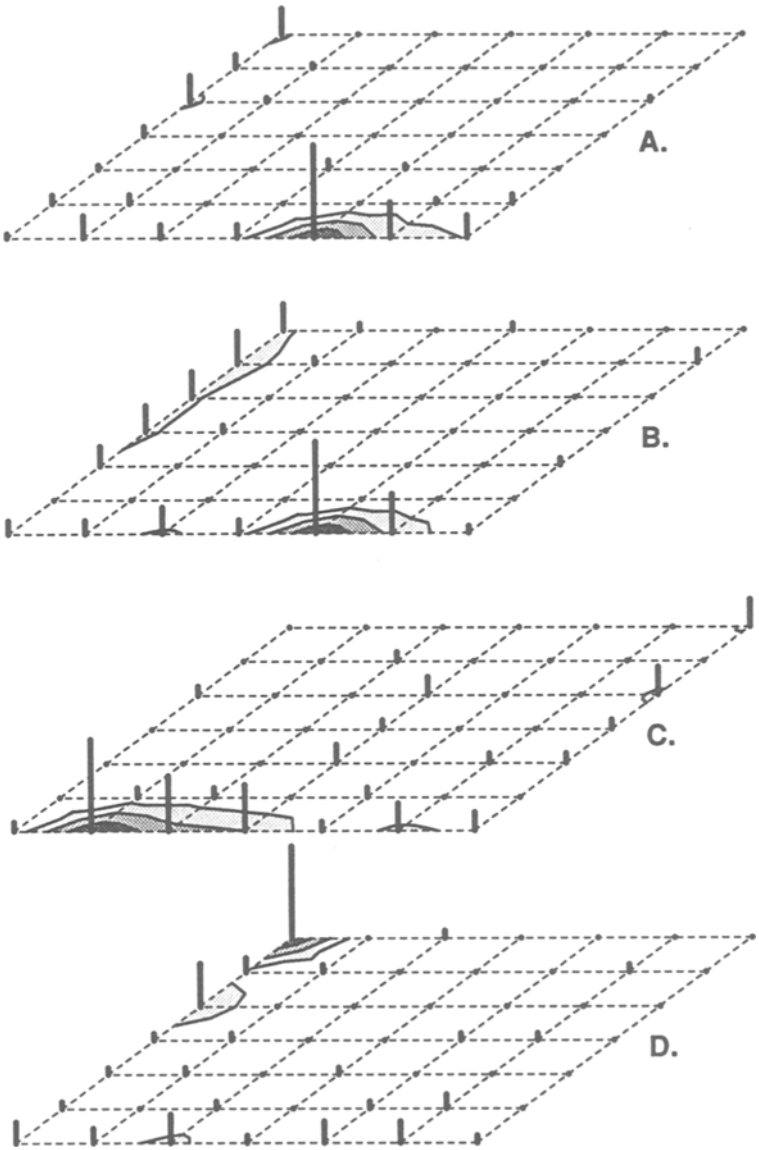

FIG. 6. (A) Catches of bark beetle *Ips typographus* (June 13, 1989) in 49 puddle traps baited with synthetic pheromone components (see text) in a grid of 6-m spacing (plot 1, Torsby, Sweden). Contour lines represent increments of 25% of the maximum trap catch (largest bar = 21). (B) Same experiment on June 14, 1989 (largest bar = 14). (C) Same experiment on June 15, 1989 (largest bar = 10). (D) Same experiment on June 16, 1989 (largest bar = 13).

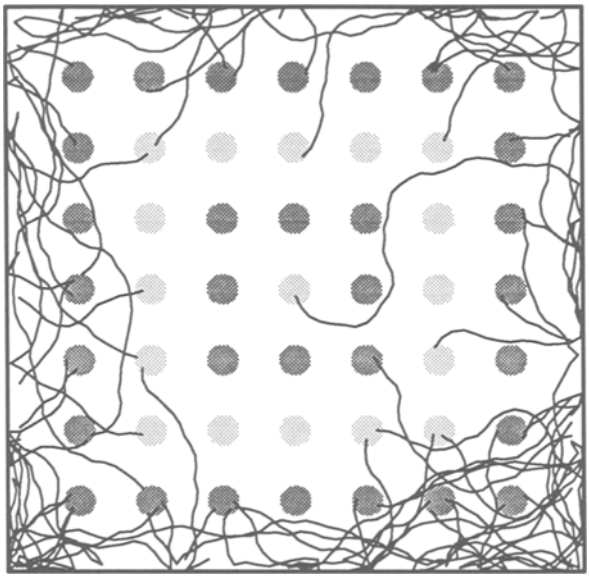

FIG. 7. Paths (wavy lines) of 60 "bark beetles" during simulation of their flight into a grid of 49 traps of effective catch radius equal to 1.3 m. The simulation area was 48 × 48 m with 6-m spacing between traps. Beetles were released at random along the edges of the area and they were not allowed to leave. The movements employed a maximum turn angle of 30° and steps of 1 m. In simulations that varied the effective trap radius, a ratio was obtained that compared the average catch per trap on the outer ring of 24 traps to the average per trap in the next inner ring of 16 traps.

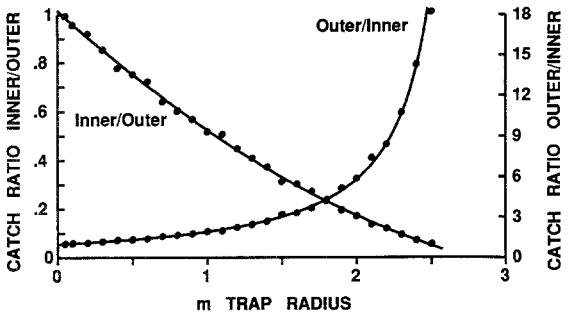

FIG. 8. Quadratic relationship between the simulated trap radius (see Figure 7) and the ratio of catch per trap on the inner ring of 16 traps and the outer ring of 24 traps (Inner/Outer) and the reciprocal quadratic relationship between the trap radius and the simulated catch ratio of the outer 24 traps and the inner 16 traps (Outer/Inner). Each point represents results from an average of four simulations each using 1000 "beetles" with model parameters as in Figure 7 (see text for details).

and $c = 1$; and for 12-m spacings: $a = 0.0242$, $b = -0.311$, and $c = 0.992$ ($22 \geq N \leq 26$ and $r^2 \geq 0.99$ for each regression).

Table 1 reports the outer/inner catch ratios (from Figures 2–6) and the corresponding expected trap radii using equation 4 for each of the quadratic equations at the 1.5-, 3-, 6-, and 12-m trap spacings. The average effective catch radius for *I. typographus* was 2.04 ± 0.66 m (±95% CL) at the 6-m grid spacing. The relationship between the spacing of traps in the grid and the effective catch radius for *P. chalcographus* is shown in Figure 9. Thus, the effective catch radius in the field was very small when traps were closely spaced at 1.5 m apart but increased linearly as the distance between traps was increased. This

TABLE 1. AVERAGE CATCHES AND CATCH RATIOS OF *Pityogenes chalcographus* AND *Lps Typographus* PER TRAP FOR TRAPS IN VARIOUS RINGS (SEE FIGURE 7) OF 7 × 7 TRAP GIRD (VARIOUS SPACINGS AND DATES, 1989).[a]

	Average Trap catch				
P. chalcographus	Outer ring	Inner ring	Center ring	Outer/inner catch ratio	Effective catch radius (m)
12-m spacing					
Figure 3A, June 10	3.7	2.3	0.9	1.65	0.81
Figure 3B, June 11	28.8	18.5	15.9	1.56	0.73
Figure 3C, June 12	118.5	77.5	80.4	1.53	0.71
6-m spacing					
Figure 2A, June 7	108.4	62.7	54.8	1.73	0.87
Figure 2B, June 10	55.5	40.8	42.9	1.36	0.54
Figure 2C, June 11	246.0	248.8	236.8	0.99	0.01
3-m spacing					
Figure 4A, June 13	28.7	23.5	16.4	1.22	0.37
Figure 4B, June 14	5.5	3.7	3.9	1.48	0.66
1.5-m spacing					
Figure 5A, June 15	4.9	5.8	3.3	0.86	0.00
Figure 5B, June 16	14.9	13.0	8.5	1.14	0.26
Figure 5C, June 16	7.0	7.0	5.0	0.99	0.02
Ips typographus					
6-m spacing					
Figure 6A, June 13	2.9	0.3	0.4	9.20	2.24
Figure 6B, June 14	2.3	0.1	0	18.67	2.48
Figure 6C, June 15	1.5	0.3	0.5	4.93	1.90
Figure 6D, June 16	1.8	0.6	0.2	3.11	1.53

[a]The ratio of the outer ring ($N = 24$) catch average divided by the inner ring ($N = 16$) catch average was used in the reciprocal quadratic equation 4, as determined by simulations, to find the effective catch radius.

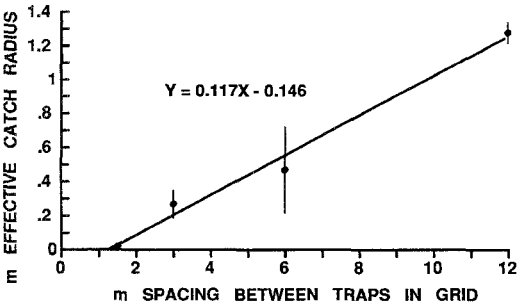

FIG. 9. Linear relationship between the spacing distance between field traps in the grid and the size of the effective catch radius for *Pityogenes chalcographus*. The effective catch radius, X, is found from equation 4, where the coefficients were obtained from simulations at the respective spacings, and Y is the ratio of catch on the outer-ring–inner-ring traps in the field. Each point represents the average from Table 1 ($\pm$SEM).

indicates that at closer trap spacings, there was significant competition between traps in attracting beetles since in principle the effective catch radius should be constant for a specific pheromone release rate.

DISCUSSION

One of the earliest traps to be used to catch bark beetles is the barrier or window trap (Chapman and Kinghorn, 1958), and many modifications of this type of trap have been used (Schlyter et al., 1987b; Tunset et al., 1988). Several large funnels at various heights in the forest canopy were used by Gara (1963) to catch *Ips paraconfusus*. A series of several funnels (the multiple-funnel trap) each directly above the other serves as both a barrier and collecting apparatus (Lindgren, 1983). The pipe trap described earlier and used in the mass trapping program in Scandinavia (Bakke et al., 1983) has served as the standard experimental trap in several subsequent studies (Schlyter et al., 1987a–c; Byers et al., 1988). The bucket trap with small holes, and similar designs, derives from the pipe trap where beetles must enter holes as if they were seeking mates and host tissue (Moser and Browne, 1978; Byers, 1983a).

The pipe trap can be used without a funnel but relatively less beetles are caught since the funnel collects falling beetles that strike the pipe barrier (Bakke et al., 1983; Regnander and Solbreck, 1981). Relatively more males of *I. typographus* are caught by pipe traps when they have a funnel since males are relatively less attracted compared to females when the concentration of aggregation pheromone increases (Schlyter et al., 1987a,b), a phenomenon found also for *I. paraconfusus* and *P. chalcographus* (Byers, 1983b; Byers et al., 1988).

Barrier traps work rather well for larger scolytids such as most *Ips* species since they often can not recover their flight ability after striking the barrier. However, for smaller scolytids such as *P. chalcographus* (2 mm long) the insects have less momentum and can more often recover after striking the barrier. Electrostatic forces at the surface of plastic barriers also can affect small insects more so than larger ones.

The sticky trap has been commonly used in semiochemical experiments for catching all sorts of scolytids and associated insects (Bedard and Browne, 1969; Browne, 1978; Byers, 1983b; Byers et al., 1989). The problem with sticky traps, of course, is that they must be picked by hand (laborious and time consuming) or cleaned with a solvent (thus the trap must be replaced). Furthermore, heavy rain, which occurs often in Scandinavia, soon reduces the trapping efficiency of Stikem Special. The problem with pipe traps, multiple-funnel traps, and window traps is that they are relatively complex to construct. Field olfactometers, consisting of a fan and formed sheet metal (Vité and Gara, 1962; Gara, 1963), mechanical rotary nets (Chapman and Kinghorn, 1958; Vité and Gara, 1962), wind-vane traps (Byers, 1988b), and mechanical, slow-rotation sticky traps (Byers et al., 1990a) are even more complex. This complexity (and expense) is appropriate for certain kinds of experimental purposes but it is a disadvantage for larger-scale experiments and control programs.

The puddle trap (Figure 1) is simple, easy to set up, and inexpensive compared to pipe (Scandinavia), multiple-funnel (U.S.A./Canada), or schlitzfalle (Germany) traps. Puddle traps are also easily transported, being constructed of wire and plastic sheeting. The traps can be reused after each replicate by simply straining the insects from the water. Rain has little effect on the trap since beetles are pounded down to the bottom, and later if the rains continue, water overflows the edges without taking the insects. Due to the low profile and heavy weight of the water pool, even strong winds of several meters per second have no effect on the trap. The puddle trap could be constructed as a broad conical dish of polypropylene and have a floating pheromone dispenser. This design would allow stacking of traps for transport and make them easy to manufacture so that many more traps could be employed in control programs for the same cost, thus increasing the prospects of success.

In the experiments reported here, synthetic chalcogran was released at about 0.15 mg/day from each trap [46% racemic *E* isomer of which half is the bioactive (2*S*,5*R*) enantiomer; Byers et al., 1989]. This could be equivalent to the release from 208 males feeding in a log [360 ng chalcogran/male/day released of which 46% is (2*S*,5*R*); Schurig and Weber, 1984; Byers et al., 1989, 1990b]. The release of methyl (*E*,*Z*)-2,4-decadienoate from *P. chalcographus* has not been determined, but in the abdomen it is present at about 10% that of chalcogran (Birgersson et al., 1990). Assuming proportional release rates for the two components, then a release of methyl (*E*,*Z*)-2,4-decadienoate of 2.4 μg/

day from the trap is equivalent to the release from 67 beetles. Individual male *I. typographus* feeding in Norway spruce trees released an average of 0.16 mg 2-methyl-3-buten-2-ol per day (Birgersson and Bergström, 1989) so the traps that released 50 mg/day were equivalent to 312 beetles. Release of *cis*-verbenol at 1 mg/day from the traps was equivalent to 178 *I. typographus* feeding in trees, while release of α-pinene at 31 mg/day corresponded to release from about 39 entrance holes (Birgersson and Bergström, 1989).

It is difficult to compare the catches in grids of the same size and plot but on different dates since the wind and temperature, as well as the population density would be expected to vary with time. Different sizes of grids on the same plot may vary in catch patterns not only due to time but also due to changes in spatial dimensions. Figures 2–6 illustrate the variation in trap catch and, presumably, the densities of flying beetles as affected by microclimate and wind patterns. In spite of the catch variation, the outer ring of 24 traps caught proportionally more per trap than the inner ring of 16 traps, and these usually caught more than a trap on the center ring (Table 1). This pattern is consistent with the expectation that beetles entering the grid would be attracted to the first traps they encountered, while the proportion not caught (due to chance and going between traps) would fly until encountering the next ring of traps where they have yet another chance of being attracted and trapped. The likelihood of beetles passing the outer ring of traps on their way through the grid is dependent on the effective size of the traps, i.e., higher pheromone releases would effectively create a larger trap. This hypothesized "filtering" effect is also evident in the results of Bakke et al. (1983), where the number of *I. typographus* caught per trap declined towards the center in a hexagonal grid of 91 pipe traps spaced 20 m apart.

The simulation model (Figure 7) varied the size of the effective catch radius from very small, so that all traps would catch about the same and thus the catch ratio of outer–inner traps would be 1, to very large, so that the ratio would become infinite (Figure 8). By comparing the catch ratio from the field trapping at a particular grid spacing to the simulation results, it is possible to estimate a theoretical effective catch radius for the field traps for each bark beetle species (Table 1). The estimated effective catch radius for *I. typographus* of 2.04 m (at the 6-m grid spacing) corresponds remarkably closely to the effective attraction radius (EAR) of 1.9 m reported earlier at the same pheromone release rate (Byers et al., 1989).

The four different spacings of traps in the grids and their catches can be used in a simulation model to estimate four effective catch radii for these traps on *P. chalcographus* (Figures 7–9). In principle, the same effective catch radius should be calculated from field catches regardless of the grid spacings of traps, as long as the true catch radii do not overlap. At the largest spacings between traps, the calculated effective catch radius was largest (Figure 9), indicating that

beetles were experiencing the least difficulty orienting to these traps. At even greater spacing between traps (not tested here), the effective catch radius should stabilize in magnitude since pheromonal interactions between traps would not occur. Thus, this estimated value should remain constant for a given pheromone release rate, regardless of the population density. Since the estimate of the catch radius decreased with closer spacing of traps, and was nearly zero at 1.5-m spacing (Figure 9), this indicates that beetles were increasingly disrupted in orientation to pheromone at closer trap spacings. The pheromone plumes from these traps probably intermingled to the extent that a significant proportion of beetles orienting first to one trap might not land but follow a coalescing plume to another trap. However, even at the 1.5-m spacing between traps, there was apparently no confusion as to where to land since the control traps (only 1.06 m from four other pheromone traps) caught no beetles.

The simulation model above was derived from another that represents graphically the movement of insects in an area where mass trapping is ongoing (Byers, 1993). The model parameters for the mass trapping are: (1) the x and y dimensions of the area, (2) the number of traps, (3) the trap's effective catch radius, (4) the placement of traps at random with a minimum spatial separation or in uniform rows and columns, and (5) the test duration. The model parameters for the insects are: (1) the number of insects, (2) the average speed, (3) the step size, and (4) the maximum angle of deviation within which a random angle is taken from the former direction at each step. Initial directions and turning angles at each step are random for each insect. The model led to discovery of iterative equations that can predict the mass trapping efficiency of a particular set of model parameters above and provide a basis for the design of mass trapping experiments and control programs (Byers, 1993).

Tilden et al. (1981) tested the effects of release of synthetic pheromone from a 7 × 7 grid of 49 release points (but no traps) at a 15-m spacing on the orientation of the bark beetle, *D. brevicomis*, to a center trap and pheromone source. They found that the many pheromone sources disrupted the orientation to the source, since 97% fewer beetles were caught at the center trap than in the control without many release points. A transect of six traps through the grid caught more beetles on the outer traps than on the inner traps. It is not certain whether beetles were experiencing sensory adaptation or were wasting time flying to one or more of the many pheromone sources (cf. Cardé, 1981; Sanders, 1981; Baker et al., 1988). In the experiments presented here, however, the wasting of time investigating sources of synthetic pheromone (false trail following) was at least partly precluded since beetles would usually be trapped. Thus, the shrinking effective catch radius with closer bait spacing might be due to sensory adaptation (Baker et al., 1988). However, the pheromone plumes (trails) at the closer spacings would also intermingle more and tend to mask or camouflage the locations of the pheromone sources.

The effective attraction radius (EAR), as well as the effective catch radius discussed above, can be used to describe the strengths of semiochemical signals within and between species, irrespective of the population level or environmental conditions (Byers et al., 1989). While calculation of the EAR uses a formula that compares the catch of the pheromone trap with that of a noninteracting, passive trap in the same area (Byers et al., 1989), the estimation of the effective catch radius compares catches on a grid of pheromone traps to results from simulations using equation 3 or 4 above. However, the two concepts of attraction strength are essentially the same, i.e., a physical trapping radius that catches all insects by interception. This does not mean that these radii describe the way a plume looks or the distance that insects are attracted, but means that a passive trap in effect must have the specified radius to catch the number it did when it was baited with pheromone. The EAR should be calculated for a release rate of semiochemical from a trap that is not in competition with other sources nearby, while the effective catch radius requires a grid of pheromone traps. However, to calculate a more accurate effective catch radius, one must space the traps sufficiently apart to minimize interactions between pheromone plumes, otherwise the radius will be underestimated.

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FLAVONOIDS RELEASED BY CARROT (*Daucus carota*) SEEDLINGS STIMULATE HYPHAL DEVELOPMENT OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI IN THE PRESENCE OF OPTIMAL CO_2 ENRICHMENT

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Abstract—Carbon dioxide has been previously identified as a critical volatile factor that stimulates hyphal growth of *Gigaspora margarita*, a vesicular-arbuscular mycorrhizal fungus, and we determined the optimal concentration at 2.0%. The beneficial effect of CO_2 on fungal development is also visible in the presence of stimulatory (quercetin, myricetin) or inhibitory (naringenin) flavonoids. Sterile root exudates from carrot seedlings stimulate the hyphal development of *G. margarita* in the presence of optimal CO_2 enrichment. Three flavonols (quercetin, kaempferol, rutin or quercetin 3-rutinoside) and two flavones (apigenin, luteolin) were identified in carrot root exudates by means of HPLC retention time. Flavonols like quercetin and kaempferol are known to have stimulatory effects on hyphal growth of *G. margarita*.

Key Words—*Daucus carota*, seedlings, *Gigaspora margarita*, fungi, symbiosis, hyphal growth, vesicular-arbuscular mycorrhizae, flavonoids, carbon dioxide, exudates.

INTRODUCTION

Plants synthesize a wide variety of phenolic compounds that are known to be of great significance in plant-soil systems (Siqueira et al., 1991b). Secondary metabolites in general, including flavonoid compounds, are reported to play an important role in protecting plants against various pests and diseases (Bailey and Mansfield, 1982; Darvill and Albersheim, 1984; Hedin, 1985). More recently,

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several studies have shown that these phenolic substances act as molecular signals in the early stages of symbiotic rhizobial colonization and parasitic agrobacterial infection. Legume–*Rhizobium* symbiotic interactions result in the formation of nitrogen-fixing root nodules. Legumes secrete specific phenolic compounds that stimulate (flavones and flavanones) or repress (coumarins and isoflavones) the transcription of bacterial nodulation genes (Firmin et al., 1986; Djordjevic et al., 1987; Peters and Long, 1988; Long, 1989). *Agrobacterium* virulence gene expression is also influenced by inducing (acetosyringone, coniferyl alcohol, coniferin, and ethyl ferulate) or inhibiting (bromoacetosyringone) phenolic compounds (Hess et al., 1991; Winans, 1992). Concerning the vesicular–arbuscular mycorrhizal (VAM) symbiosis, several reports have suggested that chemotaxis toward root exudates stimulate VAM hyphal growth before initial contact with the host plant (Elias and Safir, 1987; El-Atrach et al., 1989; Bécard and Piché, 1990). In addition, carbon dioxide (CO_2) was shown to be an important compound that stimulated hyphal growth synergistically with root exudates (Bécard and Piché, 1989a), although the optimal CO_2 concentration required in the atmosphere has not yet been determined. Actually, the new findings on the stimulatory effects of flavonoids on hyphal development of VAM fungi indicate that these compounds may regulate the earliest events of VAM symbiosis establishment (Bécard et al., 1992; Chabot et al., 1992).

The aim of the present paper was to determine the optimal CO_2 enrichment concentration required for maximal *Gigaspora margarita* hyphal growth. Special attention is given to the interaction between the level of carbon dioxide enrichment and the different flavonoids tested, in terms of their influence on the *in vitro* growth of *G. margarita* hyphae. This study also was conducted to investigate the influence of sterile root exudates from carrot seedlings on the hyphal growth of *G. margarita*. Chemical analysis of these root exudates was performed to detect and identify flavonoid compounds.

METHODS AND MATERIALS

Biological Essay

Fungal Inoculum. Spores of *G. margarita* Becker & Hall (DAOM 194757, Biosystematic Research Centre, Ottawa, Ontario) were isolated from colonized *Allium porrum* L. (leek) pot cultures, by the wet-sieving method, followed by a density gradient centrifugation (Furlan et al., 1980). After isolation, spores were surface-sterilized in a 2% (w/v) chloramine T solution mixed with 0.02% (w/v) streptomycin sulfate and 0.01% (w/v) gentamycin sulfate, according to the two-step procedure described by Bécard and Fortin (1988). Immediately after sterilization, the propagules were transferred to Petri plates containing water agar and stored in darkness at 4°C until used.

Level of CO_2 Enrichment. Hyphae from germinating spores were grown under different atmospheric concentrations of CO_2: 0.03% (control), 0.1%, 0.5%, 1.0%, 2.5%, 5.0%, and 10.0% (two spores per Petri dish, five replicates per treatment). Except for the control, these concentrations were obtained by the addition of a fresh solution of Na_2CO_3 and 1 N HCl into small vials (0.4 ml) (Figure 1). CO_2 emission was calculated for each concentration, knowing the exact residual volume in the Petri dishes. At the beginning of the experiment, CO_2 concentrations were verified by gas chromatography (GC). Cotton rolls (dental rolls, Healthco DDL, Montréal, Québec) were placed in each dish to absorb excess water. All the Petri plates were sealed with parafilm to confine the internal atmosphere. They were incubated vertically so that the germ tube elongates upward, the result of a negative geotropic mode of growth (Watrud

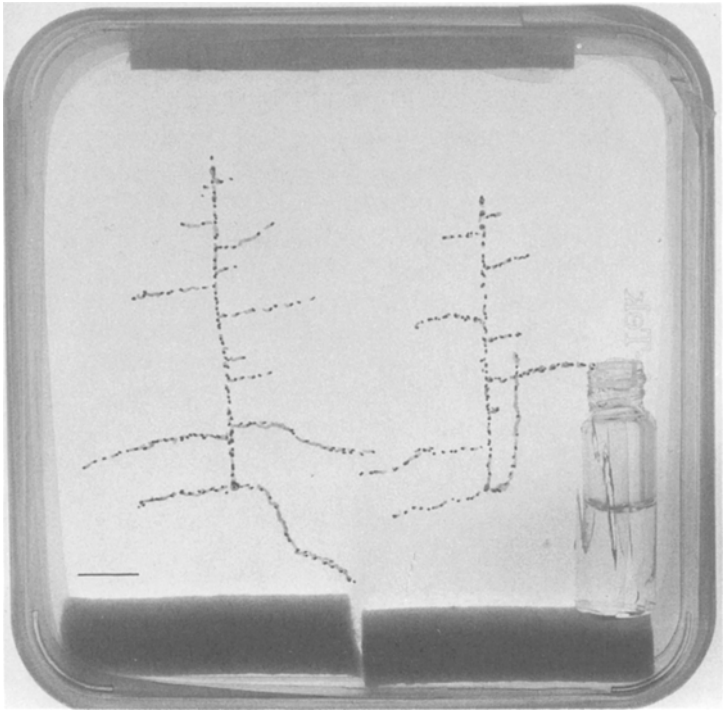

FIG. 1. Photograph of the experimental set up to study the influence of CO_2 on fungal growth. A vial containing Na_2CO_3 and 1 N HCl was placed at the bottom of the Petri plate. Two germinated spores of *G. margarita* were transferred onto minimal (M) medium. The dots traced hyphal elongation. Two cotton rolls were placed at the bottom of the Petri plate to absorb excess of water. Bar = 1 cm.

et al., 1978), and placed in the dark at 27°C for 20 days. This trial was performed twice.

Flavonoid Compounds. Three commercially available flavonoids were obtained from Sigma Chemical Co. (St. Louis, Missouri) and tested: quercetin, myricetin, and naringenin (Figure 2). The stock solutions (0.01 M) were prepared in 50% ethanol sterilized using a 0.2 μM filter (Millipore Ltd., Bedford, Massachusetts), according to the method of Gianinazzi-Pearson et al. (1989).

Microbial Essays: Synergistic Influence of CO_2 and Flavonoids on Hyphal Growth. Minimal (M) medium was used in all experiments and included the following ingredients in 1 liter: $MgSO_4 \cdot 7H_2O$, 731 mg; KNO_3, 80 mg; KCl, 65 mg; KH_2PO_4, 4.8 mg; $Ca(NO_3)_2 \cdot 4H_2O$, 288 mg; sucrose, 10,000 mg; NaFeEDTA, 8 mg; KI, 0.75 mg; $MnCl_2 \cdot 4H_2O$, 6 mg; $ZnSO_4 \cdot 7H_2O$, 2.65 mg; H_3BO_3, 1.5 mg; $CuSO_4 \cdot 5H_2O$, 0.13 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.0024 mg; glycine,

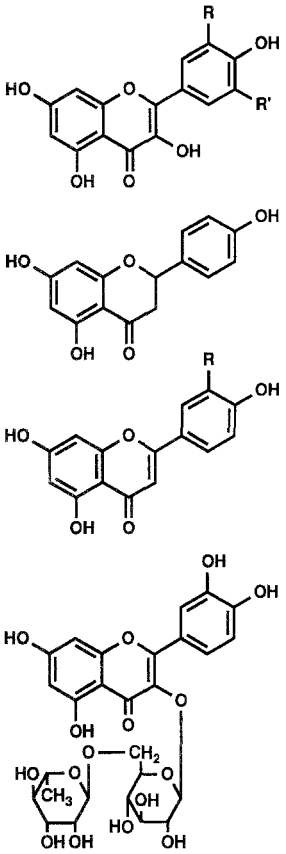

FIG. 2. Structures and names of flavonoids.

3 mg; thiamine hydrochloride, 0.1 mg; pyridoxine hydrochloride, 0.1 mg; nicotinic acid, 0.5 mg; myoinositol, 50 mg; and Gel-Gro, an agar substitute (ICN Biochemicals, Cleveland, Ohio), 5.5 g. The pH was adjusted to 5.5 before sterilization at 121°C for 16 min. After autoclaving, sterile flavonoid solutions were added to the medium to yield the final concentrations of 10.0 μM. A single spore of *G. margarita* was aseptically transferred to the bottom of a square Petri dish containing M medium, and this was counted as one experimental unit (10 replicates per treatment). Two sterile cotton rolls were placed at the base of each Petri dish to absorb excess water. All the Petri plates were placed vertically, unsealed, and exposed to an enriched atmosphere of 2.0% CO_2 in the dark at 27°C for 31 days. Atmospheric conditions were maintained by the addition of compressed CO_2 (Canadian Liquid Air Ltd. Québec) from cylinders connected to the incubators. The input of CO_2 was regulated by using a CO_2 flowmeter (Electronique M.A. Inc. Québec) and calibrated with a Fyrite gas analyzer, model 0–7, 6% $CO_2/0_2$ (Bacharach, Inc., Pittsburgh, Pennsylvania). This experiment was performed twice. The same experimental design was repeated in the presence of 0.03% CO_2.

Preparation of Sterile Plant Root Exudates. Carrot seeds (*Daucus carota* L. var. Nantaise) were surface-sterilized with a 1.0% calcium hypochlorite solution for 5 min,followed by 0.01 N HCl for 10 min and washed three times with sterile distilled water. After sterilization, the seeds were placed on a 1-mm fiberglass mesh in Magenta boxes, at a rate of 12 seeds per container (48 replicates). Each Magenta box contained 100 ml of sterile, liquid M medium (Bécard and Fortin, 1988), without sucrose. The pH was adjusted to 5.5 before sterilization at 121°C for 16 min. The seeds were incubated at 20°C and exposed under 40 $\mu E/m^2/sec$ with a 16-hr photoperiod. The M medium containing carrot root exudates was collected after 10, 20, 30, and 40 days of seedling growth. After each collection, fresh sterile M medium, without sucrose, was added. The samples sterility was checked by inoculating 0.2 ml aliquots onto Petri plates of potato dextrose agar (Difco Laboratories). The root exudate solutions were filtered using a 0.22 μm filter (Millipore) to remove cellular fragments and then lyophilized.

Incorporation of Carrot Root Exudates in Culture Medium. Before use, the lyophilized root exudates were dissolved in 95% ethanol at a concentration of 0.025 w/v and sterilized with a 0.22 μm filter (Millipore). Minimal (M) medium was used in all experiments and contained Gel-Gro (5.5 g), an agar substitute (ICN Biochemicals, Mississauga, Ontario). The sterile root exudate solution was added to the autoclaved medium to yield the final concentrations of 0.0, 0.005, 0.05, and 0.5 ml/liter of M medium. Six spores of *G. margarita* were aseptically transferred to the bottom of a square Petri dish containing M medium, and this counted as one experimental block (five blocks per treatment). Two sterile cotton rolls were placed at the base of each Petri dish to absorb excess

water. All the Petri plates were placed vertically, unsealed, and exposed to an enriched atmosphere of 2.0% CO_2 in the dark at 27°C for 30 days. Atmospheric conditions were maintained by the addition of compressed CO_2 by following the same method previously described in this section. This experiment was performed twice.

Assessment of Fungal Growth. The linear growth of hyphae emerging from germinating spores was observed under a binocular microscope and measured using the grid intersect method (Bécard and Piché, 1989b). A scale with 2 mm divisions was used to measure hyphal growth. Ungerminated and contaminated spores, although low in number (1.5%), were discarded for the calculation of average hyphal growth. All experimental data were statistically treated by an analysis of variance and the multiple comparison Waller-Duncan test.

Chemical Analysis

General. Samples of quercetin, kaempferol, rutin, and apigenin were purchased from Sigma Chemical Co., and luteolin was prepared by acid hydrolysis and demethylation of diosmin (Aldrich Chemical Co.) (Figure 2).

Extraction. The medium containing exudates was freeze-dried, crushed, and extracted with ethanol for one week (1.5 liters ethanol for each gram of dried exudates). The mixture was filtered, and this extract was evaporated to a small volume (2 ml). The exudate residue was successively extracted with 200 ml of ethyl acetate for three days followed by 200 ml of petroleum ether again for three days (bp 35–60°C). These extracts were filtered and evaporated to 2.0 ml volumes. All the three extracts were analyzed by high-performance liquid chromatography (HPLC) on reverse phase.

Chromatography. Following extraction, the organic phase of freeze-dried carrot root exudates was analyzed by HPLC in a reverse-phase mode (Beckman C18 column, 250 mm × 4.6 mm, 5 μm) employing a monitoring flow system (280 nm) at flow rate of 1.0 ml/min[1] and equipped with a UV detector. The eluent was methanol–water–acetic acid (50:45:5) in isocratic mode. R_t (minutes) are the following: quercetin: 7.25, kaempferol: 12.08, rutin: 3.76, luteolin: 8.93, apigenin: 9.27.

RESULTS AND DISCUSSION

Bécard and Piché (1989a) reported that a stimulation of *G. margarita* hyphal growth during the precolonization stage requires the synergistic action of carbon dioxide and root exudates, when cocultured with Ri T-DNA transformed carrot roots. Several authors have suggested that specific compounds contained in root exudates have the capacity to stimulate the hyphal growth of VAM fungi (Elias and Safir, 1987; El-Atrach et al., 1989; Bécard and Piché, 1990). More recently,

flavonoid compounds alone have been reported to modify the rate of spore germination and hyphal growth of VAM fungi (Gianinazzi-Pearson et al., 1989; Nair et al., 1991; Siqueira et al., 1991a; Tsai and Phillips, 1991). Two studies have shown that hyphal growth of *G. margarita* is influenced by both stimulatory and inhibitory flavonoids commonly found in plants (Bécard et al., 1992; Chabot et al., 1992). However, no attempts were made in these studies to evaluate the importance of CO_2 concentration. In this present paper, special attention has been given to determining the optimal CO_2 enrichment required for maximal *G. margarita* hyphal growth. The concentrations of ambient air (0.03%) and excess of carbon dioxide (5.0% and 10.0%) both decreased fungal growth, when compared to CO_2 concentrations in the range of 0.1–2.5% (Figure 3). There was a significant stimulation of fungal growth at 1.0% and 2.5% CO_2, clearly the optimal concentration range in our studies. This level of CO_2 probably reproduced the conditions found in the rhizosphere environment of actively growing roots. Carbon dioxide is a product of root respiration and is known to affect the metabolism of fungi (Tabak and Cooke, 1968). Its effect on spore formation, spore germination, and hyphal growth has been studied for some species of fungi. Different concentrations of CO_2 may have either stimulatory or inhibitory effects, the response varying among organisms (San Antonio and Thomas, 1972; Zadrazil, 1975; Le Tacon et al., 1983). Beneficial effects of optimal carbon dioxide concentrations were clearly demonstrated in our study.

Based on these results, subsequent experiments were conducted to test the influence of CO_2 provided in combination with flavonoids. Results presented in Figures 4a and b show the beneficial effects of 2.0% CO_2 on hyphal elongation of *G. margarita* after 31 days of culture with flavonoids. In the presence of 0.03% CO_2, quercetin or myricetin (10.0 μM) improved the growth of the hyphae, when compared to the control (Figure 4b). However, a substantial

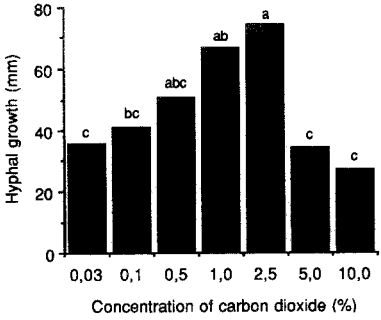

FIG. 3. Hyphal growth from germinated spores of *G. margarita* after 20 days of culture under different CO_2 concentrations. Significant differences in hyphal growth are indicated by different letters (Waller-Duncan $P < 0.05$).

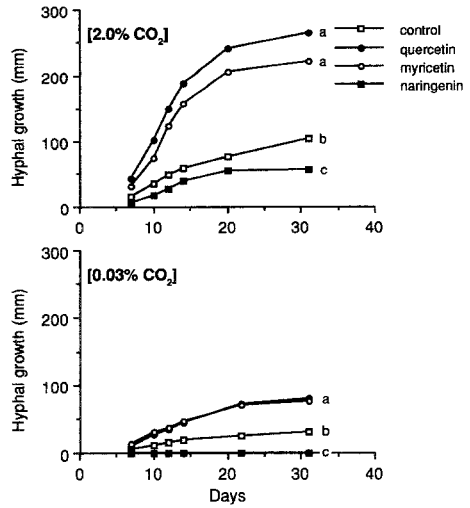

FIG. 4. Hyphal growth from germinated spores of *G. margarita* in the presence of either 2.0% CO_2 (a) or 0.03% CO_2 (b) environment, with or without flavonoid treatments at 10.0 μM. Significant differences in hyphal growth are indicated by different letters (Waller-Duncan $P < 0.05$).

increase in hyphal growth was observed when either of these substances was provided concurrently with 2.0% CO_2 in the Petri plates (Figure 4a). In the presence of 0.03% CO_2, naringenin (10.0 μM) inhibited totally the germination of spores, while significant inhibition, although less than the control, was observed in a 2.0% enrichment.

Root exudates from carrot seedlings were tested for their biological activity on the VAM fungus, *G. margarita*. These carrot root exudates produced under sterile conditions were free of microbial metabolites that could influence hyphal development. The phosphorus-deficient medium used to prepare the exudate is a prerequisite for the stimulation of VAM fungi (Elias and Safir, 1987). Results obtained in the presence of 2.0% CO_2 showed that carrot root exudates collected after 10 days of seedling growth stimulated significantly hyphal growth of *G. margarita*, after 37 days of incubation (Figure 5). Concentrations of 0.5 ml and 0.05 ml/liter of M medium stimulated hyphal growth, while root exudates at the lower concentration of 0.005 ml/liter did not influence hyphal growth, as compared to the control treatment. Root exudates collected after 20, 30, and 40 days of seedling growth had no significant effect on hyphal development. These results suggest that optimal quantities of root exudates are required for a significant enhancement of hyphal growth. They also confirm that increased plant age may influence the quality and the composition of root exudates (Hale et al.,

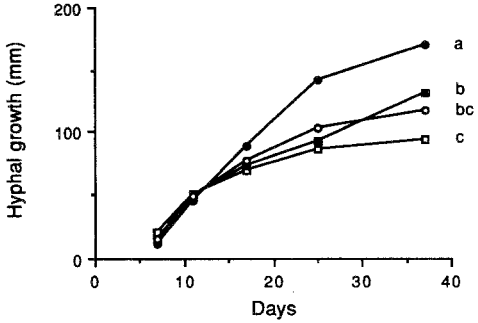

FIG. 5. Effect of carrot root exudates (collected after 10 days of seedling growth in M medium deprived of sucrose) on hyphal growth from spores of *G. margarita*, in the presence of 2.0% CO_2. Root exudates were added to 1 liter of M medium in concentrations of 0.0 ml (—□—), 0.005 ml (—○—), 0.05 ml (—■—), or 0.5 ml (—●—). Significant differences in hyphal growth are indicated by different letters (Waller-Duncan $P < 0.05$).

1971; Hamlen et al., 1972) and subsequently modify the response of VAM fungi to root exudates. Elias and Safir (1987) have shown that clover root exudates from 2-week-old seedlings stimulated more hyphal growth than did root exudates from 4- to 6-week-old seedlings. This previous study and our current results suggest that mycorrhizal establishment is initiated more readily during the early stages of plant growth by molecular signals. Carrot root exudates collected after 10 days of seedling growth were consequently subjected to chemical analysis.

Flavonoids were identified by the comparison of their retention times with authentic standards and a mixed injection technique. Two flavonols (quercetin and kaempferol), two flavones (luteolin and apigenin), and a flavonol glycoside (rutin) were identified. These flavonoids have also been isolated (free or as glycosides) from various parts (seeds, leaves, and roots) of nontransformed carrot plants (El-Moghazi et al., 1980; Gupta and Niranga, 1982) and from T-DNA-transformed carrot roots (Bel-Rhlid et al., 1992). Several authors have suggested that specific compounds contained in root exudates have the capacity to stimulate the hyphal growth of VAM fungi (Elias and Safir, 1987; El-Atrach et al., 1989; Bécard and Piché, 1990).

Among the five flavonoids detected in the exudate, quercetin and kaempferol are known to be stimulatory, apigenin is inhibitory, while rutin and luteolin have no significant influence on the hyphal growth of *G. margarita* (Bécard et al., 1992; Chabot et al., 1992). Exudates from T-DNA transformed carrot roots (Bel-Rhlid et al., 1992) contained the same flavonoid compounds, while the extracts showed the additional presence of chrysin. It has been previously demonstrated that chrysin has an inhibitory effect on the hyphal growth of *G. mar-*

garita (Bécard et al., 1992; Chabot et al., 1992). The presence of this compound in carrot root extracts may explain the ineffectiveness of the extracts to stimulate *G. margarita* (unpublished results).

In the present study, quercetin appeared to be the most prominent flavonoid detected in the carrot root exudates. This compound is commonly found in vascular plants. Tsai and Phillips (1991) reported that quercetin-3-O-galactoside is the principal flavonoid contained in exudates of alfalfa seeds, and it promotes spore germination of two *Glomus* species. Thus, quercetin and other related flavonoids may be important regulators of the early stages of VAM symbiosis development. Little is known about the underlying mechanism by which flavonoids stimulate the hyphal growth of VAM fungi. These secondary compounds from plants are of great importance in regulating rhizosphere microbial ecology and, moreover, could lead in the case of VAM fungi to the successful pure culture of these obligate biotrophs.

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POSTBITE ELEVATION IN TONGUE-FLICKING RATE BY AN IGUANIAN LIZARD, *Dipsosaurus dorsalis*

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Abstract—The herbivorous iguanid lizard *Dipsosaurus dorsalis* exhibited PETF (postbite elevation in tongue-flicking rate), an increase in tongue-flicking rate after experimental removal from the mouth of food that had been bitten. This was demonstrated by a significantly higher tongue-flick rate after having bitten food than in three experimental conditions controlling for responses to the experimental setting, sight of food, and mechanical disturbance caused by the experimental removal of food from a lizard's mouth. As in most other families of lizards, PETF was brief, occurring only during the first minute. Lizards are divided into two major suprafamilial taxa, Iguania and Scleroglossa, consisting of carnivorous species characterized by two major foraging modes, ambush and active, and of herbivores and omnivores. PETF is absent in the two families of carnivorous iguanian lizards studied that are ambush foragers but present in three families of scleroglossan lizards that are active foragers. However, PETF is absent in the two species studied in a scleroglossan family, Gekkonidae, which forages by ambush, and present in an iguanian herbivore, as reported herein. We propose that the presence or absence of PETF, in addition to its phylogenetic determinants, is adaptively adjusted to foraging mode.

Key Words—Tongue-flicking, foraging, Lacertilia, Iguanidae, *Dipsosaurus dorsalis*.

INTRODUCTION

Foraging squamate reptiles use the tongue in several contexts to gather chemical information about food. However, the uses differ greatly among major taxa and

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ecological types. In Scleroglossa, highly venomous snakes that voluntarily release prey exhibit an increase in tongue-flicking rate and perform searching movements to relocate the released prey (Chiszar and Scudder, 1980). This behavior pattern, called strike-induced chemosensory searching (SICS), was initially described in venomous snakes and is employed by both ambush foragers such as rattlesnakes and active foragers such as cobras (Chiszar and Scudder, 1980; O'Connell et al., 1985). The increase in tongue-flicking rate is called the post-strike elevation in tongue-flicking rate (PETF) (Cooper, 1991).

Lizards usually do not voluntarily release soft-bodied food that has been bitten, but representatives of scleroglossan lizard families that forage actively exhibit SICS (Cooper, 1989, 1991, 1992a, 1993a,b) after food has been experimentally removed from their mouths and presumably after food has escaped or been dropped. In contrast, representatives of the iguanian lizard families Chamaeleonidae and Phrynosomatidae, which are ambush foragers, do not exhibit SICS or PETF (Cooper, 1993b). Most iguanian families consist of insectivorous ambush foragers, but Iguanidae is exceptional. As herbivores, they are neither ambush foragers nor active foragers, but must search for immobile plants. One iguanid species, the desert iguana (*Dipsosaurus dorsalis*), discriminates preferred food from nonfood chemical cues by lingually mediated vomerolfaction (Cooper and Alberts, 1990, 1991). Because these lizards presumably locate plants by visual cues, we hypothesized that tongue-flicking may be used to chemically evaluate food quality rather than to locate food (Cooper and Alberts, 1990).

In this study, we report the results of an experiment designed to examine the possibility that PETF and perhaps SICS may occur in *D. dorsalis*. We predicted that PETF exists in the desert iguana because this species is capable of prey chemical discrimination, forages for plant food that can be sought and evaluated by chemosensory means after an animal has eaten one bite or dropped food, and is not constrained from tongue-flicking to avoid detection by prey as are lizards in related iguanian families. In the Discussion we consider the hypothesis that PETF and SICS are adaptively adjusted to foraging mode.

METHODS AND MATERIALS

Sixteen adult *Dipsosaurus dorsalis* were collected in Riverside County, California. They were transported first to the University of California at San Diego, where they were used as subjects of experiments on pheromonal communication and prey chemical discrimination, and second to Auburn University at Montgomery (AUM), Alabama. At AUM they were held for three weeks prior to testing, during which time the experimenter regularly approached and placed his hand in the cages to habituate the lizards further to the close proximity

of an experimenter. The lizards were habituated to the presence of an experimenter and to the new laboratory conditions when the experiment was conducted (April 24–27, 1989). Each lizard was housed alone in a 50 × 30 × 26-cm glass terrarium containing a 6-cm-deep layer of sand, a food bowl, and a ceramic shelter site. Lizards were fed to satiation three times per week. The diet consisted of a mixture of fresh, grated vegetables, including carrots, zucchini, yellow squash, turnips, spinach, and broccoli. Crickets were provided once per week. Because the lizards remained well hydrated using water in their food, it was not necessary to place water bowls in their cages. Fluorescent lighting was provided during daylight hours and each cage was warmed by an undercage heater and a 75-W heat lamp. Temperatures in the cages were held at 33–35°C.

To test for PETF, the experimenter observed the responses of the desert iguanas in four experimental conditions, using an experimental paradigm that has been successful in detecting PETF in several families of lizards (Cooper et al., 1989; Cooper, 1989, 1991). In an experiment having a randomized blocks design, the experimenter observed the responses of each lizard in all four stimulus conditions, presented one per day in randomized sequence. To ensure high, fairly uniform responsiveness to food chemicals, the lizards were not fed for three days before the experiment was begun or during the four experimental days.

To begin a trial, the experimenter slowly approached a lizard's cage, removed its top, and lowered the experimental stimulus slowly into the cage. The effect of biting on subsequent tongue-flicking rate was assessed by counting numbers of tongue-flicks emitted in each of five consecutive minutes in the four conditions. Recording of numbers of tongue-flicks began as soon as an experimental stimulus was removed. Trials were conducted between 1100 and 1500 hr CST.

The four conditions were the forceps, sight, mechanical disturbance, and bite conditions. In the forceps condition, empty forceps were held in view of the lizard for 10 sec and then removed. This is a control for responses to the general experimental setting. In the sight condition, a freshly cut carrot slice held in forceps was positioned 10 cm anterior to a lizard's snout for 10 sec and then removed. The sight condition is a control for effects of seeing food and possible airborne chemical cues on tongue-flicking. If a lizard approached the stimulus in either the forceps or sight condition, the stimulus was removed before 10 sec, thereby preventing lingual contact. In the mechanical disturbance condition, a carrot slice was held 10 cm anterior to the lizard's snout until the lizard approached and appeared to be ready to bite the carrot. When a bite was imminent, the experimenter removed the carrot with one hand and simultaneously pushed the lizard to simulate the mechanical disturbance caused by the next condition. Although the exact type of mechanical stimulation could not be duplicated, the degree of overall mechanical displacement to the body was similar

in the two conditions. The bite condition was similar to the mechanical disturbance condition except that the lizard was allowed to bite the carrot slice, which was then removed from its mouth. Most individuals released the carrot slice when the experimenter pulled on the forceps; the experimenter could induce a few others to release the carrot slices only by pushing the lizard away from the carrot with one hand and using the forceps to pull the carrot in the other direction.

The experimental design is not ideal because the lizards do not voluntarily release bitten prey, necessitating use of force to remove the food from a lizard's mouth. Although the mechanical disturbance condition controls for most of the effects of forced removal, it cannot simulate the effects of mechanical stimulation of the mouth, either from the food or its removal. To address this problem, an additional experiment was conducted in two other studies (Cooper et al., 1989; Cooper, 1992a). In that experiment, a squamate, either *Thamnophis sirtalis* (Cooper et al., 1989) or *Eumeces laticeps* (Cooper, 1992a), was grasped and a cotton swab bearing prey chemicals and water or water only was inserted into its mouth. Both species tongue-flicked at higher rates when the swab bore chemical prey stimuli, suggesting that the increased tongue-flicking in experiments on PETF is a response to chemical prey cues rather than to mechanical disturbance to the head or mouth.

Friedman two-way analysis of variance was used to test for a main condition effect in each minute. This was necessary because the presence of many zero values, especially in the forceps and sight conditions, produced extreme heterogeneity of variance and nonnormality, violating the assumptions of parametric tests. For minutes in which the condition effect was significant, individual comparisons between pairs of stimulus conditions were made using procedures for nonparametric comparisons outlined by Zar (1984). Because these procedures are very conservative, a sign test was conducted for comparison. Because the hypotheses tested were directional, one-tailed tests of significance were used, with $\alpha = 0.05$.

RESULTS

Eleven of 16 individuals completed the experiment, the remaining five failing to respond at all during the 5-min interval in either the mechanical disturbance or bite conditions. Five of the 11 lizards tongue-flicked in all conditions, but several did not emit any tongue-flicks in the forceps condition (five) or in the sight condition (two), including one that did not tongue-flick in either the forceps or the sight condition. Locomotion and other movements besides tongue-flicking were not routinely recorded, but some movement was observed in the bite condition, and one individual that moved appeared to be searching and even dug into the sand in the area where it bit the carrot.

Tongue-flicking rates were substantially higher in the bite condition than in the remaining conditions in the first minute, remained somewhat elevated in the second to fourth minutes, but fell to control levels in the fifth minute (Figure 1). For the first minute, the stimulus effect was highly significant ($X_r^2 = 22.01$, $df = 3$, $P < 0.001$). PETF was present in minute 1 because the number of tongue-flicks emitted in the bite condition was significantly greater than in the mechanical disturbance ($P < 0.025$), sight ($P < 0.001$), and forceps ($P < 0.001$) conditions (Figure 2). Using one-tailed sign tests, the number of tongue-flicks emitted in the bite condition was significantly greater than each of the other three conditions at $P < 0.0005$; the number in the mechanical disturbance condition was significantly greater than that in the sight condition at $P < 0.02$ and in forceps condition at $P = 0.006$. There were no significant differences between the sight and forceps conditions using either test, suggesting that airborne chemical cues did not produce elevated tongue-flicking.

By the second minute, the numbers of tongue-flicks in the bite and mechanical disturbance conditions were virtually equal and the main stimulus effect was not significant. However, the less stringent sign tests revealed some differences among pairs of stimulus conditions. Numbers of tongue-flicks in the bite condition were significantly greater than in the sight ($P = 0.008$) and forceps ($P = 0.035$) conditions. Moreover, significantly more tongue-flicks were emitted in the mechanical disturbance condition than in either the sight condition ($P = 0.035$) or the forceps condition ($P = 0.008$). The remaining differences were not significant. In the third through fifth minutes, there were no significant differences in either main effects or paired comparisons.

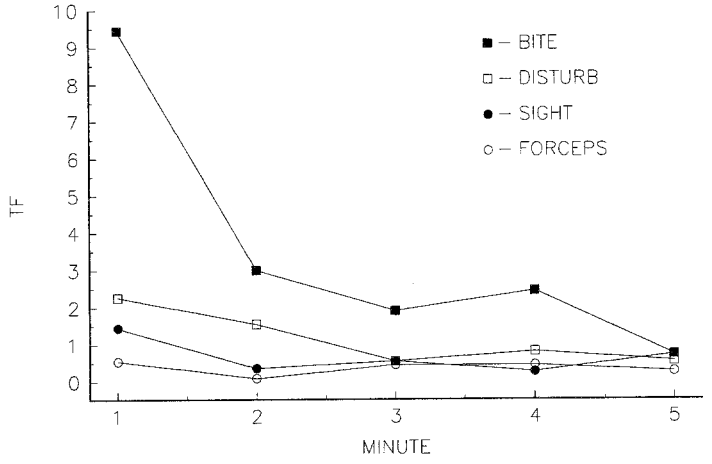

FIG. 1. Tongue-flicks (TF) emitted by *Dipsosaurus dorsalis* in each of five successive minutes in several experimental conditions.

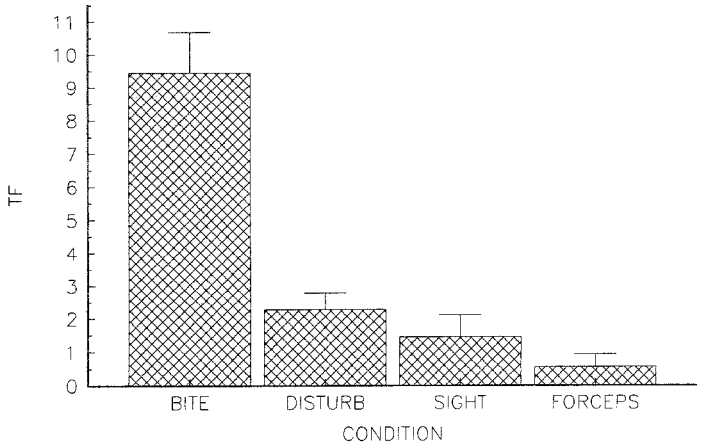

FIG. 2. Number of tongue-flicks (TF, with standard errors) emitted by *Dipsosaurus dorsalis* in several stimulus conditions during the first minute.

DISCUSSION

The desert iguana exhibits PETF, as shown by the significantly greater tongue-flicking rate in the bite condition than in any of the other conditions during the first minute. Whether SICS is also present is uncertain because the presence or absence of locomotion and movements other than tongue-flicking were not systematically recorded. The single instance in which a lizard was noted to dig in the spot at which it had bitten the carrot suggests the possible use of chemical cues to help relocate lost food (SICS), but other cues including vision and touch could be used to initiate digging and examine food discovered during digging. Further study is needed to determine whether SICS is present.

Both PETF and SICS are present in all four scleroglossan families studied that are active foragers—Varanidae (Cooper, 1989, 1993a), Lacertidae (Cooper, 1991), and Scincidae (Cooper, 1992a)—but representatives of one scleroglossan family, Gekkonidae, are exceptional among scleroglossans in being ambush foragers that lack PETF and SICS (Cooper, unpublished data). In Iguania, PETF and SICS are both absent in the two previously studied families, Chamaeleonidae and Phrynosomatidae, both of which are ambush foragers (Cooper, 1993b). The present finding that PETF occurs in at least one species in Iguanidae may prove to be exceptional for iguanian families. Because foraging mode and its relation to PETF and SICS are rather stable in Iguania and Scleroglossa (Cooper, 1993b, present paper), phylogeny presumably accounts for much of the distribution of PETF and SICS.

Despite the strong influence of phylogeny, the occurrence of both excep-

tions in families that have foraging modes differing from other families in their taxon suggests that the chemosensory behaviors PETF and SICS may be adaptively altered to optimize foraging when evolutionary changes in foraging mode arise. An important function of PETF and SICS in active foragers is relocation of prey (Chiszar and Scudder, 1980; Cooper, 1989). Because dropped plant food is unable to escape by locomotion and unlikely to be lost, possible functions of PETF in herbivores include continuing evaluation of food quality after food has been dropped and detection of nearby foliage. In addition to tongue-flicks directed to the air or substrates, licking the labials and surrounding scales after dropping food or swallowing might help remove excess plant material.

The duration of PETF in desert iguanas is quite brief, being limited to the first minute. This finding is similar to that for actively foraging scleroglossan lizards belonging to families lacking deeply forked tongues, but much shorter than in varanid lizards (about 30 min; Cooper, 1989) and crotaline snakes (sometimes over 2 hr; Chiszar and Scudder, 1980; Chiszar et al., 1983, 1985). The great duration of SICS in crotalines is undoubtedly attributable to the high probability that the envenomated prey has succumbed nearby and can be relocated. For varanid lizards, it is also likely that relatively large, mobile prey may best be relocated by scent-trailing. On the other hand, for insectivorous lizards, it may be profitable to make a brief chemosensory search of the immediate vicinity for escaped insects, but not to attempt to follow their scent trails. This reasoning applies especially to flying insects, for which scent-trailing may be difficult, if not impossible. A similar consideration may apply to herbivores because the dropped food is immobile.

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LIPOXYGENASE-DERIVED ALDEHYDES INHIBIT FUNGI PATHOGENIC ON SOYBEAN[1]

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Abstract—Several unsaturated aldehydes are produced from polyunsaturated fatty acids via the lipoxygenase pathway when soybean (*Glycine max*) plants are wounded mechanically or by pathogens. The effects of four of these aldehydes were examined on the growth of isolated fungal cultures of *Colletotrichum truncatum*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. (*E*)-2-Hexenal, (*E*)-2-nonenal, and (*Z*)-3-nonenal inhibited the growth of *R. solani* and *S. rolfsii* at 35 μmol added per liter or greater when applied as volatiles, although higher levels were required for inhibition of *C. truncatum*. (*E*)-4-Hydroxy-2-nonenal was the most inhibitory compound when applied directly in the growth medium, but it had the least effect as a volatile.

Key Words—Aldehydes, (*E*)-2-hexenal, (*E*)-2-nonenal, (*Z*)-3-nonenal, (*E*)-4-hydroxy-2-nonenal, lipoxygenase, disease resistance, *Glycine max*, *Colletotrichum truncatum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, soybean, fungal pathogens.

INTRODUCTION

Plant tissues respond to mechanical damage or attack by pathogens through physical and chemical changes, including rapid cell and tissue death, the formation of physical barriers, enzyme induction, and the production of secondary compounds (Misaghi, 1982). The enzyme lipoxygenase (LOX) catalyzes the oxygenation of the (*Z*,*Z*)-pentadiene moiety of polyunsaturated fatty acids

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(Gardner, 1991). LOX activity appears to be required for elicitation of hypersensitivity in plants exposed to fungal fatty acids such as arachidonic acid (Vaughn and Lulai, 1992). LOX expression has been shown to be stimulated in plant tissues undergoing infection or exposed to fungal chemical elicitors (Bostock et al., 1992; Ohta et al., 1991; Yamamoto and Tani, 1986). Lipid components of damaged cells may be oxidized via the LOX pathway to form lipid hydroperoxides, which rapidly break down to form many compounds, including aldehydes (Gardner, 1991). These aldehydes, although highly reactive, are sufficiently long-lived to diffuse from the sites of origin to reach extracellular targets (Esterbauer et al., 1991). Several of these aldehydes, including (*E*)-2-hexenal, (*Z*)-3-hexenal, and (*E*)-2-nonenal, were found to be inhibitory to seed germination (Bradow, 1991; Gardner et al., 1990), pollen germination (Hamilton-Kemp et al., 1991) and pathogenic fungi (Hamilton-Kemp et al., 1992) and bacteria (Deng et al., 1993). (*E*)-4-Hydroxy-2-nonenal (HNE) is also found in soybean preparations and may be formed from (*Z*)-3-nonenal (Gardner et al., 1991). A pathway to HNE from (*Z*)-3-nonenal has been recently described in *Vicia faba* microsomes (Gardner and Hamberg, 1993). HNE has been shown to be highly toxic to mammalian cells and tissues (Esterbauer et al., 1991). To the best of our knowledge, HNE has not been tested against plants or fungi.

Soybean (*Glycine max* L.) plants release many of these volatile C_6 and C_9 aldehydes derived from lipid oxidation when tissues are damaged (Gardner et al., 1991). These aldehydes may be extremely inhibitory to pathogenic fungi in enclosed airspaces such as those that occur in intracellular spaces in leaves or in air pockets under the soil surface. Thus aldehydes may play a major role in disease resistance. In this study we examine the activities of several aldehydes produced by soybean against the soybean pathogens, *Colletotrichum truncatum*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. In particular, we wished to compare the toxicities of (*E*)-2-nonenal and (*Z*)-3-nonenal with that of the newly discovered LOX pathway metabolite, HNE.

METHODS AND MATERIALS

A fungal culture of *C. truncatum* (NRRL #13737; collected and identified by C.D. Boyette, USDA/ARS, Stoneville, Mississippi) was obtained from the culture collection at the National Center for Agricultural Utilization Research. Cultures of *R. solani* and *S. rolfsii* isolated from peanut were obtained from Dr. T. Brenneman, University of Georgia. All cultures were found to exhibit positive pathogenicity on soybean. All fungi were maintained in the dark at 25 $\pm$ 1°C on V-8 juice agar (pH 5.8), augmented with 0.3% $CaCO_3$.

(*E*)-2-Hexenal, (*E*)-2-nonenal, and DMSO used in the experiments were obtained from a supplier (Aldrich) and used without further purification. (*Z*)-3-

Nonenal was synthesized from (*Z*)-3-nonenol, as described previously (Gardner and Hamberg, 1993); and the structure was verified by ^{1}H NMR spectroscopy. HNE was synthesized from (*Z*)-3-nonenol, as previously described (Gardner et al., 1992), and its identity was verified by GLC of its *O*-benzyloxime-trimethylsilyloxy ether by comparison with a standard.

The bioassay system used to test the compounds as volatiles consisted of 75-ml jars into which sterilized V-8 juice medium (10 ml) was added. These jars were placed in 275-ml jars that contained one 5.5-cm filter paper disk (Whatman No. 1) at the bottom, to which appropriate amounts of the test compounds dissolved in DMSO were added (controls consisted of DMSO only). Aluminum foil cap liners were used to create an airtight seal. The total gas headspace in the system after the addition of all components was 250 ml. Bioassays were initiated by placing 7-mm plugs of media taken from the growing margins of 7-day-old cultures. These plugs were placed, mycelial surface down, on the surface of the agar in the 75-ml jars, and the DMSO stocks were then added to the 275-ml jars. Five jars of each treatment per fungus were incubated at 25 $\pm$ 1°C in the dark until control cultures reached the edges of the 75-ml jars (96 hr). Each experiment was repeated and results were pooled (N = 10) before statistical analysis of variance.

Compounds were also tested as suspensions in agar media to test the hypothesis that these compounds might be active when present in cell and tissue fluids as well as in the volatile phase. Appropriate amounts of the compounds were added to cooled sterilized V-8 medium in 9.0-cm plastic Petri dishes. Experiments were initiated with 7-mm plugs of mycelia that were taken from the growing margins of 1-week-old cultures and placed mycelial side down on the medium. The progress of fungal growth was determined by measuring the radial advancement of the mycelial mat (from the edge of the inoculum disk to one typical point on the growing margin) when the fastest-growing cultures reached the edge of the Petri plates. Treatments are reported as percentages of the (untreated) control. Each experiment consisted of five plates per treatment per fungus and was repeated. Results were pooled (N = 10) before statistical analysis of variance.

The volatilities of test compounds were assessed by GLC analyses of 1-ml headspace samples. Since volatility is usually decreased by its partition into the aqueous phase, headspace was analyzed under three conditions: (1) "neat" in a dry vessel (60 ml), (2) with the test compound applied directly to agar media (60 ml), and (3) with the test substance applied to filter paper in vessels containing agar media (250 ml). The test compounds were applied to the containers at the rate of 0.1 μl/ml headspace, and incubated at 26°C for 2–5 hr before sampling. Unexplained isomerization of (*E*)-2-alkenals in the neat treatments caused the headspace concentration of these compounds to approach a maximum at about 0.5 hr and to decrease with time. Thus, the (*E*)-2-alkenals were equil-

ibrated for only 0.5 hr. Headspace samples were taken with a 1-ml Pressure-Lok gas syringe (Precision Sampling Co., Baton Rouge, Louisiana), either through rubber septa (250 ml jars) or through Teflon mininert valves (Pierce Chemical, Rockford, Illinois) from the 60-ml vessels. Volatiles were separated and analyzed by a Spectra-Physics (San Jose, California) model SP-7100 gas chromatograph equipped with an open tubular capillary column (12 m × 0.22 mm) coated with SE-30 (Spectra-Physics). The carrier gas flow was about 1 ml/min, and the temperature was programmed from 100 to 125°C at 5°C/min for separation of the nonenal series [retention time (minutes) (*Z*)-3-nonenal, 1.3; (*E*)-2-nonenal, 1.7; HNE, 4.0]. For analysis of (*E*)-2-hexenal, the temperature was programmed from 45 to 55°C at 5°C/min (retention time, 1.6 min). The concentrations of volatiles in the test vessels were calculated from gas chromatographic analyses of known weights of aldehyde standards injected on the column in hexane solution.

RESULTS AND DISCUSSION

The bioassay system used in these experiments provided a convenient method for testing large numbers of volatile compounds rapidly against the growth of these fungal species. Rubber septa can be placed in the caps of the large jars, allowing headspace gases to be sampled for GLC analysis. Aluminum foil cap liners prevented loss of test compound and withstood autoclaving. Teflon cap liners did not satisfactorily withstand autoclaving.

The results obtained by exposing cultures of the three fungal species to the volatilized aldehydes are shown in Table 1. For *R. solani* and *S. rolfsii*, 35 μmol added per liter or greater of (*E*)-2-hexenal, (*E*)-2-nonenal, and (*Z*)-3-nonenal completely inhibited fungal radial growth. *C. truncatum* was more tolerant of these three compounds; 175 μmol added per liter were required for complete inhibition. As a volatile, HNE did not significantly inhibit either *R. solani* or *S. rolfsii* but the highest level (600 μmol added per liter) tested did decrease *C. truncatum* growth. This suggests that uptake or metabolism of these aldehydes by *C. truncatum*, a foliar pathogen, differs from these processes in the other two fungi, which primarily attack plant tissues below or at the soil level.

When applied directly to V-8 medium, the relative effects of the four aldehydes were somewhat different (Table 2). HNE, which had little effect as a volatile, became comparatively the most potent inhibitor of *C. truncatum* and *S. rolfsii* growth when it was included in the agar media. HNE in the media also caused significant growth inhibition of *R. solani* but was not as effective as the (*E*)-2-alkenals. *R. solani* was much more tolerant of (*E*)-2-nonenal, (*Z*)-3-nonenal, and HNE than were the other two fungi. *R. solani* was the only

TABLE 1. INHIBITION OF GROWTH OF FUNGI BY VOLATILE ALDEHYDES

Treatment (μmol added/liter headspace)	Fungal growth (% of control)[a]		
	C. truncatum	*R. solani*	*S. rolfsii*
Control	100.0 a	100.0 a	100.0 a
(*E*)-2-Hexenal			
3.5	85.0 a	100.0 a	60.8 b
35	35.8 d	0.0 b	0.0 d
175	0.0 e	0.0 b	0.0 d
875	0.0 e	0.0 b	0.0 d
(*E*)-2-Nonenal			
2.4	58.3 c	95.2 a	15.0 c
24	25.8 d	0.0 b	0.0 d
120	0.0 e	0.0 b	0.0 d
600	0.0 e	0.0 b	0.0 d
(*Z*)-3-Nonenal			
2.5	65.8 bc	98.7 a	45.0 b
25	64.2 bc	0.0 b	0.0 d
125	0.0 e	0.0 b	0.0 d
600	0.0 e	0.0 b	0.0 d
(*E*)-4-hydroxy-2-nonenal			
2.4	79.2 ab	100.0 a	100.0 a
24	76.7 abc	100.0 a	100.0 a
120	72.5 abc	100.0 a	100.0 a
600	69.9 bc	100.0 a	100.0 a

[a] Mean separation among all means by Duncan's multiple-range test, $P < 0.05$.

species significantly inhibited by the lowest level (0.4 μmol/ml medium) of (*E*)-2-hexenal. Both (*E*)-2-hexenal and (*E*)-2-nonenal completely suppressed all three species at levels of 2.2 and 1.5 μmol/ml, respectively. Again *S. rolfsii* was generally more sensitive and was the only fungal species to be inhibited by 0.3 μmol/mol HNE.

The higher level of tolerance of *C. truncatum* to the three highly volatile aldehydes [(*E*)-2-hexenal, (*E*)-2-nonenal, and (*Z*)-3-nonenal] may be due to a natural selection for strains of this fungi that are resistant to these compounds. Because these compounds are green leaf/cucumber volatiles produced by aerial parts of plants (such as leaves and the surfaces of fruit), *C. truncatum*, a phylloplane pathogen, would be expected to tolerate these common allelochemicals. Large increases in LOX activity were shown to be induced in soybean leaves by mechanical wounding or by spider mite damage (Hildebrand et al., 1989). Under normal conditions, the ability of a pathogen to colonize soybean leaves would appear to depend on its ability to tolerate lipoxygenase-derived compounds.

TABLE 2. INHIBITION OF FUNGI BY ALDEHYDES INCORPORATED IN MEDIA

Treatment (μ mol added/ml medium)	Fungal growth (% of controls)[a]		
	C. truncatum	*R. solani*	*S. rolfsii*
Control	100.0 a	100.0 a	100.0 a
(*E*)-2-Hexenal			
0.4	104.8 a	81.2 b	86.3 a
2.2	0.0 d	0.0 f	0.0 d
8.7	0.0 d	0.0 f	0.0 d
(*E*)-2-Nonenal			
0.3	32.2 c	89.1 ab	13.7 c
1.5	0.0 d	0.0 f	0.0 d
6.0	0.0 d	0.0 f	0.0 d
(*Z*)-3-Nonenal			
0.3	65.1 b	89.1 ab	64.1 b
1.6	0.0 d	61.8 c	0.0 d
6.3	0.0 d	23.6 de	0.0 d
(*E*)-4-Hydroxy-2-nonenal			
0.3	41.1 c	66.1 c	0.0 d
1.5	0.0 d	35.8 d	0.0 d
6.0	0.0 d	10.9 ef	0.0 d

[a]Mean separation among all values by Duncan's multiple-range test, $P < 0.05$.

The absence of detectable HNE in headspaces (Table 3) explains its lack of effect when applied as a volatile, despite previous reports of HNE toxicity to animal cells when applied in solution. In regard to the three test compounds with significant volatility, the extent of volatilization was (*E*)-2-hexenal >> (*Z*)-3-nonenal > (*E*)-2-nonenal (Table 3). However, the effect of the three compounds on growth suppression was roughly equivalent (Table 1), indicating a higher toxicity of the nonenals compared to (*E*)-2-hexenal. This was partially confirmed when these aldehydes were included in liquid media (Table 2), but inconsistent results were obtained in experiments with *R. solani*, where the nonenals were equally or less toxic than (*E*)-2-hexenal. In experiments employing liquid media, the headspace concentration of (*E*)-2-hexenal was reduced to 11% of the value found when the compound was analyzed neat. This is consistent with a previous observation (Gardner et al., 1991). Compared to (*E*)-2-hexenal, the headspace concentrations of the nonenals were reduced less by aqueous media (Table 3), reflecting the lower relative water solubility or partition coefficient of these aldehydes.

When the headspace volatiles were examined after 48 hr, it was discovered that the (*E*)-2-alkenals in the headspaces were little changed, but (*Z*)-3-nonenal had significantly decreased (data not shown). Since (*Z*)-3-nonenal readily autox-

TABLE 3. EFFECT OF BIOASSAY SYSTEM ON HEADSPACE CONCENTRATIONS OF ALDEHYDES

Aldehyde	Headspace concentration (μM)[a]		
	Condition A[b]	Condition B	Condition C
(*E*)-2-Hexenal	100.0	31.6	11.2
(*E*)-2-Nonenal	1.1	1.0	0.9
(*Z*)-3-Nonenal	4.6	1.5	1.6
(*E*)-4-Hydroxy-2-nonenal	0.0	n.d.[b]	n.d.

[a] All conditions: aldehydes were added to enclosed containers at the rate of 0.1 μl aldehyde/ml headspace and headspace volatiles were equilibrated at 26°C for 2–5 hr) [neat samples were equilibrated for only 0.5 hr to avoid the appearance of unexplained early-eluting isomer with the E-2-alkenals]. Condition A, aldehydes added neat to dry, clean vessel; condition B, method used in this study (aldehyde applied to filter paper in proximity to open agar container placed within a larger enclosed vessel); condition C, aldehyde applied directly to agar surface within closed container. Headspace gases were sampled via Teflon mininert valves in conditions A and C, via a rubber septa in condition B.

[b] n.d., not determined.

idizes to (*E*)-4-hydroperoxy-2-nonenal (Gardner and Hamberg, 1993), the material remaining in the container was extracted with $CHCl_3$–CH_3OH (2:1, v/v), and the recovered material was reduced with triphenylphosphine (TPP). Subsequent conversion to the *O*-benzyloxime-trimethylsilyloxy derivative followed by GLC showed the presence of HNE from reduction of the hydroperoxide by TPP. This conversion may partially explain the observed effect of (*Z*)-3-nonenal on the growth of fungi.

It has been previously shown that many volatile compounds, including LOX-derived aldehydes, stimulated spore germination in many fungal species (French, 1992). Fungistasis, the inhibition of germination of fungal propagules by soil, has been attributed, in part, to volatile compounds present in the soil (Hora and Baker, 1970; Liebman and Epstein, 1992). In our study none of the levels of any of the compounds tested stimulated fungal growth, but it is conceivable that lower concentrations of these aldehydes would be stimulatory. In fact, several aldehydes not examined in this study (hexanal, nonanal) promoted hyphal growth of *Alternaria alternata* and *Botrytis cinerea* (Hamilton-Kemp et al., 1992). It is interesting to note that both (*E*)-2-hexenal and (*E*)-2-nonenal only inhibited growth in the latter study. This may indicate that certain plant-derived volatiles act by stimulating the germination of the relatively resistant spores, allowing other volatiles to act against the more vulnerable hyphae.

In conclusion, several unsaturated aldehydes derived from LOX-catalyzed lipid oxidation were found to inhibit the growth of isolated fungal cultures of *C. truncatum*, *R. solani*, and *S. rolfsii*. These aldehydes were inhibitory both

as volatiles and as media constituents. In general, *R. solani* cultures were more resistant to volatilized aldehydes than were the other two fungi. *C. truncatum* was the most tolerant to the aldehydes added to the media. HNE, which was not inhibitory as a volatile, was the most inhibitory aldehyde tested when added to the media.

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MALE RESPONSE TO NATURAL SEX PHEROMONE OF *Migdolus fryanus* WESTWOOD (COLEOPTERA: CERAMBYCIDAE) FEMALES AS AFFECTED BY DAILY CLIMATIC FACTORS

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Abstract—Males of the sugarcane borer, *Migdolus fryanus* (Coleoptera: Cerambycidae), are attracted to females by means of a sex pheromone. Mating usually occurs during a few days from October to March under field conditions in São Paulo State, Brazil. This work reports on mating of this species as affected by daily climatic factors, during a single nuptial flight. Maximum male capture by the natural sex pheromone occurred from 10:00 to 11:00 AM at air and soil temperatures of 30.0°C and relative humidity of 57.0%. As these temperatures increased, females burrowed into the soil, as they are more sensitive to heat than males. Thus, it was concluded that sex pheromone-mediated mating in this cerambycid is directly affected by temperatures of air and soil.

Key Words—*Migdolus fryanus*, Coleoptera, Cerambycidae, mating behavior, sex pheromone, climatic factors.

INTRODUCTION

Adult males of *Migdolus fryanus* respond strongly to the female sex pheromone (Bento et al., 1992). This sexual attraction can be observed during several consecutive days in the same area (Arrigoni et al., 1986). Observations in many localities have indicated differences in mating time relative to the local climatic

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conditions so that mating occurs from October (Novaretti et al., 1983) through March (Roccia, 1977; Arrigoni et al., 1986) in São Paulo State, Brazil.

Mating is apparently restricted to a narrow range of time during each day, independent of the place. Adult males fly to the ground to locate the apterous female, which remains at soil level with only head and thorax exposed (Roccia, 1977). Then she emerges for mating, possibly with more than one male (Roccia, 1977; Terán et al., 1984). She again burrows into the soil to various depths for egg-laying. Bento et al. (1992) have suggested that females can not tolerate hot and sunny weather and that they die if they remain exposed at the soil surface for more than 24 hr during mating days.

Very little has been done on the behavioral aspects of this species, which is the key pest of sugarcane in South America. Thus, our work was conducted with the following objectives: (1) to determine the peak of response of *M. fryanus* males using traps baited with the natural sex pheromone; and (2) to relate the catch to weather factors such as relative humidity and soil and air temperatures.

METHODS AND MATERIALS

The experiment was set up in Olímpia County, São Paulo State (latitude 20°46′S; longitude 49°40′W) on February 22, 1992, in sugarcane plantation areas. The number of males captured was determined for each hour during 24 consecutive hours using the UFV design traps as described in Bento et al. (1992). This trap consists of a plastic water container, 20-liter volume, with sections of the large sides cut out, leaving two rectangular windows. In the center of this, a PVC tube (85 mm diameter × 40 mm long), which had the ends covered with a nylon screen, was hung by wire. Four treatments in four replicates were set up in a completely randomized experimental design as follows: (A) two virgin females; (B) rubber septum impregnated with material extracted from virgin females + hexane; (C) rubber septum + hexane (control); and (D) unbaited (control).

All traps were placed at the soil level with a distance of 22 m between traps in an area prepared for sugarcane planting which had a high incidence of *M. fryanus* in previous years. The extraction of natural sex pheromone was carried out as in Bento et al. (1992) using hexane instead of dichloromethane as the solvent. Thirty females were aerated through 1 g of Porapak Q for 24 hr and 2.0 female equivalents were used on each septum.

Local air temperature, relative humidity, and soil temperature at 5 cm depth were taken hourly during the 24-hr period beginning at 6:00 PM.

RESULTS

Field trapping of *M. fryanus* males occurred from 7:00 AM to 3:00 PM. During this period, the air and soil temperatures increased from 23.0 to 33.0°C and from 26.3 to 32.9°C, respectively, and there was a reduction in relative humidity from 82.0 to 55.0% (Figure 1).

Maximum male capture occurred from 10:00 to 11:00 AM at air and soil temperatures of approximately 30°C and relative humidity of 57.0%.

The time of male and female occurrence in the field was also recorded. Females were found in the field from 8:00 AM until noon, when the air temperature was 27.0–30.0°C, soil temperature 28.2–30.3°C, and relative humidity 68.0–57.0%. Males were found from 7:00 AM to 3:00 PM.

DISCUSSION

Females remained on the soil surface for a shorter period of time, suggesting that they are more sensitive to higher temperatures than males. Males penetrate into the soil or die when air or soil temperature reaches 33.0°C. They live for two or three days (Roccia, 1977). Their energy reserves are apparently insufficient for soil excavation or for returning to the soil surface to compete further for females.

There was no significant difference between the number of males captured by traps baited with two females or with the rubber septum + extract + hexane during the peak of capture. This may suggest that pheromone release by the females was more intense from 10:00 to 11:00 AM. As the males come to the surface, they are able to respond to the female sex pheromone. Early catches were low (Figure 1B) due to the absence of females.

The natural sex pheromone in the septum has attracted the males with a similar intensity as virgin females and during the same period. This indicates that the cessation of male response is associated with environmental factors such as an increase in air and soil temperatures.

The male catch demonstrates that the highest period of attractiveness occurs from 8:00 AM to noon. This is the period in which the females are above the soil surface. We believe that females start releasing pheromone immediately after emerging from soil. They probably stop pheromone release only because they reenter the soil in response to high temperature (Bento et al., 1992). Another possibility is that females release pheromone continuously, but it is not detected by the males due to the soil acting as a physical barrier as females reenter the soil.

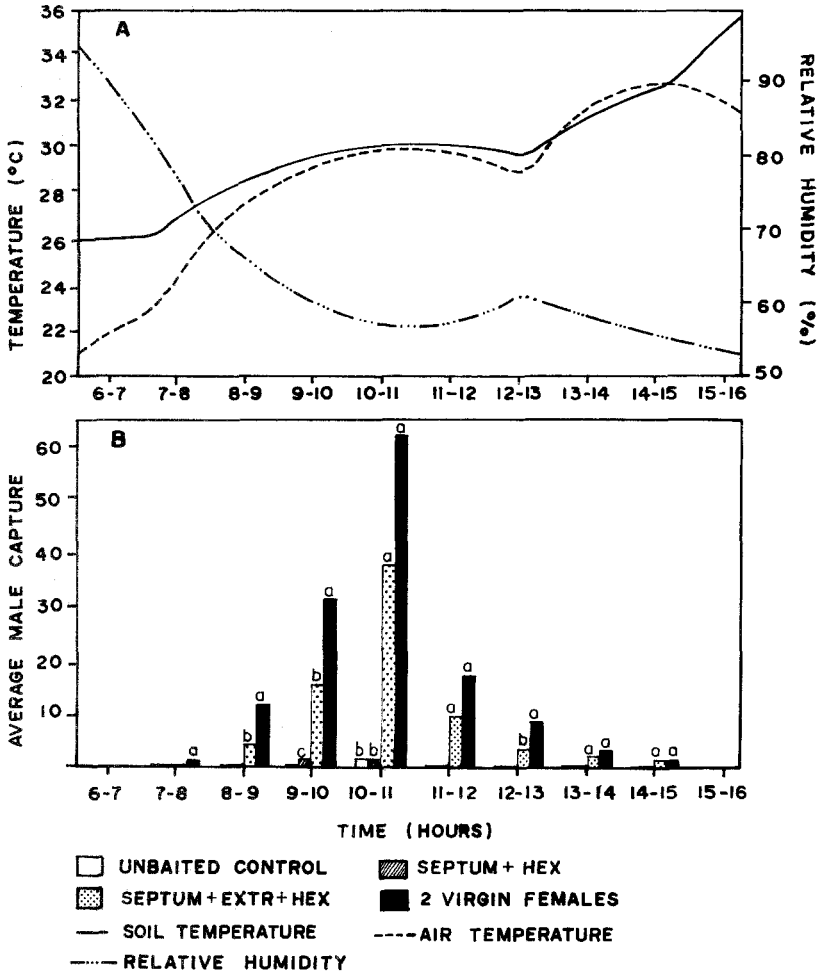

FIG. 1. Average catch of *Migdolus fryanus* using traps baited with two virgin females; rubber septum + female extract + hexane; rubber septum + hexane (control) and no bait (control), and relative humidity, and air and soil temperature during 24 consecutive hours of the trapping period. (Means followed by the same letter within time periods are not different $P < 0.05$ by Ducan's multiple-range test.)

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PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF CONIFERYL BENZOATE ON AVIAN REPRODUCTION

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Abstract—Various plant secondary metabolites related to cinnamic acid are of interest because of their repellency to birds and their occurrence in ecologically important food items. Coniferyl benzoate (CB), a phenylpropanoid ester that occurs in quaking aspen (*Populus tremuloides*) is of particular ecological interest because of its effect on ruffed grouse (*Bonasa umbellus*) feeding behavior and its possible influence on the population dynamics of this bird. During detoxification processes, CB and other analogous compounds are metabolized into by-products, such as ferulic acid (FA), that can cause antireproductive effects. We tested whether consumption of CB produces antireproductive effects similar to FA using male and female Japanese quail (*Coturnix coturnix*) as avian models for ruffed grouse. The parameters we investigated included: the production, morphology, and development of eggs; reproductive characteristics influenced by estrogen; serum prolactin levels; and male reproductive behavior. Dietary CB did not produce antireproductive effects similar to FA at intake levels that Japanese quail and ruffed grouse would freely consume. Consumption of CB by Japanese quail significantly reduced egg production and body mass but did not affect male reproductive performance. Coniferyl benzoate's effect on egg production may be explained by lower energy acquisition and retention rather than endocrine changes per se. Contrary to previous reports, it is unlikely that FA, or similar compounds act directly as estrogen mimics or antagonists. Although, CB did reduce egg production in quail, it is unlikely that it would affect egg production in wild

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ruffed grouse. Detoxification costs and the effects of CB on nutrient utilization may explain why ruffed grouse avoid high dietary levels of CB.

Key Words—Ruffed grouse, *Bonasa umbellus*, Japanese quail, *Coturnix coturnix*, ferulic acid, prolactin, estrogen, plant secondary metabolite, feeding repellent, reproductive toxicity.

INTRODUCTION

Many plant secondary metabolites related to cinnamic acid, such as coniferyl benzoate, vanillin, and methyl cinnamate, are repellent to birds and other animals (Crocker and Perry, 1990; Shah et al., 1991; Jakubas et al., 1992; Avery and Decker, 1992). These compounds are of interest because of their ecological significance (Berger et al., 1977; Buchsbaum et al., 1984; Jakubas and Gullion, 1991) and potential use as pest repellents (Crocker and Perry, 1990; Jakubas et al., 1992; Avery and Decker, 1992). Although some of these compounds have been shown to have aversive sensory properties (e.g., Jakubas and Mason, 1991), little is known about how their biological activity is linked to their repellency. Structural similarities among these compounds suggest that their biological activity and hence the mode of their repellency may be similar. One common link among phenylpropanoid compounds may be in their postingestional biotransformation. The biotransformation of different phenylpropanoid compounds often results in the production of similar phenolic acids (e.g., *p*-coumaric, caffeic, and ferulic acids) (Solheim and Scheline, 1976; Scheline, 1978). Some of these compounds [e.g., *p*-coumaric and ferulic acid (FA), Figure 1] are known to have adverse physiological effects (see below) and consequently may manifest these effects before further biotransformation occurs or they are excreted from the animal. Consequently, animals may learn to associate the adverse effects of these metabolites with the ingestion of the original compound (i.e., learned avoidance) or natural selection may favor the avoidance of compounds that ultimately lower an animal's reproductive success.

Ruffed grouse (*Bonasa umbellus*) are herbivorous birds that occur throughout most of the northern forests of North America (see Bump et al., 1947; Jakubas and Gullion, 1991). In many northern regions, the population level of this species undergoes 8- to 10-year cyclic fluctuations (Keith, 1963; Gullion, 1970, 1984). These population fluctuations have been associated with annual changes in the suitability or availability of the staminate flower buds of quaking aspen (*Populus tremuloides*) (Jakubas and Gullion, 1991)—one of the principal winter foods for ruffed grouse (Gullion, 1969, 1977). The suitability of these buds is partially determined by their coniferyl benzoate (CB) concentration [mean concentration: ca. 2.5%; range 0–9% (dry mass)] (Jakubas and Gullion, 1991; C. Vispo and W.J. Jakubas, University of Wisconsin, unpublished data). Ruffed

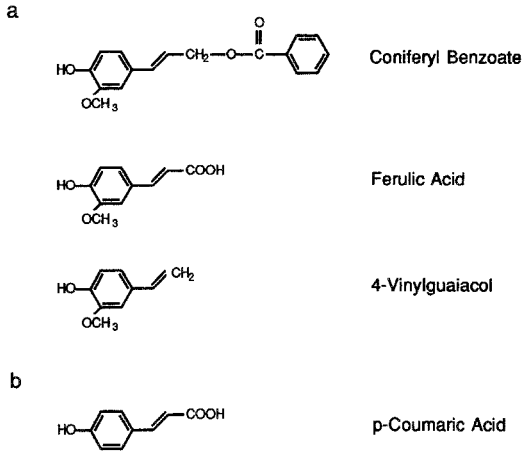

FIG. 1. Chemical structures of (a) coniferyl benzoate and two of its biotransformation products that have been shown to cause antireproductive effects and (b) a phenylpropanoid exhibiting hormonal and antireproductive effects similar to ferulic acid.

grouse selectively feed on buds having low concentrations of this compound (Jakubas et al., 1989) and appear to utilize aspen buds less frequently when annual CB levels are high (Jakubas and Gullion, 1991). Moderate increases in CB intake (e.g., <2.0 g/kg/day) may occur when annual CB levels are high and if other food items are scarce or are unsuitable for consumption. Studies on the physiological effects of CB may clarify mechanisms affecting ruffed grouse population fluctuations, reproductive behavior [e.g., an explanation for reduced drumming activity during periods of low grouse densities (see Gullion, 1984)], and feeding behavior [e.g., an evolutionary explanation for their ability to differentiate between slight changes in CB concentrations (see Jakubas and Gullion, 1990; Jakubas et al., 1993a)].

Relatively little is known about the toxicant-related effects of CB. Coniferyl benzoate is one of the principal skin allergens in gum benzoin Siam and jasmine oil (Hjorth, 1961; Kato, 1984), is a feeding deterrent for birds and insects (Jakubas and Gullion, 1990, 1991; Jakubas et al., 1992), and may result in a negative nitrogen balance (due to ornithine and ammonium excretion) when consumed by grouse (Jakubas et al., 1993b). Ruffed grouse will consume up to ca. 2 g/kg/day of CB before rapid weight loss ensues because of food rejection (Jakubas et al., 1993a). Jakubas and Gullion (1990) speculated that dietary CB may be hydrolyzed to coniferyl alcohol and subsequently oxidized to FA by detoxification mechanisms such as P-450 enzymes or hepatic oxidoreductases. Furthermore, they speculated that if FA were produced, CB might manifest the same antireproductive properties as FA. Free (nonconjugated) FA and trace

amounts of 4-vinylguaiacol have been confirmed as biotransformation products of CB (Jakubas et al., 1993a) (Figure 1).

Dietary FA may affect the physiology of a number of animals (e.g., Berger et al., 1977; deMan and Peeke, 1982; Buchsbaum et al., 1984). Berger et al. (1977) reported that consumption of FA and one of its biotransformation products, 4-vinylguaiacol (also see Scheline, 1968), by female *Microtus montanus* resulted in decreased uterine weight, decreased the number of small to intermediate follicles, and for 4-vinylguaiacol (FA not reported) lowered the number of litters produced. For male *Microtus montanus* fed FA, Sakanari (1983) observed a decreased number of copulatory mounts, intromissions, and a lower number of mature spermatids. Neither Sakanari (1983) nor Berger et al. (1977) proposed a mechanism for these antireproductive effects. deMan and Peeke (1982), working with Japanese quail (*Coturnix coturnix*), suggested that FA had antiestrogenic activity based on decreased cloacal diameters (females) and the inhibition of male copulatory behavior (both responses are partially estrogen mediated).

In addition to its possible antiestrogenic effects, FA may affect reproduction by altering the production or activity of prolactin and other hormones. Gorewit (1983) reported that intravenous administration of FA in Holstein heifers temporarily increased serum levels of prolactin and growth hormone (GH) but did not affect the serum levels of luteinizing (LH) or thyroid hormones. Similarly, Okamoto and Takahashi [1974, as cited in Gorewit (1983)] reported that FA stimulated pigeon crop activity (a prolactin mediated response). However, when FA was administered to male rats, serum levels of follicle-stimulating hormone (FSH) increased, LH decreased slightly, and prolactin decreased significantly (Okamoto et al., 1976). An apparent decrease in prolactin levels was also observed when a closely related compound, *p*-coumaric acid, was administered orally to male and female rats (Pakrashi et al., 1979, 1981) (Figure 1). In addition, oral administration of *p*-coumaric acid resulted in complete loss of male libido, decreased testicle and secondary sex organ weight, and decreased levels of plasma testosterone levels (Pakrashi et al., 1981). For birds, higher prolactin levels are believed to affect parenting behavior [e.g., as in willow ptarmigan (*Lagopus lagopus*), Pedersen, 1989] and may depress estrogenesis by inhibiting the steroidogenic action of LH on the ovary (Sharp, 1980). Lower testosterone levels generally decrease courtship and copulatory behavior (Johnson, 1986), while exogenous testosterone can cause the testes to regress and lower spermatocyte numbers (Balander et al., 1980).

The objectives of our study were to test the hypothesis that dietary CB interferes with male and female avian reproduction, to directly determine whether CB alters prolactin production, and to indirectly determine its estrogenic or antiestrogenic activity. To achieve this, we presented feed treated with CB to Japanese quail and determined the compound's effect on the production, mor-

phology, and development of eggs; sex organ (primary and secondary) characteristics; serum prolactin levels; and male reproductive behavior. Although, ruffed grouse would have been the ideal species to test because of the ecological significance between CB and these birds, Japanese quail were chosen as our model species because: (1) The antiserum used in our prolactin radioimmunoassays may not cross-react with ruffed grouse prolactin. This antiserum was developed using chicken prolactin as the antigen and is known to cross-react with Japanese quail prolactin. (2) The mating behavior of male Japanese quail is well defined (see Wilson and Bermant, 1972). (3) Japanese quail have well-developed ceca, as do ruffed grouse, which may be critical for the absorption and microfloral biotransformation of FA to 4-vinylguaiacol. (4) Comparisons could be made between our results and those of deMan and Peeke (1982). (5) Logistically, it would be very difficult to do this study with ruffed grouse. All animal care procedures were approved by the University of Wisconsin's College of Agriculture and Life Science Animal Care Committee.

METHODS AND MATERIALS

Diet Preparation

The maintenance diet consisted of a laying diet for quail (formulated by the Poultry Science Department, University of Wisconsin) that contained 25% protein and 2600 kcal/kg of available metabolizable energy. Control diets were prepared by mixing the laying ration with ethyl ether and evaporating the ether under a fume hood. For treatment diets, the laying ration was impregnated with CB by dissolving crystalline CB in ethyl ether, thoroughly mixing the feed with the ether solution, and evaporating the ether under a fume hood. Treated and control diets were kept in sealed plastic bags at $-17°C$ until utilized. Coniferyl benzoate was extracted and crystallized from benzoin Siam tears #3 (Alfred Wolff, Paris France) as described in Jakubas et al. (1992, 1993a).

Birds

Japanese quail were obtained from the Department of Poultry Science, University of Wisconsin. For the female reproductive study, laying Japanese quail (8–10 weeks of age) were arbitrarily chosen from communal cages, monitored for egg production, and 24 consistent layers selected for the study. For the male reproductive study, birds were screened (see below) for their reproductive performance, and 20 were selected for the study. All birds were housed individually in 27.5 × 46-cm stainless steel cages under constant temperature conditions (18.5°C and 22°C, females and males, respectively) and kept on the same light cycle (14:10 hr, light–dark) that they were accustomed to prior to

these studies. During the pretrial acclimation period, birds were provided untreated feed (maintenance diet) and tap water ad libitum. Acclimation time for females was at least one month, except for two control and two treatment birds (pairs) that were only acclimated eight days. Acclimation time for males ranged from one week to one month. Males were visually isolated from stimulus females except during behavior trials.

Reproductive toxicity trials incorporated a pair-fed design to control for physiological effects associated with the decreased food intake that often occurs with CB consumption. Therefore, females (all wild-type color phase) were paired according to their mass, egg productivity, and food consumption, while males were paired according to their mass, color phase (i.e., wild-type and recessive white), and food consumption. Individuals from each pair were randomly placed into treatment and control groups, and housed in separate cage banks under equal light intensities.

CB Metabolism

Coniferyl benzoate biotransformation products were determined by comparing the chemical composition of excreta from female Japanese quail fed a 1.5% CB diet to that of females fed the maintenance diet. Excreta were immediately frozen and lyophilized within 10 days after collection. For free FA determinations, lyophilized excreta (5 g) were extracted (2×) in darkened containers with ethyl ether (50 ml) by rapidly stirring the ether–excreta mixture for 30 min. Extracts were combined, filtered, and concentrated by evaporation. Ether extracts were analyzed for FA by high-pressure liquid chromatography (HPLC) (see below). The presence of conjugated FA, occurring as feruloyl glucuronide, was determined by enzymatically hydrolyzing the glycosidic bond following Voight and Schmidt (1974), extracting the free acid, and analyzing the extract by HPLC. Briefly, lyophilized quail excreta (100 mg) were extracted by vortexing the excreta with 10 ml of 0.3 M Tris buffer (pH 10) for 30 min. The extract was filtered, acidified to pH 6.5 with acetic acid, and boiled for 15 min. After the extract had cooled, β-glucuronidase (type IX-A; from *E. coli*; Sigma) was added and the mixture, incubated overnight at 37°C. Following incubation, the aqueous hydrolysate was extracted with methylene chloride (3×) and the organic phase dried with magnesium sulfate. Biotransformation products were separated by HPLC (Beckman 110 B pumps coupled to a 167 scanning detector and a 406 analog interface module) using a Waters μBondapak C_{18} column (3.9 × 300 mm) and detected at 295 nm. Chromatographic conditions were T_0 (time = 0 min) flow rate = 1 ml/min, solvent mix = 70% A; T_1 = 10 min, flow rate 1 ml/min, 40% A, ramp time = 2 min; T_2 = 35 min, flow rate = 2 ml/min, 70% A, ramp time = 2 min; T_3 = 42 min, flow rate = 1 ml/min, 70% A; where solution A is 2% tetrahydrofuran in water and solution

B is acetonitrile. Ferulic acid was identified by comparing sample peaks to that of a commercial FA standard (Sigma, St. Louis, Missouri). Additional biotransformation products were identified by gas chromatography (GC) (these products were previously identified using GC–mass spectrometry and known standards) following the methods in Jakubas et al. (1993a).

Female Reproductive Studies

Initial Screening. Twelve days prior to the start of reproductive toxicity trials, a two-day pilot study (food palatability trial) was conducted to determine the level of dietary CB that Japanese quail would tolerate. Standard 2-hr, one-cup tests were used to evaluate food palatability (e.g., Mason et al., 1989), with the exception that pretreatment and treatment periods were only one day in length. Briefly, 12 birds from the treatment group were randomly assigned to three groups (four birds/group), and each group assigned a test regime. On day 1, all birds were deprived of food overnight and for 3 hr after light onset (2000 to 0900 hr) to encourage food consumption. At 0900 hr all birds were given 25 g of the control diet. Food consumption was measured at 1100 hr, and birds were allowed to feed ad libitum on their maintenance diet until lights out. On day 2, the same food deprivation routine was followed. However, at 0900 hr, each group received 25 g of one of three treatment diets (1.5%, 2.0%, 3.0% CB). At 1100 hr, food consumption was measured and the birds were allowed to feed ad libitum on their maintenance diet. Each bird's food consumption (2-hr) on day 1 was compared to its 2-hr food consumption on day 2.

Following the palatability trials, pretrial (i.e., the reproductive toxicity trial) feed intake (grams per day) was measured for 48 hr.

Reproductive Measurements. Pretrial egg production and morphological measurements were made from eggs collected during an eight-day period immediately prior to the reproductive toxicity trial. Pretrial eggs were compared to trial eggs (12-day period) for morphological measurements including mass, color, specific gravity of (dry mass − mass of water displaced by the egg), and shell thickness (following Ratcliff, 1970). For egg color, four or more pretrial eggs, from an individual bird, were compared to the color and texture of its trial eggs by a single observer.

Each bird's pre- and post-trial blood samples (1 ml) were taken from the jugular vein at the same time of day. Pretrial samples were collected six days prior to the reproductive toxicity trial (three birds were sampled one day prior to the trial), and posttrial blood samples were taken one day after the end of the feeding routine (day 13 for treatment birds and day 14 for control birds). Serum samples for prolactin determinations were stored at −17°C until analyzed. Packed cell volumes (PCV) were determined from blood samples collected in heparinized capillary tubes that had been centrifuged at 3000 rpm for 7 min. A

bird's PCV was calculated as the ratio of red blood cells to plasma and expressed as a percentage.

For female Japanese quail, cloacal diameter is a sensitive indicator of ovarian and estrogen activity (Noble, 1973). Cloacal diameter (length) and bird mass were measured on the same days that blood collections were made. Other reproductive measurements were made after sacrificing the birds. Birds in treatment and control groups were sacrificed on day 13 and day 14, respectively. Mass measurements were made on the oviduct, entire ovary, the ovary without the three largest follicles, and the three largest follicles. In addition, the diameter of the three largest follicles and the number of intermediate size follicles (i.e., 1.5–3.0 mm diameter) were determined.

Prolactin Assay. Prolactin (AFP-10328B) was iodinated using a modification of the chloramine-T method of Hunter and Greenwood (1962). Incorporation of ^{125}I into protein was determined by first adding 1 μl of the iodination mixture to 1 ml of buffer [0.1 M phosphate buffer (PBS), pH 7.4, containing 5% thin egg albumin] and subsequently adding 2 ml of 1% (mass/v) phosphotungstic acid. The mixture was then centrifuged at 3000 rpm for 3 min, and a 300-μl aliquot of the supernatant measured for radioactivity using a Micromedic gamma counter. The labeled hormone was isolated from the iodination mixture in two steps using gel chromatography. For the initial cleanup, a 15-cm × 0.5-cm column of Sephadex G-25, overlain with 25% bovine serum albumin (BSA) (1:2, iodination solution–BSA) was used and eluted with 0.05 M (pH 7.4) sodium phosphate buffer. Fractions having high radioactivity counts and containing the marker dye were combined and further purified using a 100-cm × 1.5-cm column of Sephadex G-75 and the same eluent as above. The purified labeled hormone was diluted with the PBS–5% albumin buffer and stored under refrigeration until used in the assay.

For the assays, antiserum 1, AFP-151040789 (developed by A. Parkow, Harbor-UCLA Medical Center) was diluted (1:32,000) with PBS (pH 7.4) containing 0.5% normal rabbit sera and 0.05 M of disodium ethylene diaminetetraacetate and brought to a final tube dilution of 1:320,000. Antiserum 2 (sheep anti-rabbit) was diluted (1:40) with PBS and brought to a final tube dilution of 1:400. A standard curve was developed from 14 log increment concentrations (0.31–40 ng) of reference prolactin (AFP-10328B) as described in Wentworth et al. (1976). Standards and 100-μl aliquots of quail serum were incubated 24-hr at room temperature (ca. 20°C) with [^{125}I]prolactin (30,000 counts/min), antiserum 1, and PBS–5% albumin. Final tube volume for standards and unknowns was 1 ml, and all determinations were done in duplicate. Following the incubation, antiserum 2 was added, the mixture incubated at room temperature overnight, centrifuged, and analyzed for radioactivity as described above. Specific binding was determined by subtracting the mean nonspecific binding ([^{125}I]prolactin present, antiserum 1 absent) from total binding

([^{125}I]prolactin and antiserum 1 present). Cross-reactivity was checked by assaying three concentrations (50, 100, 200 ng/ml) of chicken LH, FSH, and GH, along with the serum from a hypophysectomized chicken (hypox. serum). Parallelism was checked by determining if the slope of the standard curve developed from reference prolactin differed significantly (95% confidence interval) from the slope of the regression derived from four concentrations of hypox. serum spiked with reference prolactin.

Reproductive Toxicity Trials. Trials consisted of presenting 10 pairs of birds (four birds were removed from the experiment because they were inconsistent layers) matched amounts of food over a 12-day period. On day 1, at 1600 hr, 10 birds in the treatment group were given 40 g of treatment diet (CB treated feed). On day 2 (1600 hr), 10 birds in the control group were allowed to consume the same amount of feed (control diet) as their counterpart in the treatment group consumed (CB diet) on day 1. This staggered feeding routine continued until the end of the trial. Food intake during the reproductive toxicity trial was compared daily to pretrial intake levels to assure adequate food consumption.

Initially, a 2% CB diet was selected for the reproductive toxicity trial based on pilot study results (see Results). However, after day 3 of the trial, it was apparent that at least four birds in the treatment group would not consume enough of the treatment diet (i.e., their consumption rates were less than 80% of their pretrial levels) to maintain egg production. Therefore, in order to improve food consumption, the CB concentration of the treatment diet was lowered to 1.5% for all birds in the treatment group for the remainder of the trial (day 4 through day 12).

Male Reproductive Studies

Initial Screening. All males were evaluated for aggressiveness and libido using female conspecifics prior to their inclusion in the reproductive behavior study. Males were isolated (visually and physically) from females 24 hr prior to testing. The observation arena consisted of an introductory chamber and an observation box. The observation box (57.5 × 40 × 38 cm) was constructed out of plywood, with a hardware cloth floor and a clear Plexiglas front. The back of the observation box was a sliding door that permitted access to the interior. A swinging door on the side of the observation box permitted the attachment of a Plexiglas introductory chamber (20 × 15 × 16 cm). The introductory chamber was translucent except for a clear sliding door that could be raised and lowered by a pulley system. Opposite from the introductory chamber and centered 29 cm from the floor of the observation box was an 11 × 7-cm hole covered with hardware cloth, from which a 100-W incandescent lamp illuminated the box. All observations were made from a blind located approximately 3 m from the observation box. For the initial screening, one to two

actively laying females were placed in the observation box. A male was then placed in the introductory chamber, and the chamber attached to the observation box. The birds were allowed to observe each other through the clear door of the introductory chamber for 30 sec before the door was raised. After the door was raised, the male was given 3 min to complete a sequence of mating behaviors. Mating behaviors included head grab, mount, and cloacal contact movement, as defined by Wilson and Bermant (1972). If the male did not complete this sequence of mating behaviors in the allotted time, it was not used in the study. Stimulus females were generally replaced with new females after they had endured three or four matings or if they became overly aggressive.

Prior to the reproductive behavior trials, food acceptability was assessed using 2-hr food palatability trials following the same protocol as described for the females, with the following exceptions. Only 11 treatment males were used for these trials (one bird was later excluded from the study), the trials were conducted nine days prior to the birds being presented their treatment diet in the behavior trials, and only 15 g of CB-treated feed was presented to each bird. Pretrial food intake (24-hr) was determined immediately following the palatability trials.

Behavioral and Reproductive Measurements. The pretrial mating behaviors of 10 pairs of control and treatment males were assessed over three days (one mating trial per day for each bird) immediately prior to the reproductive toxicity trials. Reproductive behavior trials started between 1230 and 1300 hr, and consisted of presenting a male to a single female in the previously described observation arena. Seven actively laying females were used as stimulus birds. Paired males were tested consecutively and generally presented the same female. A different female was presented to each pair of males on each of the three days, with the exception of the few males that would only mate with a specific female. Those males were only mated with one female over the three-day testing period. In general, only males and females of a like color phase were mated (see Results). Males and females were given 30 sec to observe each other before the door of the introductory chamber was opened. Mating behaviors were recorded for 4 min after the introductory chamber's door was opened. These behaviors included strut, head grab, mount, and cloacal contact movement (latency, duration, and frequency) (see Wilson and Bermant, 1972). Latency, in general, was considered to be the period of time from the opening of the chamber door to the initiation of a head grab by the male. Males were allowed to complete any behavior initiated before the end of the 4-min period. Behaviors were recorded using an Atari Portfolio computer (programmed by J. Coleman, University of Wisconsin). If a male repeated a single behavior in less than one second, it was counted as a single event. A bird's pretrial mating performance, therefore, was determined by the mean latency, frequency, and duration of each mating behavior during the pretrial period (three days).

Blood samples were taken between 0930 and 1100 hr one day prior to the birds receiving the treatment diet, and PCVs determined using the same protocol as given for the females. On the same day, body mass and the width and depth of the proctodeal gland (synonymous with the foam gland or cloacal gland) was measured. Proctodeal gland measurements are reported as proctodeal gland area (width × depth). Proctodeal gland development is under androgen control, and consequently its size is closely correlated with testicular size (Klemm et al., 1973).

Reproductive Toxicity Trial. Reproductive toxicity trials incorporated a pair-fed design similar to that described for the females. Ten treatment males were presented 25 g of the 2% CB diet each day. Control birds ($N = 10$) were allowed to consume the same amount of feed (control diet) as their paired counterpart consumed when it was given the treatment diet. All birds were kept on this feeding routine for 13 days. On days 10–12 (three days) of the trial, reproductive behavior was assessed following the same protocol described for the pretreatment period. Males were mated with the same females used in the pretreatment period. On day 14 the birds were bled and proctodeal gland measurements taken. On day 15, birds were measured for body mass, euthanized, and their testes and proctodeal gland weighed.

Statistical Analyses

Palatability trials were analyzed using a two-factor analysis of variance (ANOVA) with CB concentration as the independent factor and repeated measures between testing periods. For egg production and food consumption, the daily differences between paired birds (i.e., treatment − control) were summed for the trial and analyzed using paired t tests. Coniferyl benzoate intake (grams per kilogram per day) was calculated using daily food consumption and a bird's mean pre- and posttrial body mass. Male pairing assignments were primarily made to ensure equal light conditions and food consumption, rather than equivalent reproductive behaviors. Therefore, the mating behaviors were analyzed using unpaired t tests (two-tailed). Physiological responses (male and female) and differences between treatment periods were compared using paired t tests (two-tailed). Simple linear regressions were used in the prolactin assay to calculate standard curves. For all cases, differences were considered significant if $P < 0.05$. Means ± standard error (SE) are reported.

RESULTS

CB Biotransformation

As expected, the biotransformation of CB resulted in the excretion of ferulic acid and its conjugated form feruloyl glucuronide, when female quail fed on a 1.5% CB diet. Other biotransformation products that appeared to be similar to

those from ruffed grouse included: benzoic acid, eugenol, vanillin, coniferyl alcohol, and acetovanillone (see Jakubas et al., 1993a).

Palatability Trials

Both male and female quail consumed less feed during the treatment period (all CB concentrations) than during the pretreatment period (control diet) of the palatability trials ($F = 48.02$; 1,8 *df*; $P < 0.01$ and $F = 4.07$; 1,9 *df*; $P = 0.02$; respectively) (Table 1). However, birds did not discriminate between CB concentrations, as indicated by the nonsignificant interaction term (concentration × period) of the ANOVA ($F = 0.75$; 2,8 *df*; $P = 0.50$ and $F = 0.03$; 2,9 *df*; $P = 0.98$; males and females respectively). Since birds did not appear to discriminate among any of the CB concentrations, a CB concentration of 2% was chosen for the reproductive toxicity trials. We felt that 2% concentration was ecologically realistic, would provide a CB intake similar to that of ruffed grouse in the wild, and would be sufficient to induce antireproductive effects.

Female Reproduction

Food Intake and Body Mass. Female consumption of the 2% CB diet during the first three days of the reproductive toxicity trial was 73.6% ± 4.8 of their pretrial food intake and consequently was considered unsatisfactory for maintaining egg production (i.e., we did not want food-intake levels to drop below 80% of pretrial levels). Daily food consumption during this period for birds in the treatment group was 17.1 ± 0.9 g as compared to 22.8 ± 1.0 g during the pretrial period. Therefore, on day 4, all birds in the treatment group were put on a 1.5% CB diet. Food consumption increased to 85.9 ± 1.1% of pretrial levels on the 1.5% CB diet, and mean daily food intake over the remaining nine days of the trial was 19.2 ± 0.3 g. However, food intake on the 1.5% CB diet was lower than during the pretrial period (Table 2). Coniferyl benzoate intake

TABLE 1. MEAN 2-hr FOOD CONSUMPTION (±SE) FOR MALE (M) AND FEMALE (F) JAPANESE QUAIL DURING PRETREATMENT (CONTROL DIET) AND TREATMENT PERIOD (3 CONCENTRATIONS OF CB) OF CB PALATABILITY TRIAL

Sample size (M/F)	Pretreatment consumption (g)		Treatment consumption (g)		Percent CB
	Male	Female	Male	Female	
3/4	2.3 ± 0.5	5.7 ± 0.6	1.3 ± 0.5	4.9 ± 0.3	1.5
4/4	2.2 ± 0.4	5.0 ± 0.8	1.4 ± 0.1	4.3 ± 0.6	2.0
4/4	2.2 ± 0.4	3.9 ± 0.1	1.0 ± 0.2	3.1 ± 0.2	3.0

TABLE 2. FOOD INTAKE AND BODY MASS (±SE) OF MALE AND FEMALE JAPANESE QUAIL (CONTROL AND TREATMENT BIRDS) PRIOR TO AND DURING CB FEEDING ROUTINE

Parameter	Pretrial		Trial		P^a	
	Control	Treatment	Control	Treatment	Control	Treatment
Females						
Food Intake (g/day)	22.6 ± 1.0	22.8 ± 1.0	18.4 ± 0.3	18.7 ± 0.4	0.002	0.003
Body mass (g)	147.1 ± 3.1	148.7 ± 4.2	147.9 ± 4.9	138.9 ± 2.4	0.879	0.009
Males						
Food Intake (g/day)	13.6 ± 0.6	13.1 ± 0.7	11.19 ± 0.2	11.39 ± 0.3	0.002	0.005
Body mass (g)	128.7 ± 3.4	127.7 ± 4.4	126.3 ± 3.4	121.9 ± 3.4	0.077	0.004

[a] Indicates difference between pretrial and trial periods. Within trial differences are all nonsignificant (P values are given in the text).

on the 2% and 1.5% CB diets was 2.38 ± 0.14 and 2.01 ± 0.03 g/kg/day, respectively.

As per study design, feed consumption did not differ ($P = 0.422$) between treatment and control birds over the 12-day CB feeding routine (Table 2). The body mass of birds in the control group was slightly higher than that of birds in the treatment group after the CB feeding routine, but this difference was not significant among pairs ($P = 0.125$) (Table 2). However, it was evident over the course of the CB feeding routine that birds in the treatment group lost a significant amount mass, compared to their pretrial mass, while birds in the control group maintained their pretrial mass (Table 2).

Reproduction Indicators. Pretrial egg production was similar ($P = 0.270$) among control and treatment bird groups. Egg production during this period was 0.79 ± 0.03 and 0.74 ± 0.05 eggs/day/bird for control and treatment groups, respectively. Egg production during the CB feeding routine was significantly lower (ca. 15% lower) for birds receiving the treatment diet than for the pair-fed controls (Table 3). Mean egg production for birds in the treatment group decreased slightly from the pretreatment period while egg production in the control group increased. Follicle development appeared to be significantly different between control and treatment groups. The mass of the three largest follicles was significantly greater in treatment birds than controls (Table 3). The

TABLE 3. MEAN EGG PRODUCTION, EGG MORPHOLOGICAL MEASUREMENTS, AND REPRODUCTIVE TRACT MEASUREMENTS FOR PAIR-FED TREATMENT AND CONTROL JAPANESE QUAIL

Parameter	Treatment	Control	P^a
Daily egg production (eggs/birds)	0.68 ± 0.06	0.8 ± 0.05	0.051
Total egg production (eggs/group/12 days)	82	96	
Egg mass (g)	8.97 ± 0.20	8.92 ± 0.17	0.842
Egg specific gravity	1.018 ± 0.005	1.021 ± 0.003	0.579
Egg shell thickness index	0.97 ± 0.02	0.99 ± 0.02	0.306
Oviduct mass (g/kg)	46.24 ± 1.82	43.42 ± 2.10	0.283
Whole ovary mass (g/kg)	40.86 ± 3.15	34.31 ± 2.61	0.083
Ovary mass minus 3 largest follicles (g/kg)	5.96 ± 0.61	6.08 ± 0.50	0.892
Largest follicle (mm)	17.4 ± 0.5	15.1 ± 0.6	<0.001
Second largest follicle (mm)	14.3 ± 0.5	14.1 ± 0.3	0.198
Third largest follicle (mm)	10.7 ± 0.5	10.5 ± 0.5	0.211
Three largest follicles (g/kg)	34.80 ± 2.70	29.88 ± 1.41	0.011
Number of intermediate follicles (1.5–3.0 mm)	16 ± 1.8	20 ± 1.7	0.124
Cloacal diameter (mm)	9.3 ± 0.4	9.5 ± 0.4	0.667

[a] P values are for paired comparisons between treatment and control groups.

greater follicle mass in treatment birds was primarily attributed to the significantly larger F1 follicle in these birds (Table 3).

There were no significant differences between control and treatment birds in parameters that are readily influenced by estrogen production, including: egg color, shell thickness, and specific gravity; oviduct mass; cloacal diameter; and PCV (Table 3). Blood analyses indicated that PCVs were similar among control and treatment birds ($P = 0.580$). The percentage of red blood cells in the serum was 47.4 $\pm$ 1.6% and 48.6 $\pm$ 0.8% for treatment and control birds, respectively, following the 12-day feeding routine of the reproductive toxicity trial and was similar to pretrial levels (45.4 $\pm$ 1.3% and 47.0 $\pm$ 1.3% for treatment and control groups, respectively).

Serum prolactin levels were not significantly different between treatment and control females before ($P = 0.447$) or after ($P = 0.263$) the reproductive toxicity trial. Serum prolactin levels were 78.7 $\pm$ 6.3 and 85.8 $\pm$ 9.0 ng/ml for posttrial treatment and control birds, respectively, and 83.2 $\pm$ 6.6 and 79.9 $\pm$ 7.3 ng/ml for pretrial treatment and control birds. Individual birds did not differ in their serum prolactin levels pre- or posttrial in either the treatment ($P = 0.353$) or control ($P = 0.472$) groups. Tests for cross-reactivity with other hormones (i.e., LH, FSH, GH) indicated that the first antibody did not bind with these hormones at any of the three concentrations tested. Similarly, the first antibody showed no affinity for hypox. serum. Tests for parallelism indicated that the slope of the regression developed from hypox. serum spiked with reference prolactin was within the 95% confidence interval of the slope of our prolactin standard curve.

Male Reproductive Studies

Food Intake and Body Mass. Unlike the females, male food consumption of the 2% CB diet over the first three days of the trial did not drop below 80% of pretrial levels. Treatment males consumed 10.5 $\pm$ 0.7 g of food per day for the first three days of the trial or 80.3% $\pm$ 5.3 of pretrial levels (i.e., 13.1 $\pm$ 0.7 g/day). Over the entire 13-day CB feeding routine, treatment males consumed 87.8% $\pm$ 2.1 of their daily pretrial food intake. As per study design, food consumption of paired birds did not differ ($P = 0.274$) over the 13-day trial (Table 2). Coniferyl benzoate intake over the 13-day trial was 1.84 $\pm$ 0.04 g/kg/day. Body mass did not differ among paired control and treatment males before or after the 13-day CB feeding routine ($P = 0.780$ and $P = 0.177$, respectively) (Table 2). Similar to the females, treatment males lost a significant amount of mass over the course of the CB feeding routine, while the mass loss of pair-fed control males was not significant (Table 2).

Behavioral and Physiological Effects. Pretrial screening indicated that certain males were very selective in the females that they would mate with. For

example, a recessive-white male that was caged with a wild-type color phase female prior to the study would only mate with wild-type females. In particular, we found that wild-type males were not interested in recessive-white females (e.g., no mating behavior was observed in five mating attempts with different recessive-white females) but would readily mate when presented with a wild-type female. However, recessive-white males, in general, did not show a preference for a particular color phase. For these reasons, wild-type males were only mated with wild-type females in our study and males that would only mate with one or two particular females were only mated with those females.

Consumption of the 2% CB diet by treatment males did not appear to have any major effect on the number, duration, and latency of the four mating behaviors we measured (Table 4). Treatment males appeared to make fewer head grabs than control males; however, the difference was not significant (Table 4). Treatment males did not differ from controls, after the 13-day feeding routine, in the size or mass of their proctodeal glands or testes (Table 5). Control males did have larger (area, square millimeters) proctodeal glands ($P = 0.038$) than treatment males prior to the 13-day feeding routine; however, neither the control or treatment groups appeared to differ in proctodeal gland size from the pretrial to posttrial period ($P = 0.353$ and $P = 0.158$, respectively) (Table 5). Finally, there was no difference in blood PCV between control and treatment males (Table 5).

DISCUSSION

Consumption of CB by Japanese quail did not affect male reproductive performance but did have a minor but significant effect on female reproduction. Egg production for treatment females consuming 2.01–2.38 g CB/kg/day was ca. 15% lower ($P = 0.05$) than for females receiving the control diet. In addition to decreased egg production, females and males feeding on CB diets lost a significant amount of body mass over the treatment period (12–13 days), while control birds maintained or only slightly decreased their body mass. This was especially evident for females, where pair-fed control birds, which laid 15% more eggs, maintained their body mass while treatment birds lost ($P = 0.009$) body mass.

Male Reproduction

Although quail metabolized CB into FA, the amount of FA produced from consuming 1.84 ± 0.04 g CB/kg/day apparently was not sufficient to reduce male reproductive behavior. Comparatively, deMan and Peeke (1982) observed decreased reproductive behavior when their male quail fed on a 0.5% (mass/mass) FA diet. Estrogen and testosterone are the principal hormones that influ-

TABLE 4. REPRODUCTIVE BEHAVIORS (3-DAY MEANS) FOR MALE TREATMENT AND CONTROL JAPANESE QUAIL BEFORE 2% CB FEEDING ROUTINE AND AFTER 10 DAYS OF ROUTINE

	Pre-trial		Trial		
Parameter	Control	Treatment	Control	Treatment	P^a
Head grabs (*N*)	3.2 ± 0.5	3.0 ± 0.7	5.0 ± 1.1	2.8 ± 0.6	0.108
Latency (sec)[b]	19.9 ± 6.0	44.0 ± 20.6	37.1 ± 19.8	12.3 ± 3.7	0.260
Head grab duration (sec)[c]	24.8 ± 4.2	22.3 ± 4.7	28.67 ± 6.1	22.4 ± 5.1	0.444
Strutting duration (sec)	1.9 ± 1.0	3.5 ± 1.5	3.4 ± 1.5	2.5 ± 1.2	0.638
Mounts (*N*)	2.7 ± 0.4	2.7 ± 0.6	3.8 ± 0.6	2.6 ± 0.6	0.174
Mount duration (sec)	12.0 ± 1.8	10.5 ± 2.2	10.8 ± 1.7	9.7 ± 2.3	0.685
Cloacal contacts (*N*)[d]	2.1 ± 0.3	1.6 ± 0.3	2.4 ± 0.3	1.8 ± 0.3	0.222

[a] Behaviors are reported as daily means ± SE for the 4 min observation period. *P*-values are for unpaired t-tests comparing the response of control and treatment birds during the treatment period (trial).
[b] Latency is the number of seconds before the male made his first head grab.
[c] Duration connotates the total amount of time spent performing this activity.
[d] Cloacal contacts connotes the number of times a male copulated not individual cloacal contact movements.

TABLE 5. PHYSIOLOGICAL MEASUREMENTS OF MALE TREATMENT AND CONTROL JAPANESE QUAIL BEFORE 2% CB FEEDING ROUTINE AND AFTER 13 DAYS OF FEEDING ROUTINE

Parameter	Pretrial		Trial		P^a
	Control	Treatment	Control	Treatment	
PCV[b]	52.2 ± 0.9	53.0 ± 2.0	48.6 ± 1.6	49.79 ± 1.2	0.560
Proctodeal gland area (mm^2)	214.9 ± 8.7	188.0 ± 10.0	207.4 ± 9.5	206.1 ± 12.9	0.931
Proctodeal gland mass (g/kg)			14.50 ± 0.38	14.39 ± 0.73	0.898
Total testes mass (g/kg)			28.62 ± 2.49	28.22 ± 1.91	0.900

[a] P values are for paired t tests comparing control and treatment birds during the treatment period (trial).
[b] Packed cell volume is reported as percent red blood cells.

ence male reproductive behavior in Japanese quail (Adkins et al., 1980; Balander et al., 1980). Coniferyl benzoate did not appear to interfere with the functions of either of these hormones, based on male reproductive behavior, the size of the testes and proctodeal gland (influenced by testosterone), blood PCV levels (influenced by estrogen), and estrogen-mediated responses in female quail. Consumption of food treated with higher concentrations of CB could conceivably produce enough FA to elicit antireproductive effects, similar to those reported by deMan and Peeke (1982); however, it is doubtful whether quail or other birds (e.g., ruffed grouse) would consume a sufficient amount of this food to maintain their energy balance or actually increase their CB intake. For example, the highest intake of CB observed for Japanese quail (females), over a three-day period (2.38 ± 0.14 g CB/kg/day, 2.0% CB diet) was very similar to the maximum CB intake (2.3 ± 0.11 g CB/kg/day, 6.5% CB diet) of ruffed grouse over a four-day period (see Jakubas et al., 1993a). Coniferyl benzoate intake was limited in both of these instances by decreased food consumption. Therefore, although CB may have the potential to effect the reproductive behavior of male birds via the production of FA, it does not appear that Japanese quail or ruffed grouse would consume sufficient quantities of CB to elicit an "antireproductive" response.

There were marked differences in the reproductive behavior of Japanese quail in our study compared to quail in deMan and Peeke's (1982) study. Control males in deMan and Peeke's study performed two to three times the number of head grabs (nine), mounts (nine), and cloacal contacts (eight) as our birds (cf. Table 4). However, the duration of some of these behaviors (e.g., head grabs) were longer in our study. One reason for the higher frequency of these behaviors may be that their prescreening procedure selected birds that would mate more readily. They rejected males that would not go through the complete mating sequence within 1 min after being placed in the same box as a female, while

we found that a 3-min time limit was more realistic for our birds. Once exposed to the mating routine, however, our males would normally attempt to mate well within the first minute of being exposed to a female (see latency Table 4).

Female Reproduction

Our study indicates that the negative effect that CB had on egg production may be more related to its effect on energy assimilation and retention than to its effect on the endocrine system. We expected CB to have an antiestrogenic effect similar to that reported for its metabolic product, FA (see deMan and Peeke, 1982). However, we did not see any evidence that CB affected estrogen sensitive parameters such as oviduct mass, female cloacal diameters, and blood PCV (Table 3). In addition, dose–response growth assays (growth measured as total DNA) using human breast cancer cells did not indicate that *p*-coumaric acid, CB, or its metabolites FA, 4-vinylguaiacol, and coniferyl alcohol had any estrogenic or antiestrogenic activity (unpublished data, W.J. Jakubas, V.C. Jordan, and C. Parker, University of Wisconsin). Structure–activity studies (Jordan et al., 1985) also indicate that the principal metabolites of CB and FA do not have the structural configuration necessary to produce an estrogenic or antiestrogenic response (also see Scheline, 1978, p. 209; Jakubas et al., 1993a).

Coniferyl benzoate ingestion apparently did not affect prolactin production as was observed when FA was administered to other animals. However, in these other studies (i.e., Okamoto et al., 1976; Gorewit, 1983), FA was administered intravenously, and serum prolactin levels were only monitored over several hours postinjection. The oral route of administration and the prolonged exposure to CB (and hence FA) in our study may be two reasons why we did not observe a change in prolactin levels, but these factors did not appear to be critical in prolactin studies where rats were fed *p*-coumaric acid (cf. Pakrashi et al., 1979, 1981).

The significantly larger F1 follicle of treatment females as compared to controls cannot be attributed to a specific hormonal change or antireproductive factor. However, one of us (B.C.Wentworth) has observed that treatments which block ovulation often result in the F1 follicle becoming significantly larger than normal before undergoing atresia. Atretic follicles, however, did not occur more frequently in treatment birds than controls.

Dietary CB has several effects on the absorption and retention of nutrients and energy, which could affect egg production. For ruffed grouse, CB lowers energy absorption and retention efficiencies and increases the rate of nitrogen excretion (Jakubas et al., 1993b). At CB intake levels nearly identical to those of the present study, the energy lost by ruffed grouse from the excretion of ornithine and glucuronic acid conjugation products was 4.2% of their assimilated energy (Jakubas et al., 1993b). In addition to energy losses associated with

detoxification, the amount of utilizable energy that our quail could obtain from their food would be diluted by the 1.5% CB content of the feed. Assuming that detoxification costs from CB ingestion are similar for ruffed grouse and quail, female quail consuming the 1.5% CB diet should experience approximately a 6% decrease (i.e., costs of detoxification + dilution effect) in the amount of energy that they could retain from their food, as compared to pair-fed control birds.

Using this 6% energy loss as the minimum cost attributable to the toxic effects of CB, we can calculate whether it would be sufficient to account for the decreased egg production in our treatment birds. The basic premise for these calculations is that the difference between the maximum sustainable intake of metabolizable energy and basal energy expenditures determines the amount of energy available for various activities (see Karasov, 1986; Calow and Sibly, 1990). By partitioning an animal's total energy output into maintenance, extraneous activity, and reproductive expenditures, we can determine the energetic impact a toxicant has on a given activity such as reproduction. For example, if we assume that maintenance costs are fixed, a laying hen in our study could compensate for reduced energy intake (food) and increased detoxification costs by either decreasing extraneous activity (e.g., walking), increasing her use of stored energy (catabolism), by decreasing reproductive costs, or by using some combination of these factors. In our study, decreased food intake did not affect the egg production of pair-fed control birds or cause a decrease in their body mass. Applying these results to our model we would assume that control birds decreased their extraneous activity to keep egg production constant.

Similarly, our model would predict that treatment birds decreased their egg production and lost body mass in order to compensate for a drop in energy acquisition and retention. But would the above 6% drop in utilizable energy account for this drop in egg production? Laying quail consume approximately 33% more of our maintenance diet than nonlaying birds (B.C. Wentworth, personal observation). If we assume that normal maintenance costs are constant, a 33% increase in food consumption would equate to a 33% increase in energy intake to meet reproductive costs. By partitioning energy expenditures, we can see that the reproductive energy expenditure makes up 25% {25% = [33 (increase in energy intake)/133 (total energy intake)] · 100} of the total energy budget. If we assume that the other 75% of a bird's energy expenditures are fixed, the 6% reduction in utilizable energy must come from the reproductive component or from 25% of the bird's total energy intake. This translates to a 24% decrease in the amount of energy available for reproduction. Therefore, the 15% lower in egg production, and possibly the higher mass loss, of the treatment birds in our study could be explained by the predicted 24% decrease in energy available for reproduction, without implicating any other toxicological effects. In a broader sense, this example illustrates the energy constraints that

dietary plant secondary metabolites place on an animal's physiology and behavior through detoxification costs and interference with energy acquisition.

Comparisons to Ruffed Grouse

Although grouse and quail may attempt to limit their CB intake to approximately the same level (i.e., 2.0 g/kg/day), the physiological affects of CB appear to be slightly more pronounced in Japanese quail. Paired-intake feeding trials with ruffed grouse indicate that the mass loss associated with a CB intake level of 2.3 g/kg/day could be primarily attributed to reduced food consumption; however, the significant mass loss experienced by quail consuming ca. 2.0 g CB/kg/day was independent of food intake. This suggests that quail experienced different adverse effects or were more susceptible to the toxic effects of CB than ruffed grouse. Although it was unclear whether the most prevalent factor limiting ruffed grouse consumption of CB was toxicity or palatability (Jakubas, 1993a), postingestional effects were obvious in the present study with quail and likely played a role in limiting CB consumption. This raises the question of whether ruffed grouse are better adapted to the toxicity of CB because of their association with quaking aspen over evolutionary time. Excretal analyses indicate that the biotransformation of CB by ruffed grouse and quail is similar. Both grouse and quail feeding on a 1.5% CB diet produced free and conjugated forms of FA; however, preliminary data indicate that quail appear to produce fewer types of unconjugated biotransformation products than grouse (Jakubas, unpublished data; also see Jakubas et al., 1993a).

The palatability of CB-treated food appears to be similar among Japanese quail, ruffed grouse, and European starlings (*Sturnus vulgaris*) (see Jakubas et al., 1992, 1993a). This is consistent with the irritant properties of CB and the unspecialized nature of the trigeminal nerves, which sense this chemical irritation (Jakubas and Mason, 1991). Although quail did not discriminate among different CB concentrations (1.5% to 3% mass/mass in 2-hr palatability trials, food consumption was significantly lower over all CB concentrations than consumption of the control diet.

Ecological Relevance

Energetic costs associated with detoxification and lower utilization efficiencies are higher for ruffed grouse feeding on aspen buds than on CB-treated food, in part, because of the other phenolic compounds in aspen buds (Jakubas et al., 1993b; Guglielmo, 1993). Guglielmo (1993) compared the digestive efficiencies and detoxification costs of grouse feeding on quaking aspen buds having low and moderate levels of CB. In that study, he found that ruffed grouse were 24% less efficient in extracting and retaining energy when feeding on buds containing 2.4% CB than 1.3% CB buds. In addition, birds feeding on aspen

buds containing 2.4% CB had endogenous nitrogen losses, associated with detoxification, that were nearly double the endogenous nitrogen loss of birds feeding on food containing no plant secondary metabolites (Guglielmo, 1993). Consequently, if birds feed on aspen buds having average protein levels (10%) and moderate (2.4%) levels of CB, they would likely have a negative nitrogen balance (Jakubas et al., 1993b). Unsuitable levels of CB or protein in aspen buds may force grouse to seek alternative foods, thus increasing their feeding time and raising their susceptibility to predation (Jakubas and Gullion, 1991).

If natural selection pressures have produced ruffed grouse that can readily distinguish dietary CB concentrations, as is suggested by feeding trials (Jakubas and Gullion, 1990; Jakubas et al., 1993a), increased survival rather than higher reproduction rates may have been the most important selection mechanism. Ruffed grouse reproduction could have potentially been affected by CB, if CB directly affected the endocrine system and if this effect persisted over the breeding season. However, any effect that CB might have on reproduction appears to stem from factors related to energy acquisition and retention. Therefore, it would be necessary for ruffed grouse to feed on aspen buds at the time they are laying their clutches in order to be affected by CB. Although ruffed grouse make heavy use of aspen during the breeding season, the buds generally have elongated into catkins by the middle of the breeding season (late April in Minnesota) or by the time hens begin to lay their clutches. Even if birds fed on aspen catkins during clutch laying, aspen catkins have a lower CB content than flower buds because of their higher ratio of reproductive to bud scale material (only the bud scales contain CB) (Jakubas et al., 1989). In addition, these bud scales can easily fall off when the bird picks the catkin (bud scales seldom occur in catkin droppings; G.W. Gullion, personal communication) and hence, the bird is left with a relatively nutritious food source. In contrast to reproduction, a selective advantage would likely come from increased survival rates for birds that were able to distinguish between low and high CB buds during the winter. Birds that are capable of selectively feeding on only low CB aspen buds would have at least 24% higher energy utilization efficiencies (Guglielmo, 1993). Higher food utilization efficiencies would enable these birds to spend less time feeding and more time in the protective cover of their snow roosts. Less exposure to predation during the time of year when mortality rates are highest for adult grouse should confer a survival advantage, and hence select for birds that were able to distinguish between low and high CB levels in aspen buds.

CONCLUSIONS

Dietary CB does not appear to have antireproductive effects similar to FA at intake levels that ruffed grouse and Japanese quail would freely consume. Contrary to FA, CB produced a significant decrease in egg production, did not

affect male breeding behavior, female cloacal diameters (or other estrogen-mediated responses), and serum prolactin levels. Therefore, we reject the hypothesis proposed by Jakubas and Gullion (1990) that if dietary CB is biotransformed to FA it would manifest antireproductive effects similar to FA. Based on various assays for estrogenic and antiestrogenic effects, and previous studies on structure–activity relationships, it does not seem likely that CB, FA, or their known biotransformation products would directly act as estrogen mimics or antagonists. We cannot reject our hypothesis that dietary CB interferes with male or female avian reproduction. The lower egg production of birds consuming CB may be explained by lower energy acquisition and retention rather than endocrine changes per se. By partitioning energy expenditures, we illustrated how dietary plant secondary metabolites might constrain reproduction by limiting energy acquisition and retention. Although CB did reduce egg production in quail, it is unlikely that it would affect egg production in wild ruffed grouse. We did not observe any antireproductive effects in males (i.e., reproductive behavior and testes size); however, we had insufficient information on other reproductive parameters (e.g., sperm viability) to totally reject the hypothesis that CB interferes with male reproduction.

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MATHEMATICAL MODELING OF ALLELOPATHY: BIOLOGICAL RESPONSE TO ALLELOCHEMICALS AND ITS INTERPRETATION

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Abstract—Allelochemicals are assumed to possess specific biological properties and responses of an organism are external expressions of such properties. Based on this assumption, a mathematical model has been constructed to interpret the characteristic responses of an organism to allelochemicals. Several sets of experimental data have been compared with the model predictions; good agreement between the model and data is observed.

Key Words—Allelopathy, allelochemicals, mathematical model, plant defense.

INTRODUCTION

Allelopathy refers to both harmful and beneficial biochemical interactions between all types of plants, including microorganisms. Up to now, probably one of the most consistent features in studies of allelopathy has been the recognition of the characteristic responses of an organism to an allelochemical, i.e., stimulation or attraction at low concentrations of allelochemicals and inhibition or repellence as the concentration increases (Lovett, 1979, 1989; Rice, 1984). These phenomena have been widely recognized in other growth-regulating chemicals, including herbicides (e.g., Devlin and Witham, 1983; Fedtke, 1982).

In the present work, a model for allelopathic interactions is presented and

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compared with experimental data from the literature. The model also gives some insight into the underlying processes involved.

DESCRIPTION OF THE MODEL

It is hypothesized that the characteristic response to allelochemicals is a result of the character of the allelochemicals themselves. An allelochemical is assumed to have two complementary attributes: stimulation and inhibition. These attributes need not necessarily have the same sites of action. As concentration changes, the relative dominance of stimulation and inhibition by the allelochemical alters, so determining the overall property of the allelochemical. This can only be shown through the biological responses when an allelochemical acts on an organism, and is referred to as the biological property of the allelochemical (as opposed to physical or chemical).

It is assumed that both stimulation and inhibition have a sigmoidal response to allelochemical concentration. The equation used to define this response is

$$v = \frac{v_m[X]^q}{K^q + [X]^q} \tag{1}$$

where $[X]$ is the allelochemical concentration, v is the response, v_m is the response at saturating concentration, K is the concentration at which $v = v_m/2$, and q is a constant that controls the shape of the curve. Equation 1 can be derived from enzyme kinetics to represent the reaction speed of an enzyme substrate reaction with q active sites per enzyme molecule for the substrate (e.g., see Thornley and Johnson, 1990, Chapter 2). It is used here on the basis that it is a versatile curve that has the appropriate response and has some basis in biology. Equation 1 is illustrated in Figure 1.

Let the biological response to the allelochemical be $P\%$ of the control (no allelochemical present), and let S and I be biological responses to the stimulatory and inhibitory attributes of the allelochemical respectively, so that, applying equation 1,

$$S = \frac{S_m X^q}{K_S^q + X^q} \tag{2a}$$

and

$$I = \frac{I_m X^q}{K_I^q + X^q} \tag{2b}$$

Note that the same parameter q is used for both S and I; this could be relaxed if necessary.

In the study of allelopathy, biological responses to allelochemical are fre-

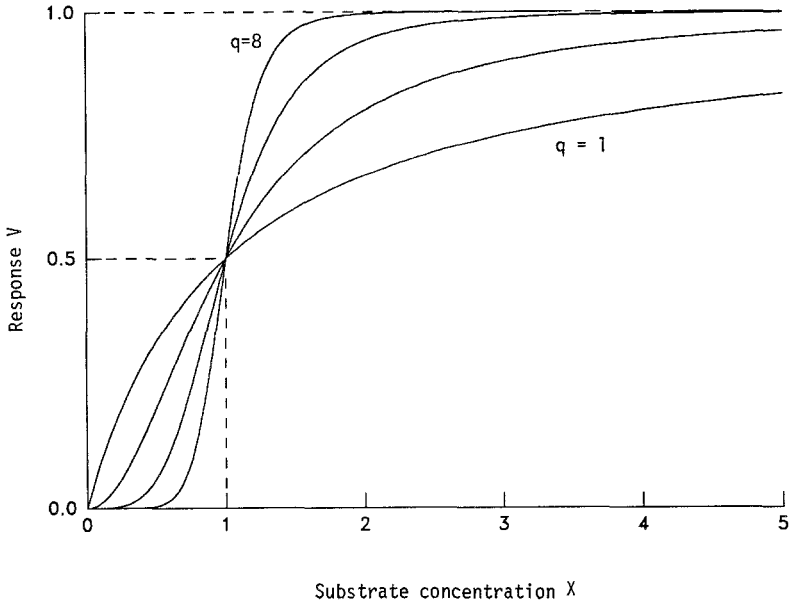

FIG. 1. The sigmoidal response curve for the reaction response v as a function of substrate concentration, $[X]$, as defined by equation 1. $q = 1$ and $q = 8$ are indicated, and the intermediate curves are for $q = 2$ and 4 as indicated. There is an asymptote at $v = v_m$; and $v = v_m/2$ when $[X] = K$ (from Thornley and Johnson, 1990).

quently expressed as percent of control. With the control set at 100% it is, therefore, hypothesized that the biological response to allelochemical, P% of control, is given by

$$P = 100 + S - I \tag{3a}$$

where S and I are given by equations 2a,b. It is further assumed that $P \to 0$ as $X \to \infty$, which implies

$$I_m = S_m + 100 \tag{3b}$$

Note that although this relationship is derived in the limit $P \to 0$ as $X \to \infty$, the equality applies since I_m and S_m are not themselves taken as limiting values. Equation 3a with 2a,b and 3b define the model.

Note that there is a maximum at

$$X^q = \frac{K_S^q \sqrt{I_m K_I^q} - K_I^q \sqrt{S_m K_S^q}}{\sqrt{S_m K_S^q} - \sqrt{I_m K_I^q}} \tag{3c}$$

although this will not exist for all parameter values. Equation 3c is derived by

differentiating equation 3a with 2a,b with respect to X, equating to zero, and solving the resulting equation for X. While we could pursue the range of parameter values for which this maximum will exist further, there is little point since we anticipate that the data to be analyzed will generally display a maximum. The parameters S_m, K_S, I_m, K_I, and q should be regarded as parameters to be derived by comparing the model to experimental data. Before doing so, it is useful to look at the general behavior of the model, as shown in Figure 2a–c for a range of parameter values.

It can be seen from Figure 2 that the model has the general characteristics of the biological response to an allelochemical. At low concentrations, stimulation is observed. As the concentration increases, the pattern changes and inhibition dominates, eventually to the extent that $P \rightarrow 0$. The biological property of an allelochemical will vary both with the allelochemical and species under consideration.

APPLICATIONS OF THE MODEL

We now turn our attention to the behavior of the model in comparison with experimental data. In the following illustrations, all direct measurements of biological responses have been converted to percent control values. All fitted curves are derived by minimizing the residual sum of squares for the difference between the actual data values and the corresponding value of the model, equations 2a,b and 3a,b.

Figures 3–6 show the model behavior compared with experiments testing morphological biological responses to single allelochemicals which are from, or contained in, living plants. Note that the data shown in Figure 6 do not show stimulation, although it is possible that stimulation may have been observed had a lower allelochemical concentration been included in the series. Figure 7 shows the emergence of wild oats (*Avena ludoviciana*) in response to wheat straw leachate, which is known to contain allelochemicals (e.g., Lynch, 1977). Figures 8 and 9 show the response to allelochemicals at the biochemical level, which is similar to that observed in gross morphological studies (Roshchina et al., 1986; Lovett et al., 1989). It can be seen from the illustrations presented here that the model behavior is in good agreement with a wide range of experimental data taken from the literature.

CONCLUSIONS

The characteristic responses of an organism to an allelochemical, namely, stimulation or attraction at low allelochemical concentrations and inhibition or repellence as the concentration increases, are almost universally observed. The

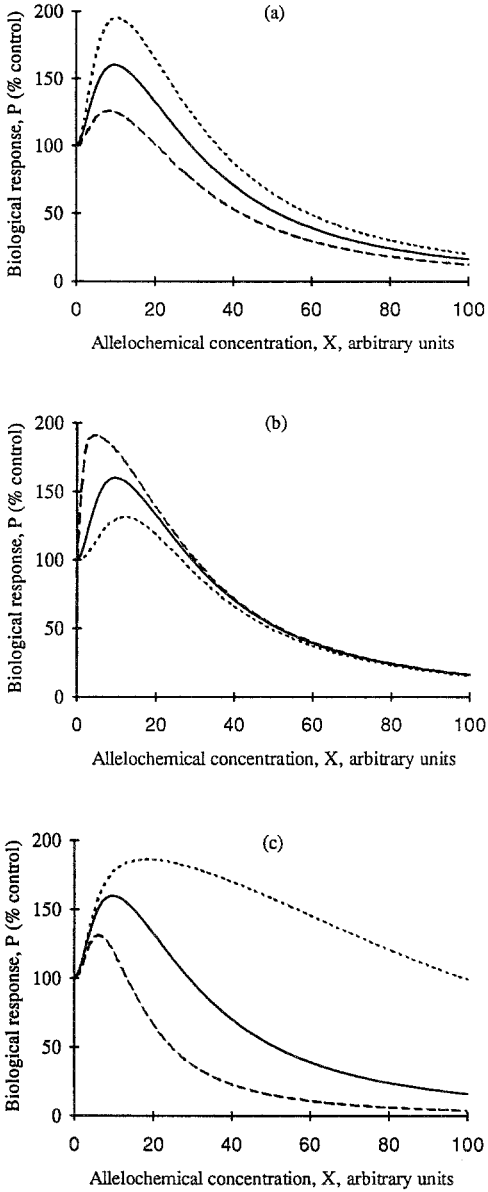

FIG. 2. Biological response, P, to an allelochemical, X, as given by equation 3a with equations 2a,b and 3b. P is measured as a percentage of control and X has arbitrary units. Default parameter values are $S_m = 100$, $I_m = 200$, $K_S = 5$, $K_I = 30$, $q = 2$, corresponding to the solid lines. The other parameter values are: (a) $S_m = 50$ (- - -), 150 (——); (b) $K_S = 1$ (- - -), 10 (——); (c) $K_I = 15$ (- - -), 100 (——).

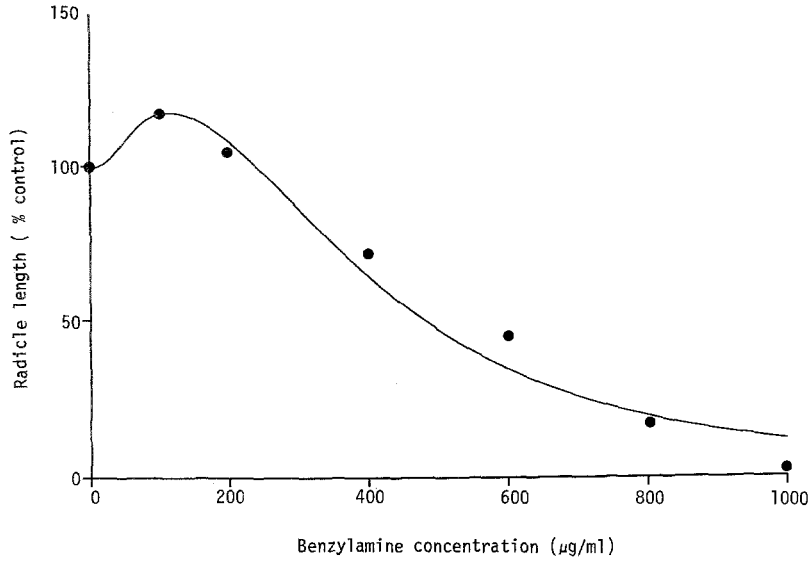

FIG. 3. Response of radicle length of linseed to benzylamine. $S_m = 30$, $I_m = 130$, $K_S = 70$, $K_I = 400$, $q = 2.5$ (data from Lovett et al., 1989).

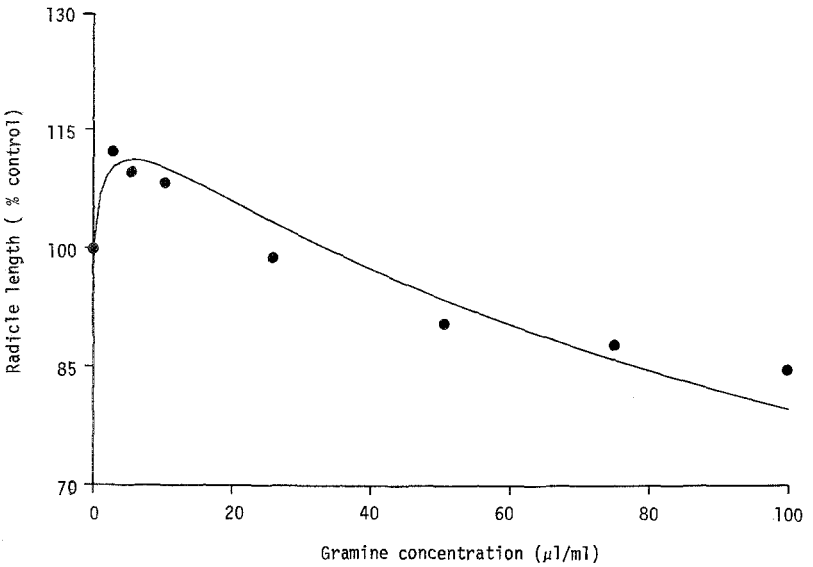

FIG. 4. Response of radicle length of white mustard to gramine. $S_m = 30$, $I_m = 130$, $K_S = 3$, $K_I = 190$, $q = 0.8$ (data from Lovett et al., 1989).

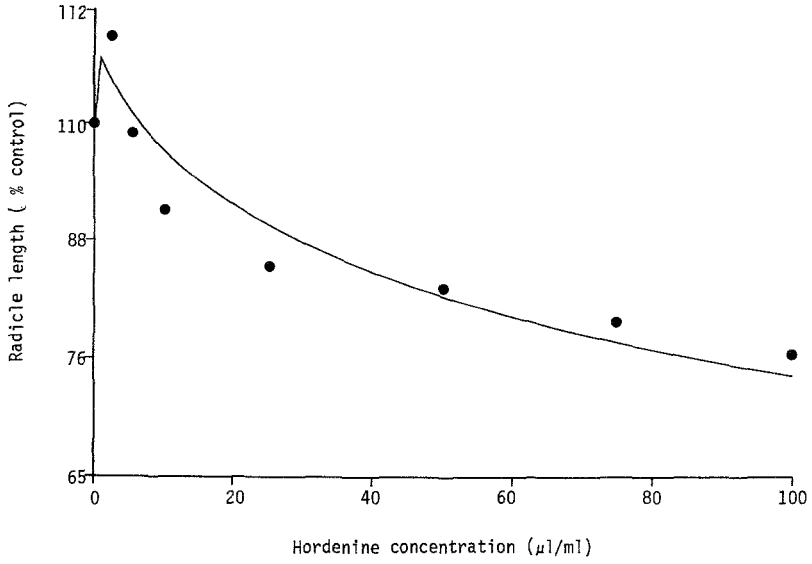

FIG. 5. Response of radicle length of white mustard to hordenine. $S_m = 40$, $I_m = 140$, $K_S = 0.6$, $K_I = 200$, $q = 0.4$ (data from Lovett et al., 1989).

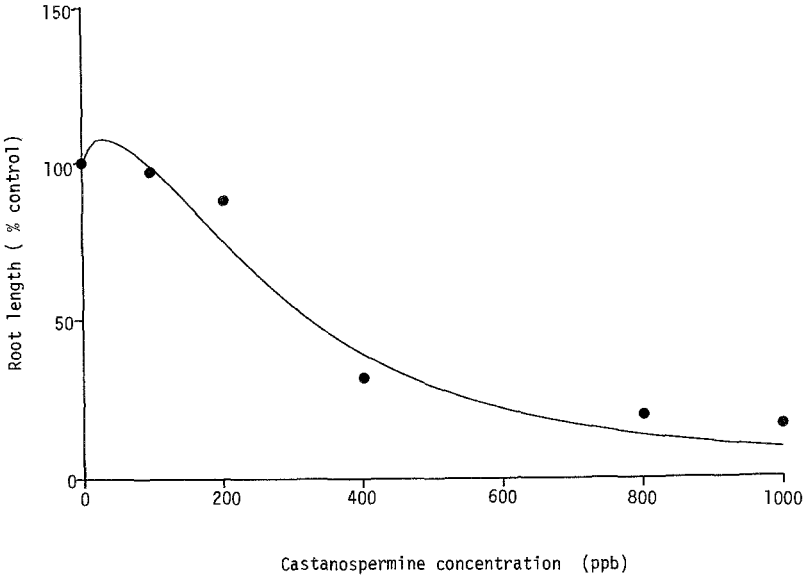

FIG. 6. Effect of castanospermine on root growth of lettuce. $S_m = 10$, $I_m = 110$, $K_S = 10$, $K_I = 300$, $q = 2$ (data from Stevens and Molyneux, 1988).

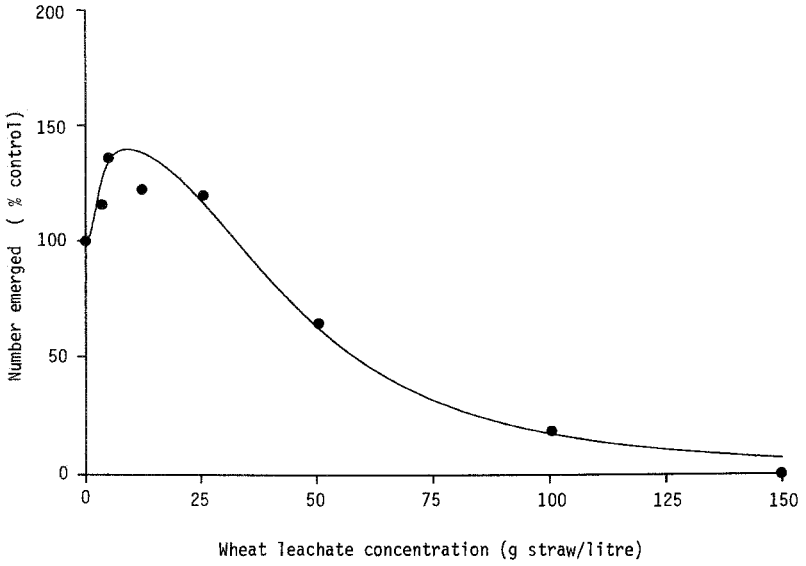

FIG. 7. Emergence of wild oats (*A. ludoviciana*) in response to applied wheat straw leachate. $S_m = 45$, $I_m = 145$, $K_S = 3$, $K_I = 45$, $q = 2.5$ (data from Purvis and Jessop, 1985).

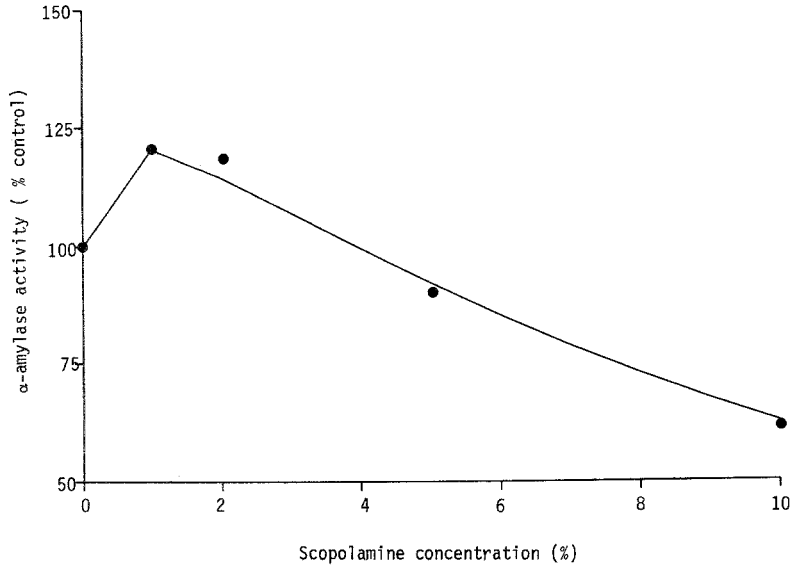

FIG. 8. Response of α-amylase activity to scopolamine. $S_m = 25$, $I_m = 125$, $K_S = 0.1$, $K_I = 10$, $q = 1.5$ (data from Lovett et al., 1989).

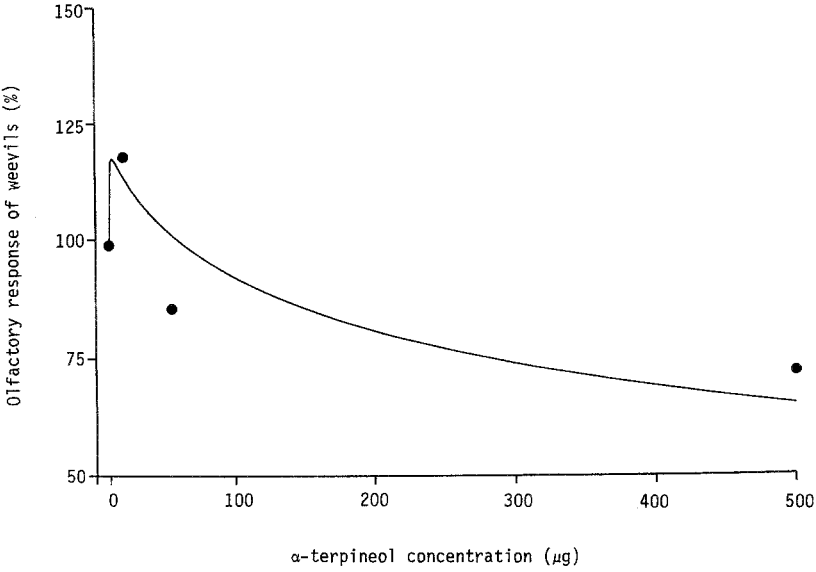

FIG. 9. Olfactory response of weevil larvae to α-terpineol. $S_m = 40$, $I_m = 140$, $K_S = 0.5$, $K_I = 400$, $q = 0.5$ (data from Selander et al., 1974).

model presented here simulates well such responses for a wide range of experimental conditions. The model is simple in structure and is based on the hypothesis that the response to allelochemicals is simultaneously stimulatory and inhibitory in nature: the balance between stimulation and inhibition alters as allelochemical concentration changes. We feel that the model provides a useful means for analyzing experimental data, and may be of value in attempts to predict allelopathic effects in practice.

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TRANSFER OF BIPIPERIDYL AND QUINOLIZIDINE ALKALOIDS TO *Viscum cruciatum* Sieber (LORANTHACEAE) HEMIPARASITIC ON *Retama sphaerocarpa* Boissier (LEGUMINOSAE)

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Abstract—Plant material of *Viscum cruciatum* Sieber contains bipiperidyl (ammodendrine) and quinolizidine alkaloids (lupanine, 5,6-dehydrolupanine, retamine, cytisine, *N*-methylcytisine). This plant obtains the alkaloids by root parasitism on *Retama sphaerocarpa* Boissier (host plant). These results have important implications for *Viscum* ecology, for the study of herbivores that are *Viscum* specialists, and in the development of systems for the investigation of the role of alkaloids as plant defenses.

Key Words—*Viscum cruciatum*, Loranthaceae, hemiparasites, alkaloids, Leguminosae, *Retama sphaerocarpa*.

INTRODUCTION

Viscum cruciatum Sieber is one of the two recognized species of *Viscum* in the European flora and is found in the eastern zone (Jerusalem, Gaza, Judea, and Samaria) (Post and Dismore, 1932) and Occidental areas (southern Iberian Peninsula and northern Africa) (Amaral Franco, 1971; Ball, 1964; Sennen and Mauricio, 1933) of the Mediterranean coast. It is an evergreen hemiparasitic plant, known in Spain as "muerdago colorado" due to its red berries. This plant is hosted by a wide range of angiosperms, among them *Retama sphaerocarpa* Boissier. We previously undertook alkaloid analyses from this parasite and

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reported the isolation of several quinolizidine alkaloids (Martín Cordero et al., 1989).

The objective of the present paper is to demonstrate the interrelation of the parasite–host with respect to the plant's alkaloid content.

METHODS AND MATERIALS

Aerial parts (stems and leaves) of *Viscum cruciatum* Sieber were collected near Cadiz, Spain, between August 1989 and May 1990 and identified for their authenticity. A voucher specimen was deposited at the Herbarium of the University of Seville.

Stems and leaves of the host plant *Retama sphaerocarpa* Boissier were collected at the same time and place as its parasite.

After air-drying at 20–25°C and pulverizing the plant material (hemiparasite and host), the powder was made alkaline by addition of NH_4OH and extracted with $CHCl_3$ in a Soxhlet apparatus. The alkaloid mixture was further purified by acid–base treatment (HCl, 2%, followed by $NH_4OH/CHCl_3$). Quantitative determination of alkaloids was done gravimetrically (Paris and Moyse, 1976).

Qualitative alkaloid analysis was determined by gas chromatography–mass spectrometry and performed using a Kratos MS80RFA mass spectrometer combined with a Carlo Erba/Kratos MFC 500 gas chromatograph with a CP-Sil 5 capillary column, 25 m × 0.32 mm. The temperature program was 100–280°C at 10°C/min. The detector and injector heaters were set at 250°C. The carrier gas was helium, at a flow rate of 1 ml/min.

Alkaloids were identified by comparing their mass spectra with those we had previously reported (Martín Cordero et al., 1989) and data reported in the literature (Neuner-Jehle et al., 1964, 1967; Fitch and Djerassi, 1974).

Gas chromatographic quantitative analysis was performed by the relative proportions method (areas), under the conditions specified in the qualitative analysis section.

RESULTS AND DISCUSSION

The data obtained in the qualitative and quantitative determinations for each sample (hemiparasite and host) are reported in Table 1.

The total yield of alkaloid for *Viscum cruciatum* and the host *Retama sphaerocarpa* fluctuated markedly. The highest percentage of alkaloids was found in *Viscum* in May and August (1.57 and 1.04%), and the lowest content in November and February (0.65 and 0.60%) when the host increased its content of alkaloids.

TABLE 1. PERCENTAGE OF TOTAL YIELD OF ALKALOIDS AND SEASONAL VARIATION OF *Viscum* AND *Retama*[a]

	Date of harvest							
	August		November		February		May	
Alkaloids	*Viscum*	*Retama*	*Viscum*	*Retama*	*Viscum*	*Retama*	*Viscum*	*Retama*
Ammodendrine	4.95	0.22	tr	18.83	7.92	11.54	tr	15.91
5,6-Dehydrolupanine	tr		tr		4.12		tr	2.27
Lupanine	6.98	8.74	5.26	0.88	32.34	11.38	9.02	9.74
Sparteine		0.22						17.53
Genisteine				11.51		31.46		
Retamine	84.70	90.81	62.20	68.78	10.89	55.85	46.34	49.19
Anagyrine	1.10		tr		3.79		tr	
Cytisine	1.22		9.73		tr		18.29	5.36
N-Methylcytisine	1.10		22.81		40.92		26.34	
Total yield (g/100 g)	1.04	0.63	0.65	1.26	0.6	1.91	1.57	1.89

[a]tr = trace (<0.1%).

A cytophotometric study of *Viscum album* L. showed that during winter (October–January) intercalary meristematic cells are blocked in the G_1 presynthetic phase of their cycle (Salle, 1983). This coincides with the high variability in alkaloid content of parasite; the lowest percentage of alkaloids for the parasite was in November and February when the intercalary meristem of secundary haustoria or sinkers is blocked.

The alkaloid patterns of *Viscum cruciatum* are not distinct from those of the host plants: all alkaloids, with the exception of *N*-methylcytisine and anagyrine, were found in the host. The *N*-methylcytisine and anagyrine found in the parasite may be derived from lupanine. A similar biotransformation can be observed in plant cell cultures that had been provided lupanine (Wink et al., 1983; Wink, 1984a; Cho and Martin, 1971).

As can be seen from Table 1, retamine was a major alkaloid during all the biological cycle in parasite and host.

Quinolizidine alkaloids are repellent to herbivores (Wink et al., 1982; Wink, 1984b; Waller and Nowacki, 1978). Toxic and teratogenic effects have been observed in mammals that had been fed α-pyridone alkaloids, especially anagyrine (Wink, 1984c).

The accumulation of high alkaloid levels may be advantageous even for *Viscum* itself.

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A BIOASSAY SYSTEM FOR COLLECTING VOLATILES WHILE SIMULTANEOUSLY ATTRACTING TEPHRITID FRUIT FLIES

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Abstract—A bioassay system was developed that permits the testing of various substrates for biological activity in a flight tunnel, while simultaneously collecting a portion of the volatiles from the attractive source for subsequent chemical identification and quantification. Bioassays of the response of virgin female Caribbean fruit flies, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), to volatiles released by calling males were conducted in a greenhouse under natural light cycles and fluctuating environmental conditions, similar to those in the field. Using this system, the periodicity of response of the female flies between 1300 and 1845 hr (EST) was tested. Fifty to 75% response occurred between 1700 and 1845 hr. Male pheromone release was greatest between 1500 and 1800 hr. Videotaped records of insects, taken between 1700 and 1800 hr as flies approached and entered the traps, were analyzed to interpret the communicative role of the volatiles released. Significantly more flies landed on and entered the pheromone-emitting trap than the control trap. There was no difference in the amount of time spent on the trap face, an indication that volatiles were attractants. The system described should be of general utility in determination of the attraction of pest fruit flies to suspected attractants.

Key Words—Bioassay system, flight tunnel, fruit flies, Caribbean fruit fly, Anastrepha suspensa, Diptera, Tephritidae, pheromone.

INTRODUCTION

The development of more effective lures for monitoring populations of pest fruit flies may be enhanced if test systems permit determination of the chemical(s)

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being released while simultaneously monitoring the relative attractive response of the insect to the chemical(s). Detailed knowledge of the environmental conditions that occur during the bioassay period may provide significant information relative to variation in fruit fly response to the chemical attractant. As part of continuing efforts to discover and improve fruit fly attractants, we designed a bioassay system that permits the testing of various substrates for biological activity in a flight tunnel, the collection of a portion of the volatiles from an attractive source for subsequent chemical identification, the automated recording of environmental conditions during the investigation, and the use of a video system to monitor fly activity on and in the trap. Our initial studies employed the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), to test the effectiveness of the bioassay system in an evaluation of response of the female fruit fly over time to pheromone released by conspecific males. Bioassays were conducted in an environment of purified air containing a minimal amount of volatile organics and under a light, temperature, and humidity regime similar to that available in the field.

METHODS AND MATERIALS

Chemical Attraction, Killing, and Evaluation Bioassay System. The bioassay system consists of five main subcomponents designed for specific tasks and allows certain parameters to be varied between experiments (Figure 1). The components include the bioassay flight tunnel (BFT), airflow system (AFS), air delivery system (ADS), insect isolation trap (IIT), and the volatile collection system (VCS). The bioassay system was contained in a greenhouse at the U.S.D.A. facility in Gainesville, FL.

Bioassay Flight Tunnel (BFT). The BFT was designed to operate similar to those used for insect pheromone research (Baker and Linn, 1984, and references therein). Briefly, the BFT is constructed out of 0.64-cm-thick clear acrylic Plexiglas and fused together using methylene chloride as a solvent making transparent and nonintruding joints (Figure 1A). The main flight tunnel body is a rectangular box 30.2 × 30.2 × 122 cm with solid fixed sides and bottom, having flanged front and back ends. Two removable top panels, each with a small sliding 10 × 10-cm door centered 15 cm from the end of each panel, allows for hand access into the tunnel. These two top panels (each 30.5 cm wide × 61 cm long) are removable for cleaning the tunnel interior between experiments. The sliding doors on the top panels allow quick access into the tunnel during a bioassay for insertion or removal of insects and/or equipment with minimal disruption and contamination of the interior environment. Temperature within the BFT and greenhouse is controlled by two independent heating and cooling subsystems. For increases in temperature, an in-house closed-

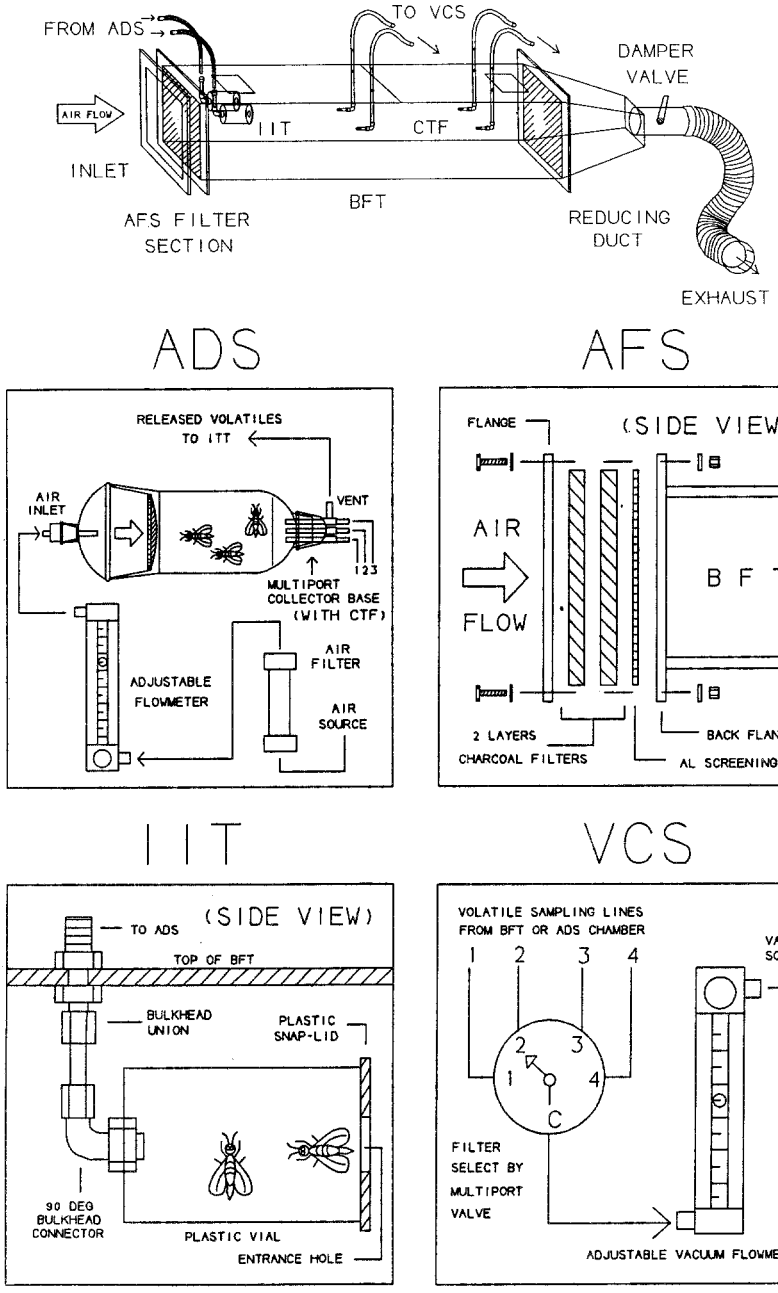

FIG. 1. The wind-tunnel bioassay system, which is comprised of (A) a bioassay flight tunnel (BFT); and (B) an air delivery system (ADS), an airflow system (AFS), two insect isolation traps (IIT), and a volatile collection system (VCS) with lines to collector trap filters (CTF) used to sample volatiles within the bioassay flight tunnel.

loop hot water circulation system with passive radiators is utilized. The lower limit for a desired temperature range is controlled by a heating-only thermostat and is used to maintain the temperature during the colder days of the year. The cooling system incorporates the use of evaporative water coolers and exhaust fans to lower temperatures to a desired setpoint. The upper limit of a temperature range is set by the cooling thermostat and, when that threshold is reached, two large exhaust blowers pull air out of the greenhouse. This causes fresh air to be drawn through evaporative coolers on the opposite wall through which water is constantly recirculated. During this process, water is vaporized into the incoming air which helps maintain a high relative humidity within the greenhouse.

Airflow System (AFS). The AFS consists of an air purification and diffusing system located at the upwind side and an air exhaust system located at the downwind side of the BFT (Figure 1B, AFS insert). The air purification method used is a modification of the charcoal-infused media system reported previously (Heath and Manukian, 1992). For the purpose of this study, two 1.27-cm-thick layers of 12.0-oz nonwoven fabric media infused with 150% activated carbon (18 oz of carbon per yard, P/N#ACF-NWPE-12-150P, Lewcott Corp., Millbury, Mississippi) were used. Each filter layer was cut to match the tunnel cross section and was secured to the intake side of the wind tunnel with a 30.5 × 30.5-cm piece of standard aluminum window screening (1.5-mm square mesh). The material provides a means for producing a uniform laminar airflow across the tunnel cross section, in addition to purifying the incoming air.

The air exhaust system is comprised of an exhaust blower assembly and a reducing duct attached to the exhaust side or back-end of the wind tunnel. This is separated by another 30.5 × 30.5-cm piece of the aluminum screening to confine insects to the tunnel, an air damper or baffle-type valve, a section of flexible corrugated plastic ducting, and finally an exhaust blower. The reducing duct is fabricated out of 0.64-cm-thick Plexiglas and serves to uniformly decrease the cross section of the tunnel down to an 8.89-cm-OD cylindrical port to which standard flexible plastic dryer-tube ducting is attached. This flexible ducting is used to connect the tunnel to the inlet of a high-capacity, 120-VAC, 1725 rpm, 28-cm-diameter radial wheel (''squirrel-cage'') type exhaust blower motor capable of moving 40,500 liters/min of air (Grainger model 2C864, Chicago, Illinois). The blower is located several meters away from the tunnel to minimize any noise and vibrational effects on the tunnel generated from the blower motor. The air speed through the tunnel is controlled by increasing or decreasing the internal diameter of the outlet port at the end of the reducing duct by adjusting a damper inside this port. The wind speed is set by measuring the average velocity in the tunnel using a hot-wire type thermoanemometer (Kurz model 1440M-4, Davis Instruments Inc., Baltimore, Maryland) and positioning the damper until the desired speed is set. Experiments were conducted using a wind speed of 0.110 m/sec. Air pressure in the tunnel was measured using an elec-

tromechanical baratron tube differential pressure transducer (MKS Instruments model 223BD, Burlington, Mississippi) to determine if negative pressures were developed by exhausting more air by the blower than could pass through the carbon filter media.

Air Delivery System (ADS). The ADS is used to deliver a constant volume of air over the test substrate (e.g., calling males) and to entrain the volatiles for delivery to the bioassay wind tunnel without contamination (Figure 1B, ADS insert). A 45.5-liter portable air compressor capable of supplying 142 liters/min of air at 8.50 kg/cm^2 was used in this investigation. The maximum air supply needed for the ADS is a flow of 5.0 liters/min at a pressure of no more than 0.70 kg/cm^2 to prevent the separation or rupture of glass and soft Teflon tubing used in connecting all the components. A pressure regulator was used to reduce the source outlet pressure down to this level. The air coming from the compressor is then pushed through two in-line charcoal filters to remove organics from the air supply. The filters were made of 1.6-cm-OD (0.05-mm wall thickness) × 12.7-cm-long stainless steel tubing and contain 28-mesh activated charcoal (Alfa Products, Danvers, Massachusetts) packed between two 325-mesh stainless steel screens held in place by 1.6- to 0.64-cm Swagelok reducing unions. Connected in line with these filters are two adjustable single-tube flowmeters hooked in parallel (Aalborg P/N# FM112-02C, Monsey, New York), which are used to set the flow rate of purified air that is delivered into the chamber containing the flies or the control chamber.

The 5-cm-OD × 30-cm-long glass chamber with glass frit used to contain male flies has been previously described (Heath et al., 1992). Purified air is introduced upwind of the frit, which provides laminar airflow over the insects in the chamber. A multiport collector base located at the downwind end of the chamber contains a vent port for introduction of pheromone into the flight tunnel and three collector trap filters (see below) for pheromone collection. Airflow to the chamber containing male flies was maintained at 1 liter/min of which 250 ml/min of air was pulled through the collector trap filter and the remaining 750 ml/min was directed into the wind tunnel. Airflow to the chamber without flies was 750 ml/min. Connections from the flowmeters to the insect-holding chamber and from the vent port to the wind tunnel were made using a 40-cm piece of 0.64-cm-OD convoluted flex Teflon tubing (Berghof/America P/N# 14610-1/4″, Concord, California) and standard 0.64-cm Swagelok fittings.

Insect Isolation Trap (IIT). Two insect isolation traps (Figure 1B, IIT insert) were symmetrically positioned 5.1 cm left and right of the centerline on the top panel of the wind tunnel 5.1 cm from the front (upstream) edge. The traps were cylindrical in shape and made from clear plastic 140-ml vials, 4.8 cm ID × 8.6 cm long, with removable plastic snap lids (BioQuip, Gardena, California). Both were mounted horizontally, centered 10.2 cm from the top of the wind tunnel and 10.2 cm from the front end. A 1.1-cm-diameter hole was

drilled in the center of the bottom of each vial and attached to a 0.64-cm-ID Teflon 90-degree bulkhead union. This union was then attached to another 0.64-cm-ID bulkhead union mounted on the top panel (point at which the volatiles from the ADS enter the BFT, see above) using a 4.0-cm piece of 0.64-cm-OD stainless steel tubing as a support. A second 1.1-cm-diameter hole was drilled in the center of each removable lid, and then placed back onto each vial, pointing downstream. Lids were orange-colored to provide a visual cue (Greany et al., 1977). Interior surfaces of the insect isolation traps were coated with approximately 0.10 ml of a sugar–pesticide solution consisting of 10 g sucrose + 200 mg methomyl (98% AI, Reference #52543-98-1, DuPont Corp., Wilmington, Delaware) in 10 ml H_2O.

Volatile Collection System (VCS). The final component of the bioassay system is the VCS (Figure 1B, VCS insert), which is used for collecting a portion of released pheromone volatiles from flies inside the insect-holding chamber, as well as the downstream collection of volatiles at various locations inside the flight tunnel. Pheromone volatiles were collected on trap filters that were prepared by packing ca. 25 mg of Super-Q (Alltech Assoc. Inc., Deerfield, Illinois) in 4-cm-long × 4.0-mm-ID glass tubes, resulting in a bed length of 5 mm. Two stainless steel frits were used to contain the adsorbent. The Super-Q filters were cleaned by Soxhlet extraction with methylene chloride for 24 hr prior to use. Volatiles collected on the filters were eluted with 100 μl of methylene chloride, and then 1 μg of *n*-tetradecane was added as internal standard for subsequent analyses.

Besides the three filters placed in the multiport collector base, additional filters were placed in the flight tunnel. These filters were attached to 0.64-cm Swagelok elbow fittings that were supported from the top of the bioassay wind tunnel at various locations by rigid 0.64-cm-OD Teflon tubing. This tubing was inserted through 0.64-cm holes cut in the top panel of the bioassay tunnel along its centerline at the front, middle, and back ends. The insertion length of the tubing can be adjusted vertically in the wind tunnel. Attached to the outside end of this tubing were various lengths of No. 15 Masterflex Tygon tubing (ca. 0.6 cm ID), which connected the filters to a VCS vacuum manifold assembly placed in the vicinity of the tunnel. This manifold assembly consisted of multiport switching valves, flowmeters, and a vacuum pump. This assembly enabled the selection of a filter to use for volatile collection and controlled the sampling rate for the collection. The selection of the individual collection filters located in the insect-holding chamber and at various locations in the BFT was done using four-way, multiport valves (Whitney #B-43ZF2, Swagelok Co., Solan, Ohio). Tygon tubing was used to make the connection from the filters to the vacuum source. The common outlet of the valve was attached to a vacuum source through a single tube adjustable flowmeter (Aalborg P/N# FM112-02C, Monsey, New

York) that was used to set the collection flow rate of the selected filter. Volatile collections made within the BFT were made with an airflow of 1 liter/min.

Analyses of Pheromone. Gas chromatographic analyses were conducted using a Hewlett-Packard model 5890A Series II gas chromatograph, equipped with a cool on-column capillary injector (septum injector) and flame ionization detector. Helium was used as the carrier gas at a linear flow of 18 cm/sec, and the column temperature was initially isothermal at 60°C for 2 min, then temperature programmed at 20°/min to 180°C. The chromatographic data was collected and processed using the Perkin-Elmer Nelson Turbochrom III software running on an IBM-type 386-PC system under MS-Windows 3.0. Capillary gas chromatography (CGC) was done using a retention gap column prior to the capillary column. This system permitted the injection of samples without concentration in 5–100 μl of solvent (Grob, 1982; Murphy, 1989). A combination of three fused silica columns connected in series using GlasSeal connectors (Supelco Inc., Bellefonte, Pennsylvania) was used. The primary deactivated fused silica column, 8.0 cm long × 0.5 mm ID, was connected between the injector and the retention gap column. This primary column permitted the use of 0.4-mm-OD stainless steel needles with a septum injector for on-column injections. The retention gap columns used were 10-m × 0.25-mm-ID deactivated fused silica (Quadrex, New Haven, Connecticut). The analytical column used for analysis was a 30-m × 0.25-mm-ID SE-30 purchased from Alltech Assoc. Inc. (Deerfield, Illinois). Confirmation of compound identity was obtained by mass spectroscopy using the SE-30 capillary column, operated as described above, coupled to a Finnigan Ion Trap mass spectrometer in either electron impact (EI-ITDMS) or chemical ionization (CI-ITDMS) mode. The reagent gas used for CI was isobutane.

Environmental and Experimental Monitoring. Environmental parameters were recorded using an Omega Engineering Inc. dual digital display thermohygrometer with linearized analog outputs for monitoring temperature and relative humidity (Omega model RH-411, Stamford, Connecticut), and a three-decade digital light meter (also with linearized analog outputs and RS-232C interface) covering a range from 0 to 50,000 lux utilizing a selenium photovoltaic detector with a 300-nm bandwidth centered at 570 nm for measuring light intensity during the experiments (Davis Instruments Inc., C/N# EH1191025, Baltimore, Maryland). The analog outputs of these instruments were connected to a Computerboards Inc. CIO-AD08 eight-channel analog to digital (A/D) data acquisition board (Mansfield, Massachusetts) inside a USDA assembled rack-mounted industrial Intel-based 80386 computer system used for data logging. The remote sensors used to measure temperature, humidity, and light intensity were connected to the A/D board through a Computerboards Inc. ISO-RACK08 signal conditioning board containing Analog Devices 5B-type analog isolation and signal conditioning modules (Norwood, Massachusetts) used to filter out

any noise from the sensors and amplify weak signals. The sampling rate of this system was 16,000 Hz (samples/sec) divided by the number of input channels used, in this case three, allowing for the collection of environmental data at 5300 samples a second per parameter. Data were averaged continuously and stored on the computer's hard disk once every 6 min, yielding 10 data points per hour for temperature, relative humidity, and light intensity.

Activity of the test insects on the trap was recorded using live remote NTSC video equipment. A Yashica model KX-90U 8-mm tape camcorder with 8× zoom, telephoto, macro, and autofocus lens (Kyocera Corp.) was mounted on a Panasonic model WV-7330 remote-controlled motorized three-axis camera pointing system (Panasonic Corp., Secaucus, New Jersey) placed next to the bioassay tunnel. This allowed for accurate pointing of the video camera at any object inside the tunnel from a remote location without disturbing insects inside the tunnel. All functions of the camera were also controlled remotely using a Sony Remote Commander model RM-95 LCD display remote control unit (Sony Corp.). The video signal of the camera was put through a Panasonic model WJ-810 time/date generator, which superimposed the video signal with accurate date and time information as well as an elapsed time counter. This combined signal was then recorded on VHS format T-160 tape on a Panasonic model PV-6000 portable VCR.

Bioassay Procedure. Caribbean fruit flies were obtained as pupae from the Florida Department of Agriculture, Division of Plant Industry in Gainesville, Florida. Adult flies were sorted by sex two days after eclosion and placed in screen cages (30 × 30 × 30 cm). Flies were maintained in the laboratory with a 12:12 hr light–dark photoperiod at room temperature and ambient relative humidity and were provided with water and food (3:1 refined cane sugar–hydrolyzed brewer's yeast). Flies were placed in the greenhouse one day prior to testing.

Bioassays were conducted using virgin sexually mature females and males (10–16-days old) during four time periods in the afternoon. An experiment consisted of placing 10 males in the insect-holding chamber at 1200 hr and venting any volatiles produced during this time period outdoors and away from the air inlet into the greenhouse and BFT. At 1300 hr, 20 virgin females were released from a vial in the BFT, while at the same time 75% of the volatiles from the 10 males were introduced into one of the insect isolation traps. Twenty-five percent of the volatiles released by males were collected on a collector trap filter for subsequent analyses of pheromonal components. At 1500 hr, females were removed from the BFT and IIT, and 20 more females were released. Pheromone collection was continued using a different collector trap filter. This procedure was repeated at 1700 hr and at 1800 hr. Bioassays were concluded at darkness (<2 lux), which typically occurred between 1830 and 1845 hr. A paired control IIT was included that had a flow of 750 ml/min of purified air

from a container similar to the insect-holding chamber, but containing only water and sugar in the same amounts as that provided to the males. This experiment was repeated six times, and each time the positions of the pheromone and control volatiles were switched to eliminate possible IIT location effects.

The relationship between male pheromone production and number of females responding was examined with correlation analysis using Proc Corr (SAS Institute, 1985). The number of females trapped per collection period was tested for correlation with amounts (nanograms per male per hour, adjusted for 1 liter/min total airflow) of each individual component and of total pheromone (i.e., sum of all individual components), and with the percent of each component produced per collection period. The behavior of the flies on the trap with pheromone volatiles versus the control trap was examined by viewing videotapes recorded during five of the trials. Data recorded included number of flies landing on a trap, amount of time females spent on the face of a trap, number of aggressive interactions between females on the face of a trap, and percent of landing flies that entered a trap. Data was analyzed by two-sample *t*-test using Proc TTest (SAS Institute, 1985).

RESULTS

System Performance. At a wind velocity of 0.25 m/sec, a volume of 1394 liters of purified air per minute (83,640 liters/hr) is passed through the tunnel. With this particular configuration, velocities up to 0.5 m/sec can be used to purify air through the charcoal filter media and maintain ambient pressures within the BFT. Measurements of pressure differences at a wind velocity of 0.5 m/sec were determined to be -1.21×10^{-5} kg/cm^2 (or -0.069 in. of H_2O) under ambient atmospheric pressure. Investigation of the airflow in the BFT using smoke demonstrated that a laminar airflow existed over the central portion of the BFT and a uniform air velocity was obtained throughout the length of the system. Plume geometry from the IITs using smoke indicated that mixing of the two plumes occurred at approximately 38 cm from the outlet holes when a flow of 750 ml/min was used through the IIT and a wind speed of 0.25 m/sec through the tunnel. Removal of organic contaminents using charcoal infused media provided a level of air purity similar to that previously reported (Heath and Manukian, 1992).

Pheromone Emission and Bioassay Results. Gas chromatographic analysis of volatiles collected from male flies contained previously identified compounds, i.e., ocimene, (*Z*)-3-nonenol, and (*Z*,*Z*)-3,6-nonadienol, suspensolide, (*E*,*E*)-α-farnesene, β-bisabolene, anastrephin, and epianastrephin (Chuman et al., 1988, Nation, 1990; Rocca et al., 1992). Chromatograms of collections made at the outlet of the insect-holding chamber were similar to that previously published

(Heath and Manukian, 1992). Analyses of the average amounts of total pheromone released and percentages of the pheromonal components released by males collected at the outlet of the insect-holding chamber are shown in Table 1. Based on analyses of volatiles collected inside the flight tunnel 30 cm downwind of the IIT, we found that approximately 4.4% of suspensolide, 2.3% of (*E*,*E*)-α-farnesene, 3.4% of β-bisabolene, 3.4% of anastrephin, and 2.5% of epianastrephin were present at this point in the BFT as compared with material collected at the outlet of the insect-holding chamber. The amounts of ocimene, (*Z*)-3-nonenol, and (*Z*,*Z*)-3,6-nonadienol collected could not be measured accurately using capillary gas chromatography; however, their presence was confirmed using mass spectroscopy. Total ion chromatograms of volatiles collected in the flight tunnel indicated that the purity of air using the charcoal-infused media was similar to that observed in our initial studies of the use of this material for air purification. No detectable pheromonal components were obtained in the analyses of collections made 105 cm downwind from the IIT.

Response of virgin females to the pheromone released by virgin male Caribbean fruit flies increased throughout the time periods tested. Based on the response of 120 female flies released during each of the four time periods, an average percent ± standard error of females trapped per time period was: 34.2 ± 2.4 (1300–1500 hr); 61.7 ± 6.14 (1500–1700 hr); 60.0 ± 4.7 (1700–1800 hr) and 50.0 ± 2.9 (1800–1845 hr). Precise comparisons of these percentages are not possible since the lengths of the time periods are not equal. However, over 65% of the total response occurred between 1500 and 1800 hr. Of the 480 females tested, only two were trapped in the control IIT.

The number of female flies caught during each time period and the total amount of pheromone released by males for the six experiments conducted is shown in Figure 2. There were no significant correlations between the number of females trapped per hour and the amount of any of the individual components or the sum of all components produced per hour. There was a significant correlation, however, between the proportion of epianastrephin in the total pheromone blend produced and the number of females trapped per hour ($r = 0.428$, $df = 21$, $P = 0.05$), and that was the only component proportion to which the female response was significantly correlated. An examination of a scatterplot of percent epianastrephin and female response indicated that the relationship was most apparent during the first two collection periods (Figure 3).

Examination of environmental data recorded during the six experiments indicated a high degree of variability in light intensity, temperature, and humidity. This was largely due to the dynamic weather changes experienced daily in Florida during the time of year the experiments were conducted, which affect the conditions within the greenhouse. The occurrence of a severe storm and consequent early onset of darkness resulted in an earlier termination of both pheromone production and female response (see Figure 2, experiment 2). Light

TABLE 1. AVERAGE PERCENTAGES (±SD) OF PHEROMONAL COMPOUNDS AND AVERAGE AMOUNTS (±SD) OF TOTAL PHEROMONE BLEND RELEASED BY MALE FLIES DURING THE AFTERNOON[a]

Time	Compound (%)							Total (ng/male/hr)
	Ocimene	Nonenols	Suspensolide	Farnesene	Bisabolene	Anastrephin	Epianastrephin	
1300–1500	3.0 ± 1.9	2.2 ± 1.7	46.9 ± 7.2	5.3 ± 1.5	30.8 ± 7.9	2.4 ± 1.0	9.4 ± 3.9	182.6 ± 56.8
1500–1700	3.8 ± 2.1	1.6 ± 1.3	35.7 ± 11.4	7.2 ± 3.1	34.4 ± 8.6	2.6 ± 1.3	14.7 ± 3.2	307.6 ± 229.8
1700–1800	3.3 ± 2.3	1.2 ± 1.0	27.6 ± 11.7	7.4 ± 2.2	38.7 ± 6.8	3.7 ± 0.7	18.1 ± 4.2	223.9 ± 53.2
1800–1845	3.7 ± 1.6	1.2 ± 1.1	18.2 ± 12.0	8.9 ± 2.1	43.5 ± 0.8	1.7 ± 2.4	22.8 ± 7.9	144.4 ± 22.0

[a]Collections were made at the outlet of the insect holding chamber.

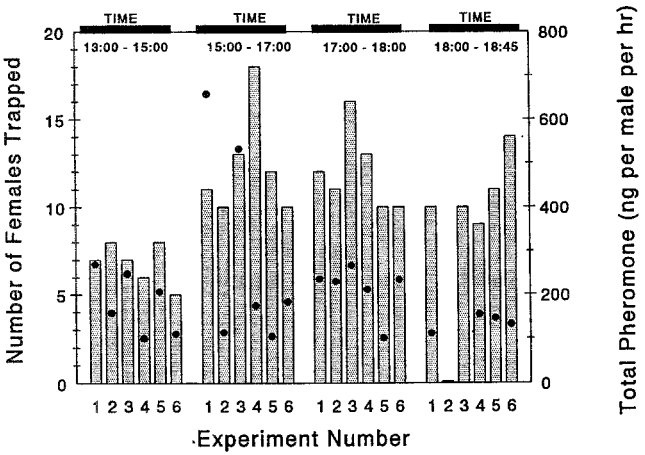

FIG. 2. The number of female *A. suspensa* trapped (bars) and the total amount of pheromone blend released by male *A. suspensa* (symbols) during six bioassays with simultaneous volatile collections. There were 20 females released during each time period.

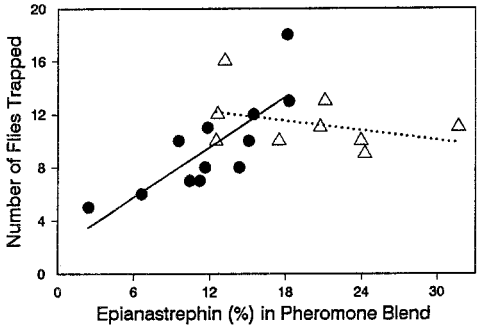

FIG. 3. Scatterplot of the number of female *A. suspensa* trapped in response to, and the percent epianastrephin in, pheromone released by male *A. suspensa*. The solid line represents tests run between 1300 and 1700 hr (two 2-hr tests), and the dotted line represents tests run between 1700 and 1845 hr (a 1 hr and a 45 min test).

intensity recorded during the 1700- to 1845-hr period for each experiment is shown in Figure 4. A representative data set of continuous measurements of temperature, humidity, and light intensity obtained during an experiment is shown in Figure 5. Considerable fluctuation in light intensity occurs between 1300 and 1600 hr. These fluctuations reflect the varying cloud coverage that occurred during a relatively sunny day. Light intensity decreased rapidly after 1600 hr, with darkness occurring at approximately 1840 hr. Temperature tended

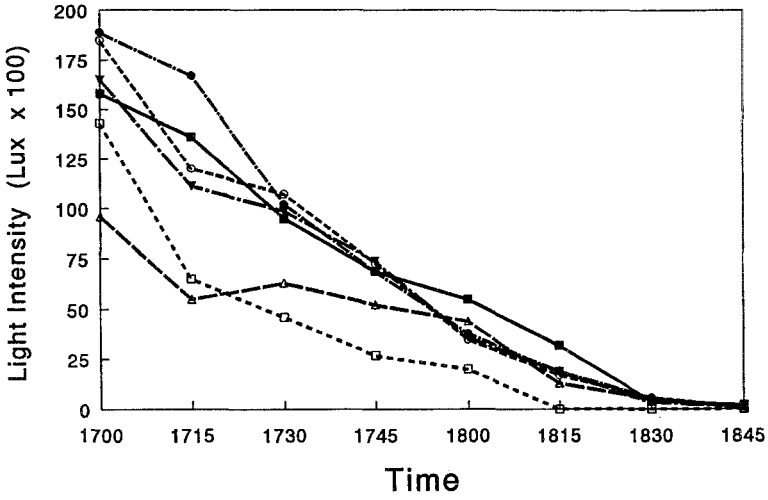

FIG. 4. Light intensities recorded at dusk during bioassays of response of female *A. suspensa* to volatiles from calling male *A. suspensa*.

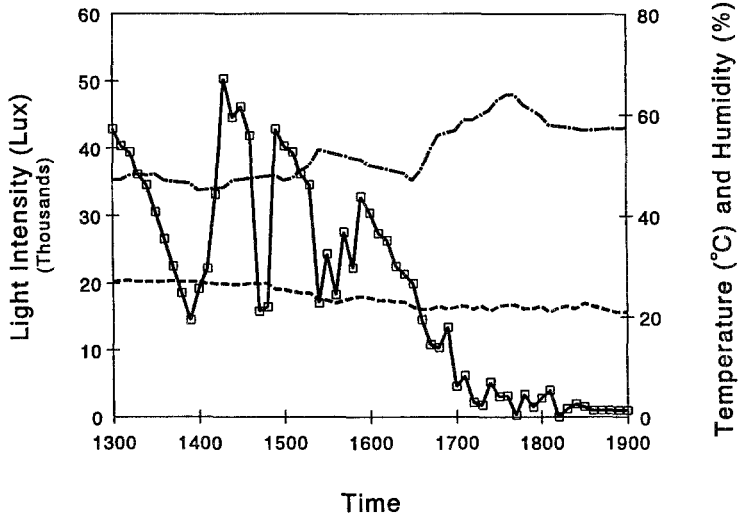

FIG. 5. An example of environmental data on temperature, humidity, and light intensity recorded during bioassays conducted in a greenhouse under simulated natural conditions.

to be fairly stable, with an overall drop in temperature throughout the test. Relative humidity tended to be inversely related to temperature, with an increase in relative humidity occurring as light intensity dropped after 1600 hr.

A review of the video taken from 1700 to 1800 hr, a time period of high female response in all trials, showed that there was a total of 109 landings on the traps. Significantly more landings occurred on pheromone-emitting traps (18.2 $\pm$ 3.8/hr) than on control traps (3.6 $\pm$ 1.1/hr; $t = 3.73$, $df = 4.7$, $P = 0.02$). There was no difference in the amount of time spent on the trap surface, with 41.3 $\pm$ 8.5 sec spent on the face of the pheromone-emitting trap versus 47.3 $\pm$ 18.2 sec on the control trap ($t = 0.3$, $df = 25$, $P = 0.77$). Females on the trap surface sometimes engaged in apparent aggressive interactions. Thirty-two percent of the landings resulted in aggressive encounters, and females sometimes left the trap surface after these interactions. Females that engaged in aggressive interactions were more likely to leave the trap than those without such encounters (48% vs. 37%, respectively). Of those flies that eventually went into the trap, those that engaged in fights spent significantly more time on the trap surface (90.1 $\pm$ 16.7 sec, $N = 15$) than those that went in without aggressive interactions (21.2 $\pm$ 4.3 sec, $N = 40$; $t = 4.0$, $df = 16$, $P = 0.001$). Sixty percent of the 91 landings on a pheromone-emitting trap ended with the fly entering the trap.

DISCUSSION

The long calling period of the male Caribbean fruit flies and the complexity of the volatile blend released by male flies during that time (Nation, 1990) have impeded the determination of the appropriate pheromone blend that could be used as an effective lure for female fruit flies in the field. Some individual components and blends have been tested in either laboratory bioassays or field trials (Nation, 1975, 1991), but only low levels of attraction have been recorded in those studies. The response of females flies was surprisingly high in the bioassay system, with 50–75% response occurring within all but the earliest testing period. The rapidity of the response enabled an examination of its periodicity and a simultaneous determination of the pheromone blend produced during the periods of greatest attraction. Environmental factors influence male pheromone production (Epsky and Heath, 1993), and these may also reduce or enhance female responsiveness regardless of pheromone blend produced.

The video records of insects as they approach and enter the traps can be used to interpret the communicative role of the chemical being tested. Simple recording of the number of insects trapped over time does not distinguish effects due to arrestants from effects due to attractants (Landolt et al., 1992): Suppose

two chemicals are being tested, neither one of which is an attractant capable of eliciting directed flight over a distance. Equal numbers of flies would land near both ports due to random movement. As flies wander about, they would be equally likely to enter either port. However, if one chemical is an arrestant, i.e., keeps insects in its vicinity, then flies in the control port are more likely to leave the area than those held by the arrestant. In this case, a chemical with no ability to attract insects over any distance could give the misleading appearance of being an attractant. Therefore, observations of behavior are essential in determining that a substance is an attractant. The significant difference in number of landings on the pheromone versus the control sites demonstrates that the male volatiles are not simple arrestants, although the data alone do not reveal over what distances the chemical signal might be effective. More information might be gained from further modification of the video system. Records of anemotaxis at further distances from traps could give insight into the range of the signal.

Numerous bioassay systems have been developed to determine behavioral response of insects to attractive sources. The system described here affords the simultaneous identification of volatile chemicals released, determination of attractiveness of these blends, automated record of environmental conditions that occurred during the investigation, and assessment of fly activity on and in the trap. Unique to this research is the adaptation of infused charcoal media for air purification of large volumes of air and the detailed design and description of the numerous components used to conduct the experiments. Additionally, this paper is the first description of response and periodicity of response by female Caribbean fruit flies to measured amounts of pheromone released by conspecific males. The use of the system described should be of general utility in determination of the attraction of pest fruit flies to suspected attractants.

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Review

NEUROTOXIC ACYLPOLYAMINES FROM SPIDER VENOMS[1]

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INTRODUCTION

In their relations with humans, spiders have acquired considerable respect, often inspiring emotions ranging from mild discomfort to genuine terror.[1-3] One of the few literary instances in which spiders are treated realistically but affectionately is E.B. White's classic, "Charlotte's Web."[4] It does not appear entirely unreasonable to attribute our tendency to avoid contact with spiders, at least in part, to the ability of some species to produce and deliver venoms that paralyze and/or kill their prey.[5] The capacity to intoxicate invertebrate and vertebrate prey is clearly an extremely important factor in the chemical ecology of this fascinating group of arachnids. It is also the reason for the recent wave of "arachnophilia" which has manifested itself in the chemical and pharmacological literature of the past decade, coinciding with the realization that spider venoms contain many neuroactive components, of relatively low (< 1000 amu) molecular weight, which may have important uses. Our own entry into this field

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has necessitated a critical examination of the relevant chemical literature.[6] It is our purpose here to present an overview of recent research on spider-derived neurotoxins, designating all those species that have been studied chemically, describing briefly the types of neurochemical activity associated with the various toxins, listing the chemical structures that have been determined to date, and outlining the syntheses that have been accomplished.

Venomous animals from a wide variety of taxa have evolved powerful arsenals stocked with chemical weapons able to stun, paralyze, and kill other organisms. Many venom components function as neurotoxins, which often exhibit remarkable specificities and binding affinities for particular neural receptors or ion channels. For example, toxins from snakes, scorpions, and predatory snails have been found to block specifically certain ligand-dependent[7,8] and voltage-dependent ion channels.[9-12] Consequently, these compounds are proving to be useful tools for neurochemical research.

There are additional reasons why neurotoxins are important. Many neural diseases and disorders are based on perturbations in the functioning of neural receptors and ion channels. As an example, nerve cell death in ischemia[13,14] (associated with strokes and heart attacks) and epilepsy[15,16] is often caused by an uncontrolled firing of the *N*-methyl-D-aspartate (NMDA) receptors, which allows toxic levels of calcium to enter the cells. Knowledge of the structures of venomous compounds that block these responses and of their mechanism(s) of action can be expected to aid in the development of drugs with which to treat these neurological problems.

Spiders are now receiving considerable attention in this context. Spiders are completely carnivorous, and many depend on their venom for survival. They are abundant throughout the world; more than 30,000 species from over 70 different families had been described by the early 1980s.[17] Until recently, however, spiders have been largely neglected by chemists because of the small size of most arachnids, coupled with the difficulty in collecting their venom. Black widow spiders (*Latrodectus mectans*), for example, are only 6–11 mm long, and contain about 0.2 mg of venom per individual.[18] Nevertheless, it is now possible to collect spider venom nondestructively,[19,20] and new methods are available to isolate, bioassay, and characterize venom components in very small amounts. These advances have facilitated the study of many new species.

NEUROTOXINS FROM SPIDERS

Research into the detailed chemistry of spider venoms began over 30 years ago when Fischer and Bohn analyzed the venoms of some very large South American bird spiders.[21] At that time, full characterization of the venom constituents was not possible. However, the venoms were shown to contain both

proteins and polyamines. Since this initial study, venom components from over 85 spider species representing 15 different families have been investigated (Table 1), although fewer than a dozen of these species have been studied in depth. The spider venoms are composed chiefly of proteins and polypeptides, but they also contain low-molecular-weight (< 1000 amu) organic compounds and inorganic salts.[22] Most recent research has been devoted to neurotoxic substances, although some attention has also been directed toward venom-derived enzymes. The venom of the aggressive brown recluse spider (*Loxosceles reclusa*), for example, contains a powerful sphingomyelinase capable of extensive tissue damage.[23]

Categorizing the different types of spider neurotoxins on the basis of their biological activity is difficult. The nervous system is complex, and determining how individual toxins act requires a variety of bioassays to probe these complexities. For example, black widow spider venom contains two large (130 and 150 kDa) neurotoxic proteins. One of these, α-latrotoxin, is very toxic to vertebrate animals but not to insects.[24] The other, α-latroinsectotoxin, is active against insects but not vertebrates.[25] Interestingly, both neurotoxins function by a similar mechanism. They bind to recognition sites along the presynaptic membrane, and then form transmembrane Ca^{2+} ion channels stimulating a massive release of neurotransmitter.[26,27] The different target selectivities seem to be due to recognition of different binding sites along the presynaptic membrane. Other than that, both neurotoxins function identically and have a high degree of sequence homology.[24,27]

Many of the earlier bioassays determined only whether a compound was neurotoxic to some animal species. Few mechanistic studies were done, and consequently the mode of action of many neurotoxins remained unknown. However, as knowledge of the nervous system has improved, so have the bioassays. The precise mechanisms by which some neurotoxins function are now known, and spider toxins are revealing new information about neural receptors and ion channels. For example, FTX, an intriguing venom component of as yet undetermined structure or homogeneity isolated from *Agelenopsis aperta*, was found to block an unknown type of calcium current in Purkinje cells; this study led to the discovery of the P-type calcium channel.[166,171,174]

COMPILATION OF STRUCTURES

Most neurotoxins identified so far are proteins or polypeptides, of which four different classes are known: (1) high-molecular-weight proteins that create divalent cation channels which cause massive transmitter release from presynaptic nerve terminals, (2) small polypeptides that function similarly to the first class, (3) polypeptide blockers of presynaptic calcium channels that inhibit signal

Table 1. Spider Taxonomy, Indicating Species Whose Venoms Have Been At Least Partially Characterized

- Araneae
  - Orthognatha
    - Mesothelae (atypical tarantulas)
      - Liphistiidae
      - Anthrodiaetidae
      - Mecicobothriidae
      - Atypidae
    - Opisthothelae (typical tarantulas)
      - Theraphosidae
        - *Acanthoscurria atrox*[21]
        - *Acanthoscurria cratus*[28]
        - *Acanthoscurria emelia*[29]
        - *Acanthoscurria* sp. (Arizona)[29-31]
        - *Acanthoscurria* sp. (Honduras)[29]
        - *Aphonopelma chalcodes*[32]
        - *Brachypelma smithii*[33]
        - *Dugesiella hentzi*[29,30,33-36]
        - *Eurypelma californicum*[37,38]
        - *Eurypelma emilia*[33]
        - *Eurypelma vellutinum*[21]
        - *Grammostola mollicoma*[21]
        - *Grammostola actaeon*[21]
        - *Grammostola pulchripes*[21]
        - *Harpactirella* sp.[32]
        - *Lasiodora klugii*[21]
        - *Pamphobeteus platyomma*[39]
        - *Pamphobeteus roseus*[21,40]
        - *Pamphobeteus soracabae*[39]
        - *Pamphobeteus tetracanthus*[21]
        - *Phormictopus cancerises*[41]
        - *Pterinochilus* sp.[42,43]
      - Paratropididae
      - Pycnothelidae
      - Barychelidae
      - Migidae
      - Dipluridae (funnel-web spiders)
        - *Atrax infensus*[44]
        - *Atrax robustus*[45-54]
        - *Atrax versutus*[55]
      - Ctenizidae (trap-door spiders)
        - *Aganippe berlandi*[44]
        - *Aptostichus schlingeri*[56]
        - *Hebestatis theveniti*[32]
        - *Phoneutria nigriventer*[57-66]
        - *Phoneutria fera*[67,68]
      - Actinopodidae
  - Labidognatha
    - Hypochiloidea
      - Gradungulidae
      - Hypochilidae
    - Neocribellatae
      - Filistatidae
      - Oecobiidae
      - Eresidae
      - Dinopidae
      - Uloboridae
      - Dictynidae
      - Amaurobiidae
      - Amphinectidae
      - Neolanidae
      - Psechridae
      - Stiphiidae
      - Tengellidae
      - Zoropsidae
      - Acanthoctenidae
    - Ecribellatae
      - Haplogynae (primitive hunters and weavers)
        - Sicariidae
        - Scytodidae
        - Loxoscelidae
          - *Loxosceles laeta*[69,70]
          - *Loxosceles gaucho*[71]
          - *Loxosceles reclusa*–brown recluse[23,72-95]
          - *Loxosceles refescens*[96,97]
          - *Loxosceles reufipes*[96]
        - Diguetidae
        - Plectreuridae
          - *Plectreurys tristes*[41,98-101]
        - Caponiidae
        - Oonopidae
        - Tetrablemmidae
        - Pacullidae
        - Ochyroceratidae
        - Leptonetidae
        - Telemidae
        - Textracellidae
        - Dysderidae
        - Segestridae
          - *Segestrie florentina*[102,103]

TABLE 1. CONTINUED

- Entelogynae
  - Trionycha (higher web weavers)
    - Pholicidae
    - Symphytognathidae
      - *Amaurobius insignis*[44]
    - Theridiidae (comb-footed) spiders
      - *Achaearanea tepidariorum*[104, 105]
      - *Episinus?*[106]
      - *Latrodectus hasselti*[44]
      - *Latrodectus hesperus*[107]
      - *Latrodectus mectans*—black widow[18, 20, 24–27, 68, 106, 108–135]
      - *Latrodectus pallidus*[106, 136]
      - *Latrodectus dahli*[136]
      - *Steatoda bipunctata*[106]
      - *Steatoda paykulliana*[106, 126, 137, 138]
      - *Steatoda triangulosa*[106]
      - *Theridion impressum*[106]
      - *Theridion varians*[106]
    - Nicodamidae
    - Nesticidae
    - Hadrotarsidae
    - Linyphiidae
    - Micryphantidae
    - Theridiosomatidae
    - Araneidae (typical orb weavers)
      - *Agalenatea redii*[139]
      - *Araneus diadematus*[139]
      - *Araneus gemma*[41, 140–142]
      - *Araneus tartaricus*[143]
      - *Argiope auranta*[41, 144, 145]
      - *Argiope bruennichi*[97]
      - *Argiope lobata*[139, 143, 146–149]
      - *Argiope florida*[140, 142]
      - *Argiope trifasciata*[140, 142]
      - *Mangora acalypha*[139]
      - *Neosocona adianta*[139]
      - *Neosocona arabesca*[41, 145, 150]
      - *Neosocona cruciferoides*[139]
      - *Neoscona nautica*[97]
      - *Nephila clavata*[97, 151–159]
      - *Nephila edulis*[44]
      - *Nephila maculata*[152, 154, 155]
      - *Nuctenea folium*[139]
      - *Zygiella caspica*[139]
    - Tetragnathidae
    - Agelenidae (funnel-web weavers)
      - *Agelena opulenta*[160]
      - *Agelenopsis aperta*[161–178]
      - *Hololena curta*[101, 179–181]
      - *Tegenaria domestica*[182]
    - Argronetidae
    - Sesidae
    - Hahniidae
    - Hersiliidae
    - Urocteidae
    - Mimetidae
    - Archaeidae
    - Mecysmaucheniidae
    - Zodariidae
    - Palpimanidae
    - Stenochilidae
    - Pisauridae (nursery-web spiders)
      - *Dolomedes okefinokensis*[6, 183]
      - *Dolomedes sulfureus*[97]
    - Lycossidae
      - *Lycosa erythrognatha*[57, 67]
      - *Lycosa godeffroyi*[44, 184]
      - *Lycosa labrea*[41]
      - *Lycosa singoriensis*[185–187]
      - *Lycosa tarentula*[188]
      - *Sosippus californicus*[189]
    - Oxyopidae
      - *Peucetia viridens*[41, 145]
    - Senoculidae
    - Toxopidae
  - Dionycha (two clawed hunting spiders)
    - Ammoxenidae
    - Platoridae
    - Gnaphosidae
      - *Lampona cylindrate*[44]
    - Prodidomidae
    - Homalonychidae
    - Cithaeronidae
    - Clubionidae
    - Anyphaenidae
    - Amaurobioidae
    - Zoridae
    - Ctenidae
    - Sparassidae
      - *Isopeda immanis*[44]
    - Thomisidae
    - Philodroimidae
    - Aphantochilidae
    - Salticidae
    - Lyssomanidae

- Unknown family
  - *Chiranthium japonicum*[97]
  - *Eriophora transmarina*[44]
  - *Namea salantri*[44]
  - *Scodra griseipes*[190, 191]

transmission, and (4) polypeptide activators of presynaptic sodium channels that stimulate repetitive nerve firings. Examples of these classes are given in Table 2.

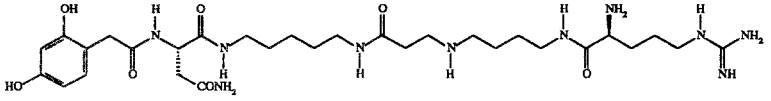
Spider venoms also contain many compounds other than proteins and polypeptides that are neuroactive. Some of these compounds are well known from other sources as neural transmitters (serotonin,[20,51,61] octopamine,[51] 5-hydroxytryptamine,[68,114] 5-methoxytryptamine,[51] histamine,[61,68] tyramine,[51] γ-aminobutyric acid,[20,21,34,47,51,68] aspartic acid,[20,21,34] and glutamic acid[20,21,34]). Only one new, nonproteinaceous class of neuroactive compounds has been discovered in spider venoms, the acylpolyamines. These all contain an aromatic acyl end group and a polyamine chain. They were first discovered in the early to mid-1980s from the spiders *Nephila clavata*[152] and *Argiope lobata*.[146] These neurotoxins function by blocking glutamate-sensitive calcium channels that are important in many neural functions including pain,[192] motor control,[193] and memory.[194] The glutamate-sensitive calcium channels have also been implicated in several neurodegenerative disorders such as amyotrophic lateral sclerosis[195] and Alzheimer's, and Huntington's diseases.[196] The desire to understand how these ion channels function has stimulated much research on spider venoms as well as on acylpolyamines from other sources. Many new acylpolyamines have now been identified in spider venoms, and quite remarkably, some closely related acylpolyamines have also been identified in certain wasp venoms.[198,199] Currently, the neuronal activity reported for all but two of these acylpolyamines is glutamate-receptor blocking. Since glutamate is the active chemical messenger in insect neuromuscular junctions, this mode of action is hardly surprising (a recent review of the activities of these glutamate receptor affecting toxins has been published by P.N.R. Usherwood and I.S. Blagbrough).[200] The exceptions are CNS 2103 (as its trifluroacetic acid salt), which blocks L- and R-type voltage-sensitive calcium channels,[183] and FTX, which blocks P-type voltage-sensitive calcium channels. Additionally, one group of acylpolyamines, the nephilatoxins, has also been shown to degranulate mast cells,[201] although the biological significance of this observation is not clear.

The acylpolyamines can be subdivided into two groups: the amino acid-containing acylpolyamines (Figure 1) and the non-amino acid-containing acylpolyamines (Figure 2). Both types function similarly as glutamate receptor blockers, and both incorporate the same family of aromatic end groups (Figure 3). The amino acid-containing acylpolyamines were the first to be discovered. To date, 34 of these compounds have been fully characterized, all from one spider family: the Araneidae (orb weavers) (Table 3). All of these possess one or two of the following basic amino acids between the acyl end group and the polyamine chain: asparagine, ornithine, and ω-*N*-methyllysine. In addition, some of these acylpolyamines possess up to three amino acids at the polyamine terminus, with arginine usually occupying the terminal position.

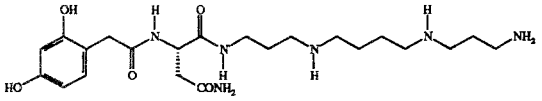
TABLE 2. EXAMPLES OF FOUR CLASSES OF POLYPEPTIDE NEUROTOXINS IDENTIFIED IN SPIDER VENOMS

Type I. Large proteins that bind to presynaptic membranes and form transmembrane, divalent cation channels, producing an influx of calcium ions that stimulates massive neurotransmitter release.		
Lactrodectus mectans		
α-Latrotoxin	130 kDa 1170 amino acid sequence	Forms channels in vertebrates but not invertebrates.[24,26,116-118]
α-Latroinsectotoxin	120 kDa partial amino acid sequence	Forms channels in invertebrates but not vertebrates.[25,27,135]
Steatoda paykulliana	110–130 kDa	Forms ion channels, toxic to insects.[137]
Type II. Low molecular weight pore forming polypeptides which cause massive transmitter release from nerve terminals.		
Latrodectus mectans, L. pallidus, Steatoda bipunctata, S. paykulliana, S. triangulosa, Theridion impressum	5 kDa	Forms ion channels through phospholipid membranes[106,126,134,197]
Sosippus californicus		
Sositoxin I	2.5 kDa 23 amino acid sequence	Forms ion channels through phospholipid membranes[189]
Type III. Peptides that suppress neurotransmitter release from presynaptic stores. These function by blocking presynaptic ca^{2+} channels.		
Agelenopsis aperta		
ω-Aga-IA	7.5 kDa (66 aa) 66 amino acid sequence	Blocks L- and N-type Ca^{2+} channels.[165,168,173]
ω-Aga-IB	7.5 kDa partial sequence	Blocks L- and N-type Ca^{2+} channels.[165,168]
ω-Aga-IIIA	8.5 kDa	Blocks N- and L-type Ca^{2+} channels.[174]
ω-Aga-IIA	11 kDa partial sequence	Blocks N-type Ca^{2+} channels. inhibits ω-CgTx binding.[168]
ω-Aga-IVA	5.2 kDa 48 amino acid sequence	Blocks P-type Ca^{2+} channels.[177]
Hololena curta		
HoTX	16 kDa two subunits	Blocks voltage dependent Ca^{2+} channels vertebrate inactive[101,179]
CT-I, II, and III	4 kDa 36–38 amino acid sequence	Irreversible presynaptic neuromuscular blockage in insects[180]
Plectreurys tristes		
α-PLTX I-III	7 kDa	blocks Ca^{2+} channels vertebrate inactive[99,101]
Type IV. Polypeptides that cause repetitive firing in presynaptic neurons. This action is caused by activation of presynaptic voltage-sensitive sodium channels.		
Agelenopsis aperta		
μ-Aga I-VI	4.2 kDa 36–38 amino acid sequences	External sodium required for activation. Excitation is abolished with the addition of tetrodotoxin.[164,167]
Phoneutria nigriventer		
Phx1	8 kDa 77 amino acid sequence	Excitation is abolished with the addition of tetrodotoxin.[63,65,66]
PhTx2	6–6.5kDa partial amino acid sequence	Excitation is abolished with the addition of tetrodotoxin.[66]

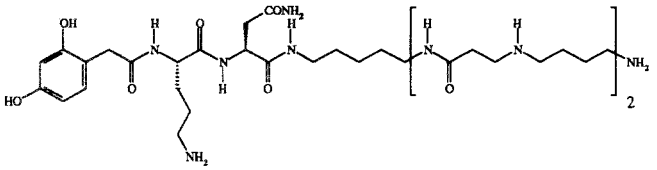
NSTX-3



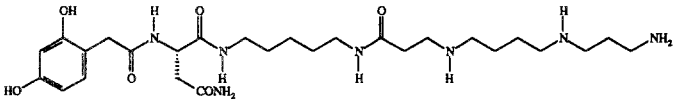
JSTX-1



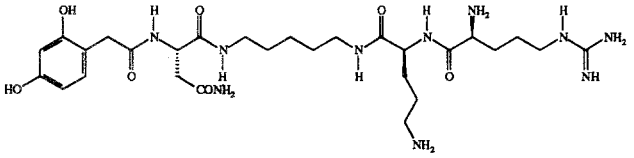
JSTX-2



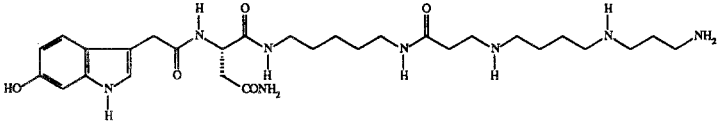
JSTX-3



JSTX-4



NPTX-1



NPTX-2

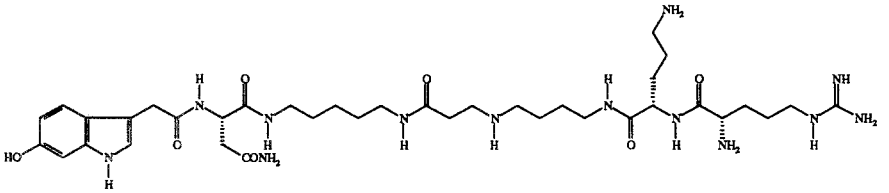
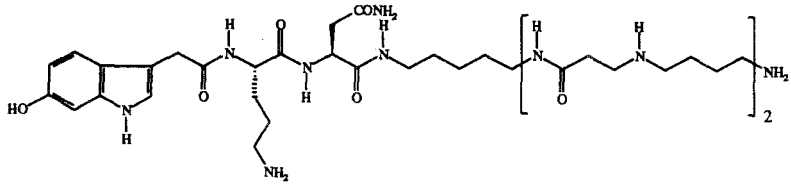


FIG. 1. Chemical structures of all the known amino acid-containing acylpolyamines identified from spider venoms.

NPTX-3



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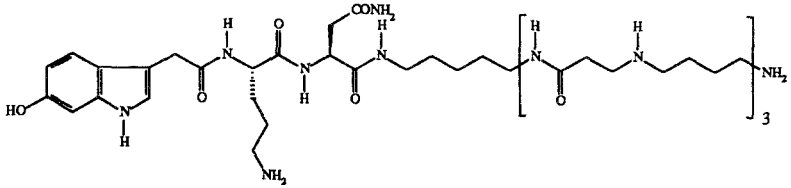
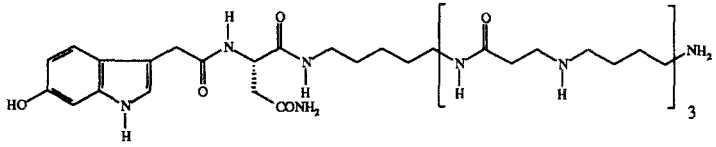
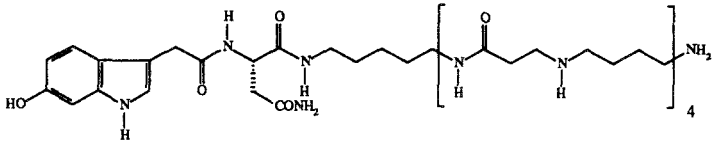


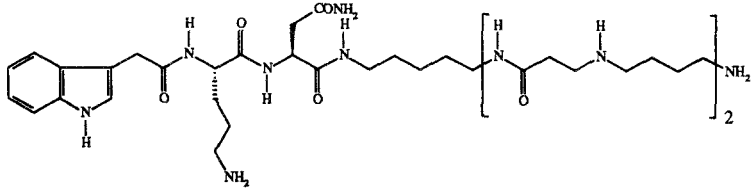
NPTX-5



NPTX-6



NPTX-7



NPTX-8

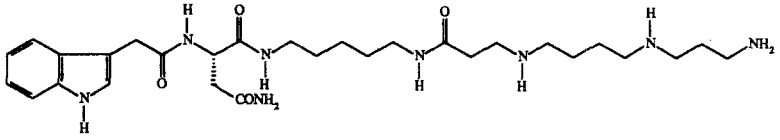
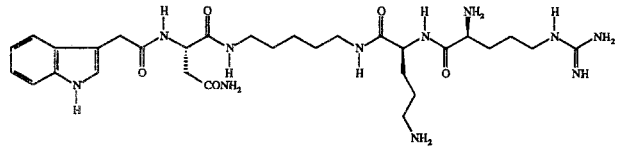
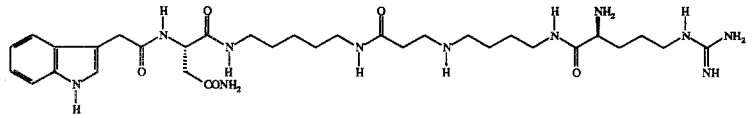


FIG. 1. Continued

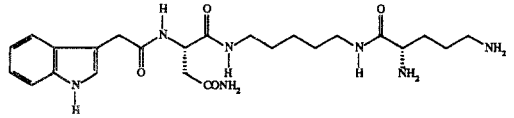
NPTX-9



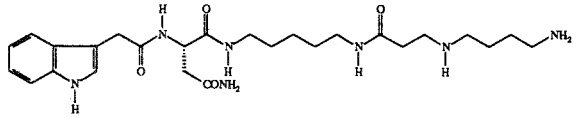
NPTX-10



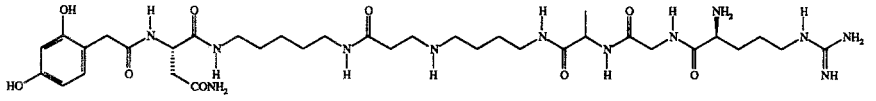
NPTX-11



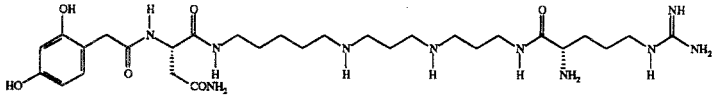
NPTX-12



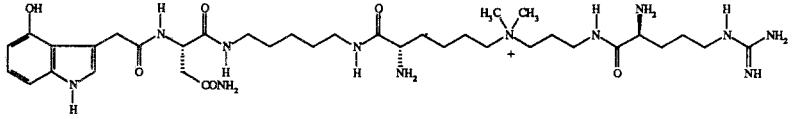
Clavamine



Argiopine or Argiotoxin-636



Argiopinin I



Argiopinin II

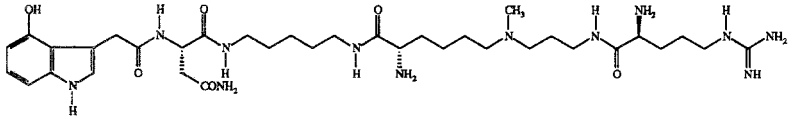
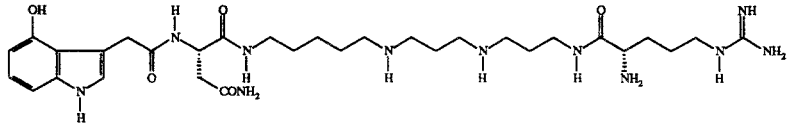
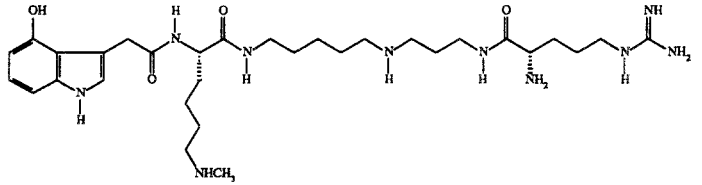


FIG. 1. Continued

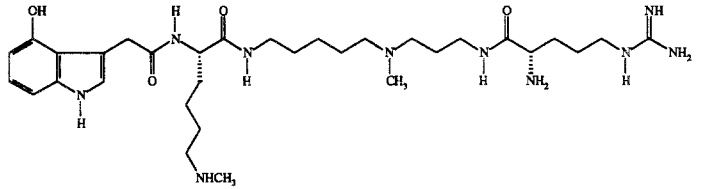
Argiopinin III or Argiotoxin-659



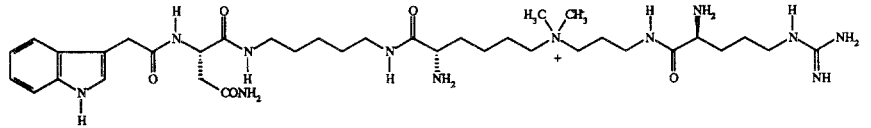
Argiopinin IV



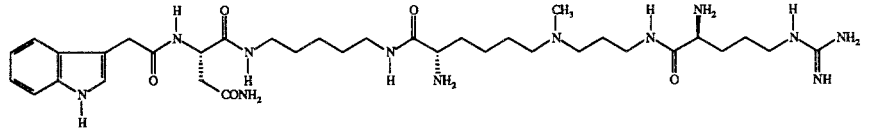
Argiopinin V



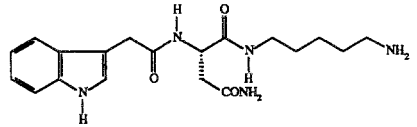
Pseudoargiopinin I



Pseudoargiopinin II



Pseudoargiopinin III



Argiotoxin-480

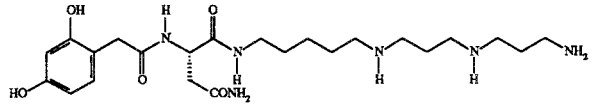
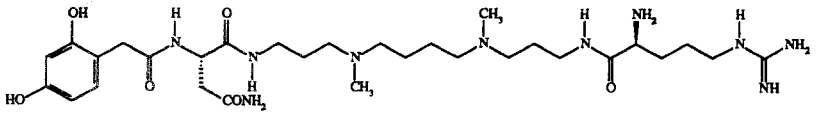
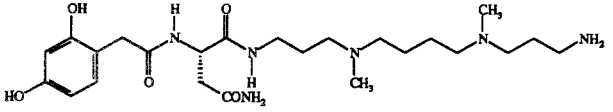


FIG. 1. Continued

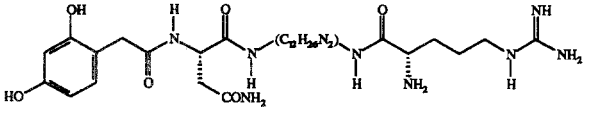
Argiotoxin-650



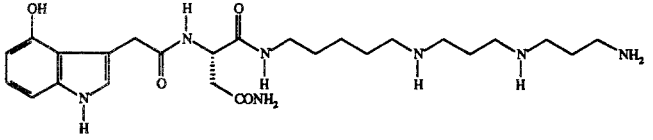
Argiotoxin-494



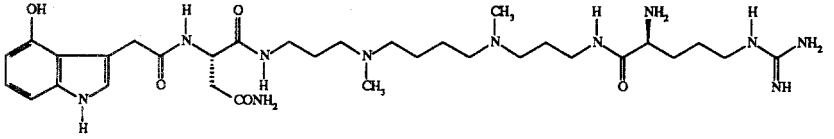
Argiotoxin-622



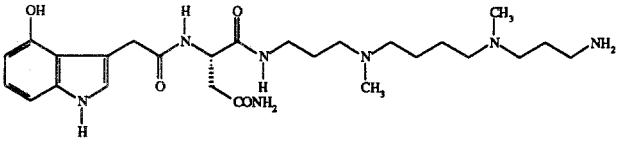
Argiotoxin-503



Argiotoxin-673



Argiotoxin-517



Argiotoxin-645

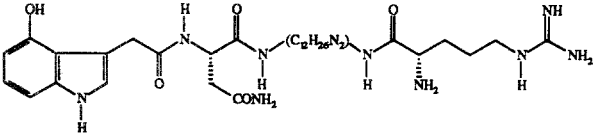
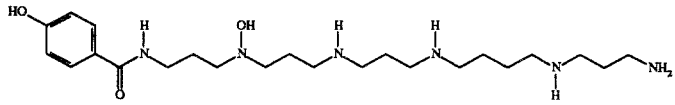
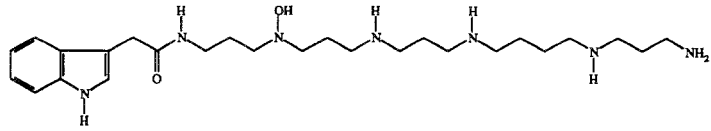


FIG. 1. Continued

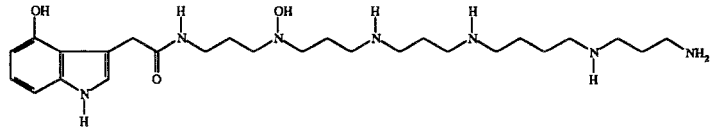
AG 452 or AGEL 452



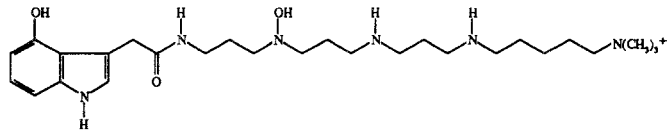
AG 489, AGEL 489 or HO 489



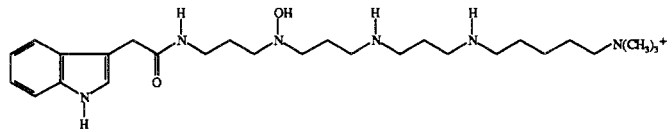
AG 505, AGEL 505 or HO 505



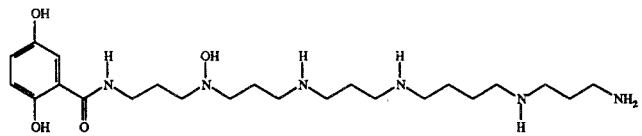
AG 504 or AGEL 505a



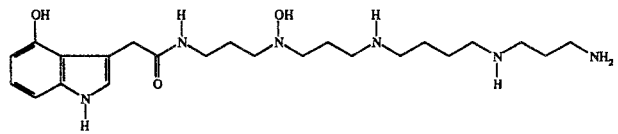
AG 488 or AGEL 489a



AGEL 468 or HO 468



AGEL 448



HO 473

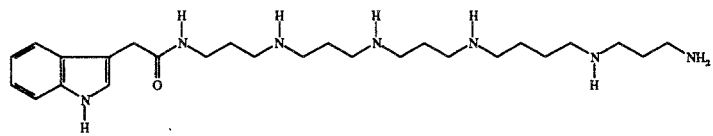
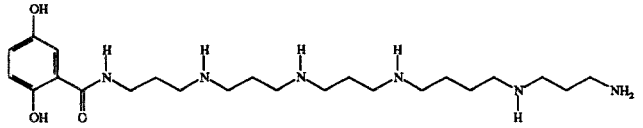
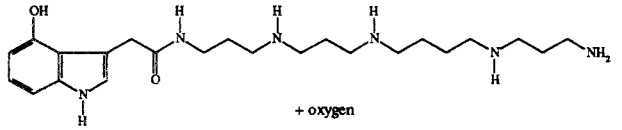


FIG. 2. Chemical structures of all known non-amino acid-containing acylpolyamines from spider venoms.

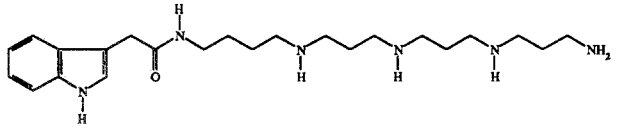
HO 452



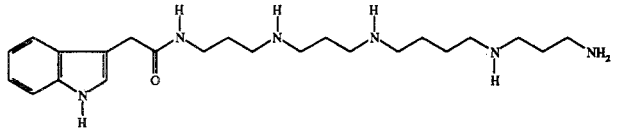
HO 448 (same as Agel 448?)



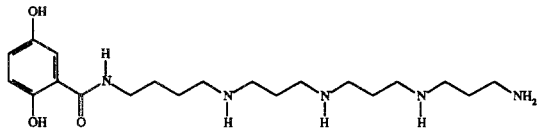
HO 395



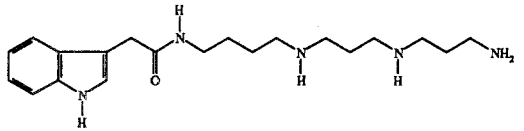
HO 416a



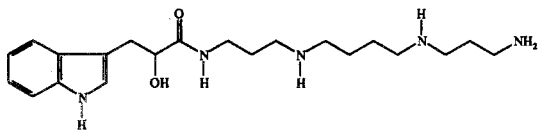
HO 416b



HO 359



Het 389



Het 403

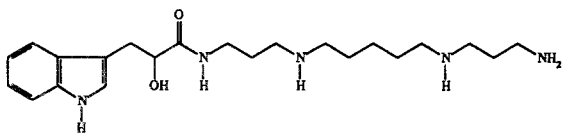


FIG. 2. Continued

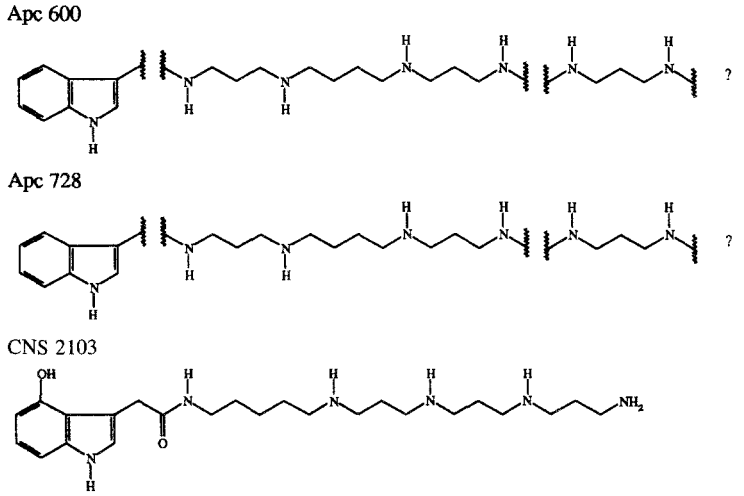


FIG. 2. Continued

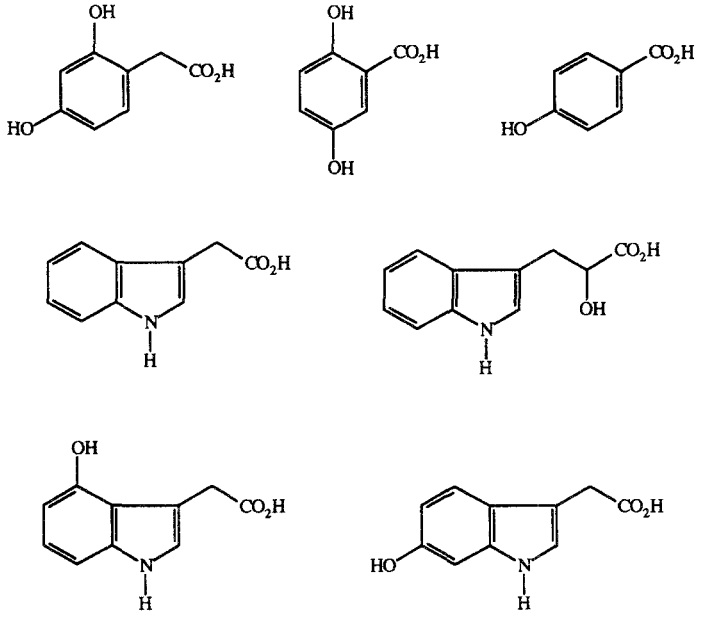


FIG. 3. The seven acids corresponding to the acyl end groups identified in spider venom acylpolyamines.

TABLE 3. AMINO ACID-CONTAINING ACYLPOLYAMINES FROM SPIDERS

Compound	*Nephila maculata*	*Nephila clavata*	*Argiope lobata*	*Argiope trifasciata*	*Argiope florida*	*Argiope auranta*	*Araneus gemma*	Synthesis
NSTX-3	152, 155							202, 203, 204, 205
JSTX-1		158						
JSTX-2		152, 158						
JSTX-3		152, 153						203, 206
JSTX-4		152. 158						
NPTX-1		157						
NPTX-2		157						
NPTX-3		157						
NPTX-4		157						
NPTX-5		157						
NPTX-6		157						
NPTX-7		156, 157						
NPTX-8		156, 157						
NPTX-9		156, 157						207
NPTX-10		157						208
NPTX-11		157						207
NPTX-12		157						208

Clavamine	159						209
Argiopine, Argiotoxin-636		146, 147	140, 142	142	145	140, 142	145, 210, 211, 212
Argiopinin I		147					
Argiopinin II		147					
Argiopinin III, Argiotoxin 659		147	142	142	145		145, 211
Argiopinin IV		147					
Argiopinin V		147					
Pseudoargiopinin I		147					
Pseudoargiopinin II		147					
Pseudoargiopinin III		147					
Argiotoxin-480			142	142			
Argiotoxin-650			142	142			
Argiotoxin-494			142	142			
Argiotoxin-622[a]			142	142			
Argiotoxin-673			142	142	145		211
Argiotoxin-503			142	142			
Argiotoxin-517			142	142			
Argiotoxin-645[a]			142	142			

[a] Structures are only partially characterized.

The lengths of the polyamine chains show considerable variation. The smallest chain is only seven atoms long (pseudoargiopinin III), whereas the longest extends for 43 atoms (NPTX-6). These chains consist of NH, NCH_3, or $^{+}N(CH_3)_2$ groups separated by segments of three to six carbon atoms. The carbon segments are linear chains of methylene groups, with the exception of some three and all of the six carbon segments, which appear as β-alanine and lysine units, respectively. Interestingly, all of the lysine's terminal nitrogens are either mono- or dimethylated, but few of the other amino groups are substituted. Finally, the first segment within the polyamine chain of most known amino acid containing acylpolyamines is a five carbon unit.

The non-amino acid-containing acylpolyamines (Figure 2), although discovered later, appear to be more widely distributed among spider species. Currently, 17 different compounds have been fully characterized from five different families: the Theraphosidae, Dipluridae, Ctenizidae, Agelenidae, and Pisauridae (Table 4). These compounds consist solely of an acyl end group attached to a polyamine chain. The carbon segments consist entirely of trimethylene, tetramethylene, and pentamethylene units. Some of the nitrogen atoms separating the first and second carbon segments occur as hydroxylamines, and a few terminal nitrogens are methylated. Unlike the amino acid-containing acylpolyamines, the first polyamine segment of the non-amino acid-containing compounds is usually a three or occasionally a four or five carbon unit.

SYNTHESIS OF SPIDER NEUROTOXINS

Largely because of the inevitably small supply of individual, biologically active components from spider venoms, synthesis is an extremely important tool for confirming postulated structures and for making useful amounts of material available for in vitro and in vivo experimentation.

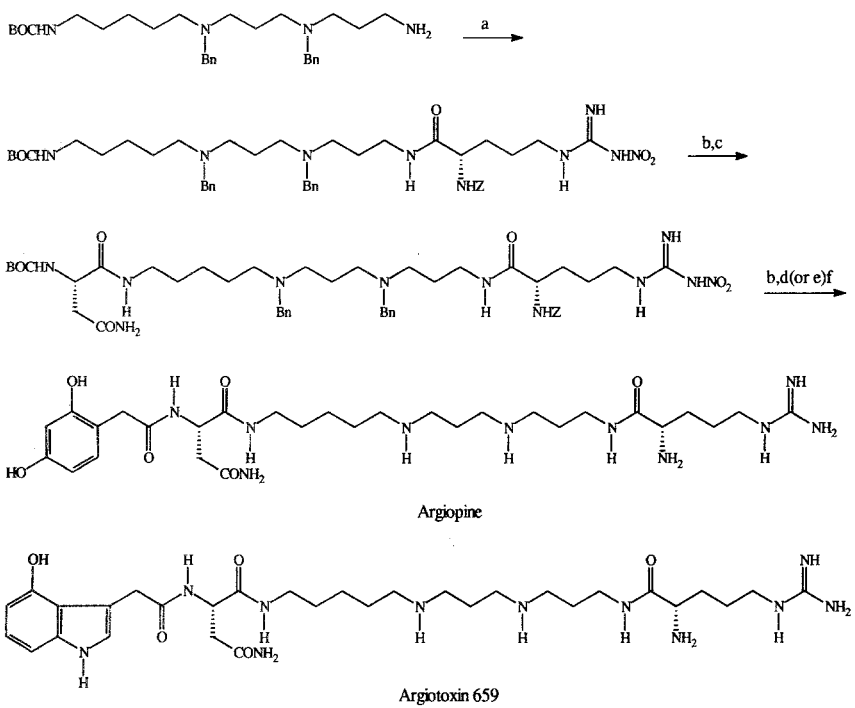
All of the successful synthetic work in this field is described in roughly a score of recent papers, already referred to in the final columns of Tables 3 and 4. The syntheses combine many well known elements of polyamine and amino acid chemistry; they illustrate the importance of specific protecting groups, and the use of particular techniques for handling strongly basic, hydrophilic compounds, some of which are also sensitive to oxygen.

To save space, each synthesis is presented simply as a chart with an indication of the reagents used to bring about each step. They are grouped in two sets: the first set (Schemes 1–14) describe polyamines containing amino acid moieties, and the second set (Schemes 15–19) describe those lacking amino acids. Within each group, the schemes are presented in alphabetical order with respect to the first author's names.

TABLE 4. NON-AMINO ACID-CONTAINING ACYLPOLYAMINES FROM SPIDERS

Compound	*Agelenopsis aperta*	*Hololena curta*	*Hebestatis theveniti*	*Harpactirella* sp.	*Aphonopelma chalcodes*	*Atrax robustus*	*Dolomedes okefinokensis*	Synthesis
AG 452	167, 170,							170
AGEL 452	171							
AG 489	164, 167,	181						170
AGEL 489	170, 171							
HO 489								
AG 505	164, 167,	181						170
AGEL 505	170, 171							
HO 505								
AG 504	164, 167,							
AGEL 505a	171, 176							
AG 488	164, 167,							176
AGEL 489	171, 176							
AGEL 468	170	181						170
HO 468								
AGEL 448	170							170
HO 473		181						
HO 452		181						
HO 448		181						
HO 395		181						
HO 416a		181						
HO 416b		181						
HO 359		181						
Het 389			32	32		? 49		32
Het 403			32					
Apc 600[a]					32			
Apc 728[a]					32			
CNS 2103							6	6

[a] Structures are only partially characterized.

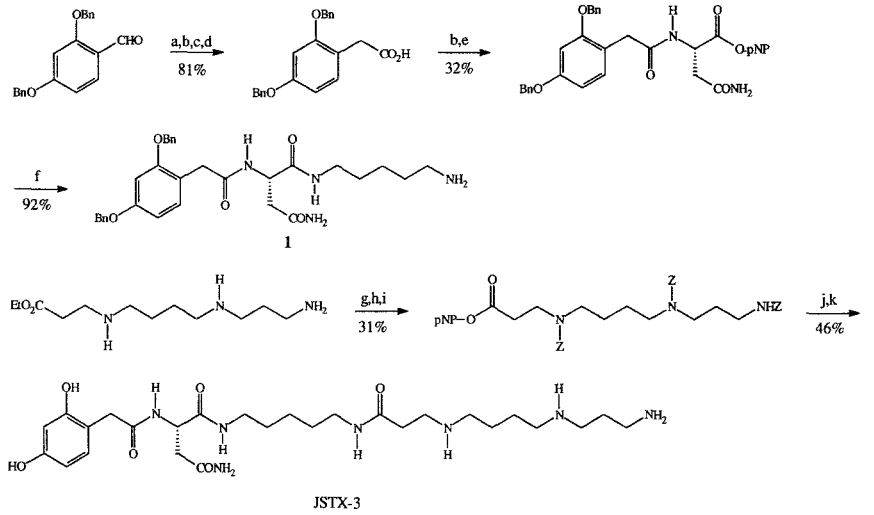


SCHEME. 1. Adams, 1987 (ref. 145). a. Nα-Z-Nω-nitro-Arg, DCC, HOBt; b. TFA; c. BOC-Asp, DCC, HOBt; e. 2,4-dibenzyloxyphenyl acetyl chloride; f. 4-benzyloxyindole-3-acetic acid, DCC, HOBt; g. H_2, Pd-C, HOAc.

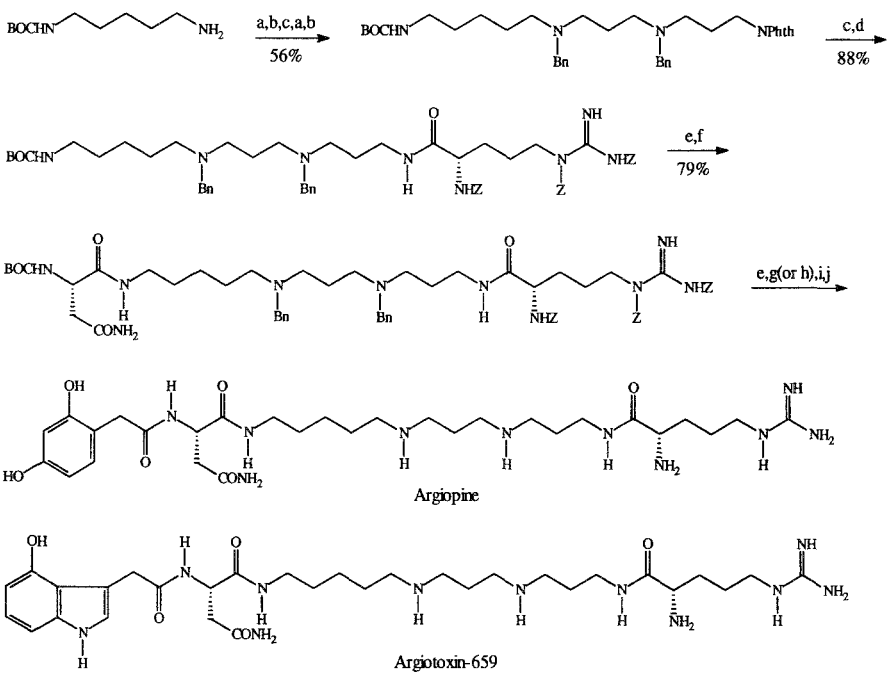
CONCLUDING REMARKS

With the structures of so many natural acylpolyamines known, and with synthetic samples of these and their analogs now becoming available in useful quantities, the stage has been set for an intensive study of the receptors at which these toxins act. How these receptors interact with polyamine neurotoxins must now become a major objective of future research. While the details of such research lie outside the scope of this review, they can be expected to illuminate what it is that the spiders, as applied neuropharmacologists, have been getting at.

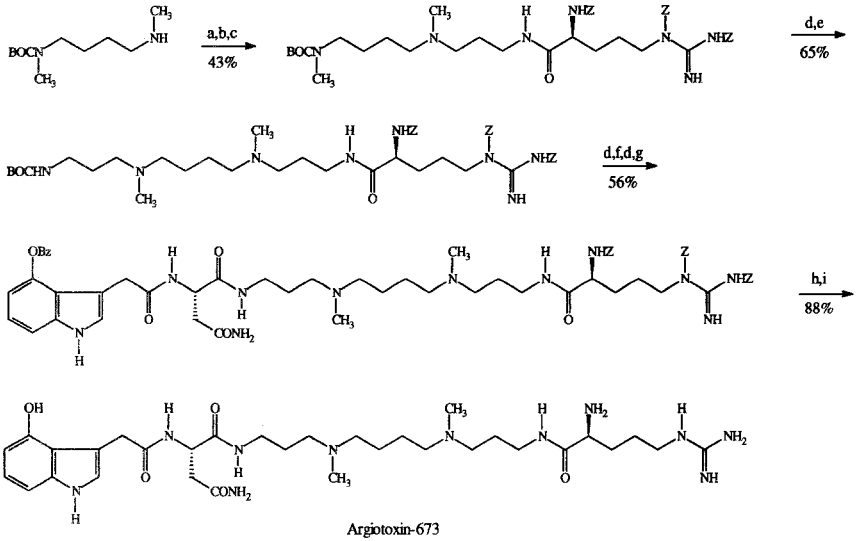
At the least, spider neurotoxins represent a powerful set of neurobiological research tools. Whether they ultimately provide a new generation of useful drugs and insecticides remains to be seen. From the viewpoint of the chemical ecologist, the chemical and biological studies that have been completed so far should certainly whet the appetite for a deeper understanding of how venoms influence the relationships between spiders and the rest of the world.



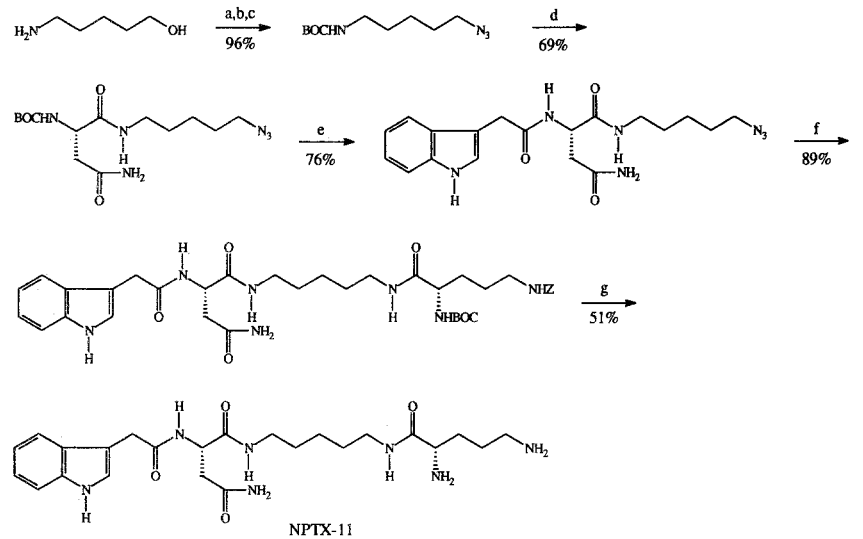
SCHEME. 2. Hashimoto, 1987 (ref. 206). a. $NaBH_4$, MeOH; b. $SOCl_2$, PhH, Δ; c. NaCN, DMSO; d. KOH, EtOH, Δ; e. L-Asn-O*p*NP. TEA, DMF; f. $H_2N(CH_2)_5NH_2$, DMF; g. Z-Cl, $NaHCO_3$, Et_2O-H_2O; h. KOH, EtOH; i. *p*-nitrophenol, DCC, DMF; j. 1, HOBt, DMF; k. H_2, Pd-C, HOAc.



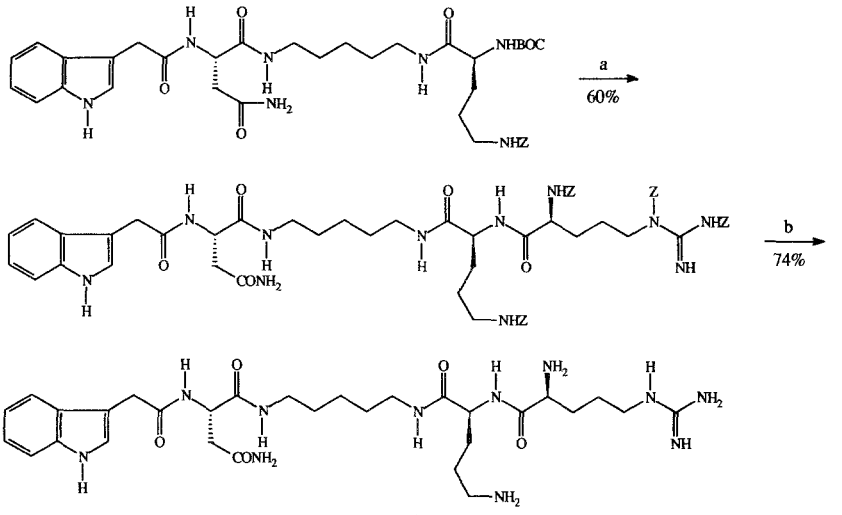
SCHEME. 3. Jasys, 1988 (ref. 211). a. $NaBH_4$, PhCHO, $MgSO_4$, MeOH; b. $PhthN(CH_2)_3Br$, KF-celite, CH_3CN, Δ; c. H_2NNH_2, MeOH, ; gD; d. Z-Arg(Z_2)-Su, CH_2Cl_2; e. TFA; f. BOC-Asn-O-*p*NP, TEA, CH_2Cl_2; g. 2,4-dibenzyloxyphenyl acetic acid, CH_2Cl_2; h. 4-benzyloxyindole-3-acetic acid, DCC, HOBt, CH_2Cl_2; i. H_2, $Pd(OH)_2$, HOAc; j. Amberlite CG-50, MeOH, HCl.



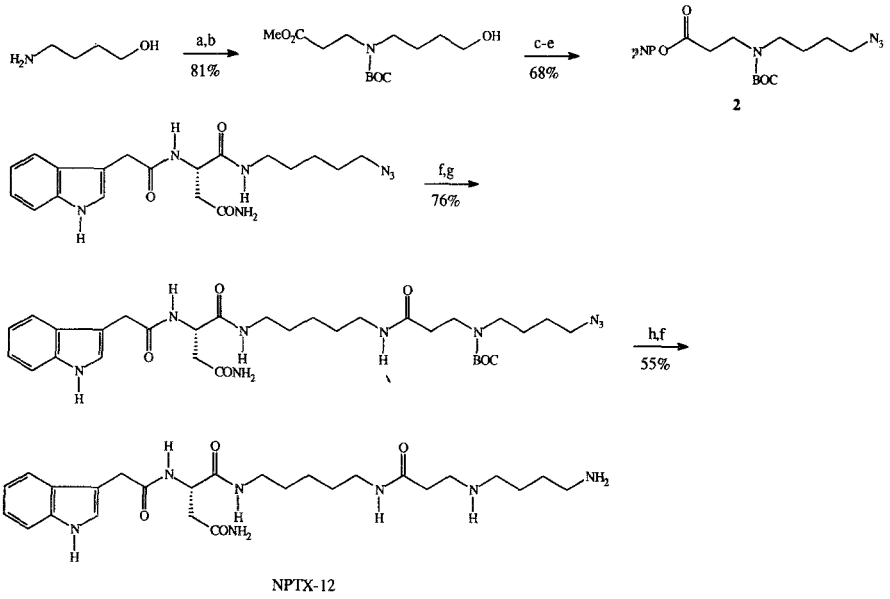
SCHEME. 4. Jasys, 1988 (ref. 211). a. $PhthN(CH_2)_3Br$, KF-celite, CH_3CN, ; gD; b. H_2NNH_2, MeOH, Δ; c. Z-Arg(Z_2)-Su, CH_2Cl_2; d. TFA; e. $BOCHN(CH_2)_3Br$, Na_2CO_3, CH_3CN, Δ; f. BOC-Asn-O-*p*NP, TEA, CH_2Cl_2; g. 4-benzyloxyindole-3-acetic acid, DCC; HOBt, CH_2Cl_2; h. H_2, $Pd(OH)_2$, HOAc; i. Amberlite CG-50, MeOH, HCl.



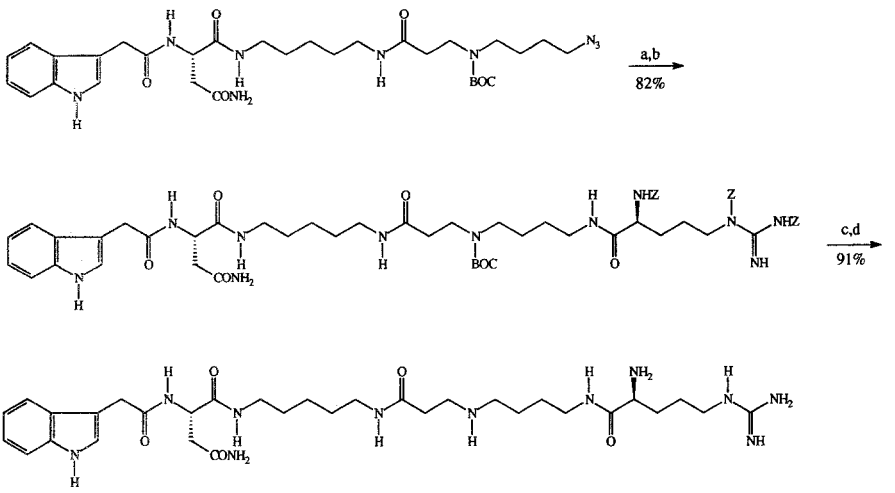
SCHEME. 5. Miyashita, 1992 (ref. 207). a. $(BOC)_2O$, aq. Na_2CO_3; b. MsCl, pyr, CH_2Cl_2; c. NaN_3, DMF; d. TFA, CH_2Cl_2; BOC-Asn-O*p*NP, TEA, DMF; e. TFA, CH_2Cl_2; indole-3-acetyl-O*p*NP, TEA, DMF; f. H_2, Pd-C, EtOH; BOC-Orn(Z)-O*p*NP, TEA, DMF; g. TFA, CH_2Cl_2; H_2, Pd-C, EtOH.



SCHEME. 6. Miyashita, 1992 (ref. 207). a. TFA, CH_2Cl_2; Z-Arg(Z_2)-ONSu, TEA, DMF; b. H_2, Pd-C, TEA, HOAc.

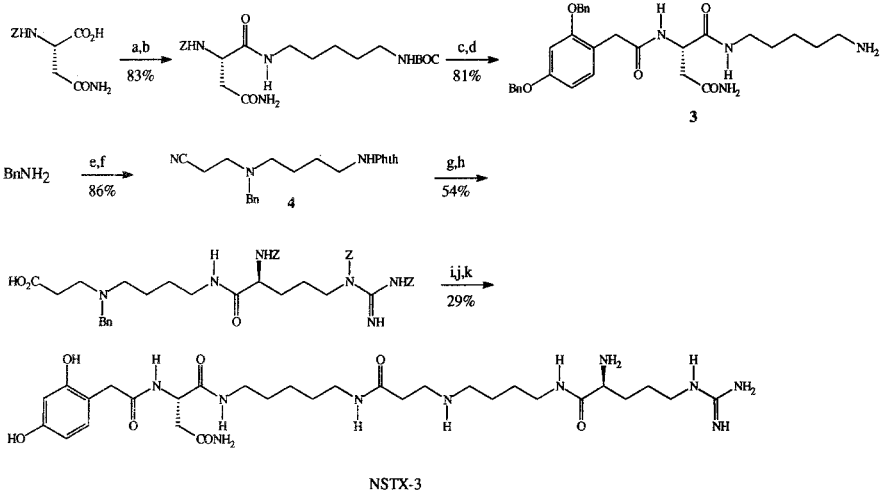


SCHEME. 7. Miyashita, 1992 (ref. 208). a. CH_2:$CHCO_2Me$, 0°C; b. BOC-ON, TEA, aq. acetone; c. MsCl, pyr, CH_2Cl_2, 0°C; d. NaN_3, DMF; e. 1N NaOH, MeOH, 60°C; *p*-nitrophenol, DCC, EtOAc; f. H_2, Pd-C, EtOH; g. 2, TEA, DMF; h. TFA, CH_2Cl_2.



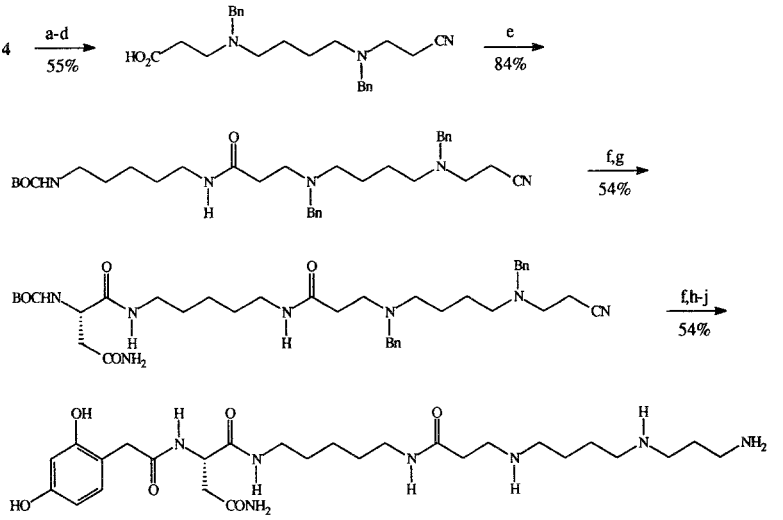
NPTX-10

Scheme. 8. Miyashita, 1992 (ref. 208). a. H_2, Pd-C, EtOH; b. Z-Arg(Z_2)-ONSu, TEA, DMF; c. TFA.

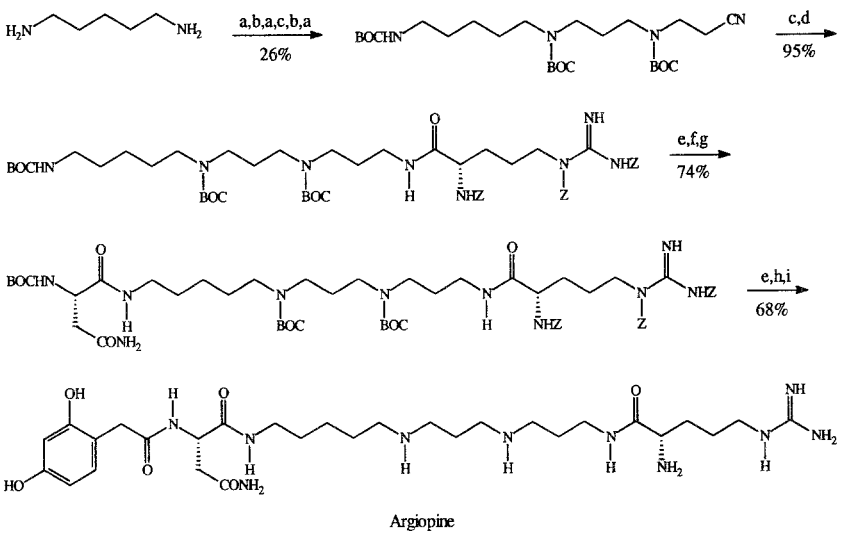


NSTX-3

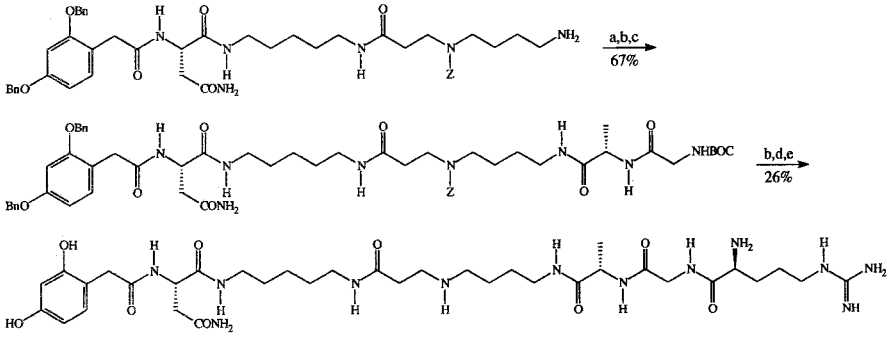
Scheme. 9. Nason, 1989 (ref. 203). a. DEC, HOBt, TEA, $H_2N(CH_2)_5NHBOC$; b. Pd-C, EtOH, cyclohexene; c. 2,4-dibenzyloxyphenyl acetyl-OSu, DMF; d, HCl, dioxane; e. CH_2:CHCN; f. $PhthN(CH_2)_4Br$, KF- celite, CH_3CN, Δ; g. 6N HCl; h. Z-Arg(Z_2)-ONSu, DMF; i. DEC, HOBt, TEA, DMF, 3; j. H_2, Pd-C, HOAc; k. Amberlite CG-50, MeOH, HCl.



SCHEME. 10. Nason, 1989 (ref. 203). a. H_2NNH_2, MeOH; b. CH_2:$CHCO_2Et$; c. BnBr, KF-celite, CH_3CN, 70°C; d. NaOH; e. $BOCHN(CH_2)_5NH_2$, DEC, HOBt, $CHCl_3$; f. TFA, CH_2Cl_2; g. N-α-BOC-L-Asp-O*p*NP, $CHCl_3$; h. 2,4-dibenzyloxyphenyl acetyl-OSu, DMF; i. H_2, Pd-C, HOAc; j. Amberlite CG-50, MeOH, HCl.

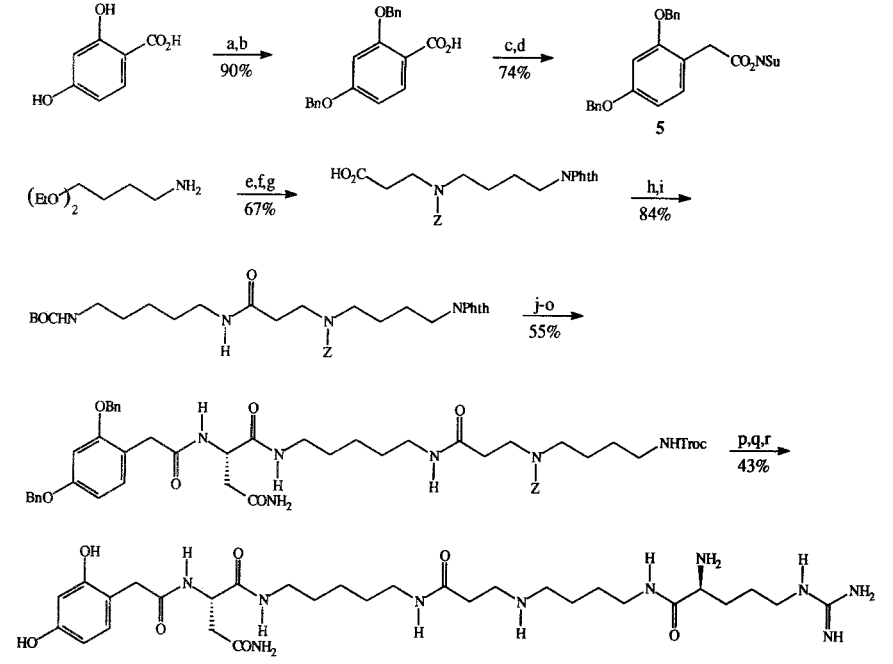


SCHEME. 11. Shih, 1987 (ref. 210). a. BOC-ON; b. CH_2:CHCN; c. RaNi, NH_3, EtOH; d. Z-Arg(Z_2)-O*p*NP, THF; e. TFA; f. TEA, BOC-Asn-O*p*NP; g. Z-Cl; h. TEA, 2,4-dibenzyloxyphenyl acetyl-O*p*NP; i. HF, anisole, −5°C.



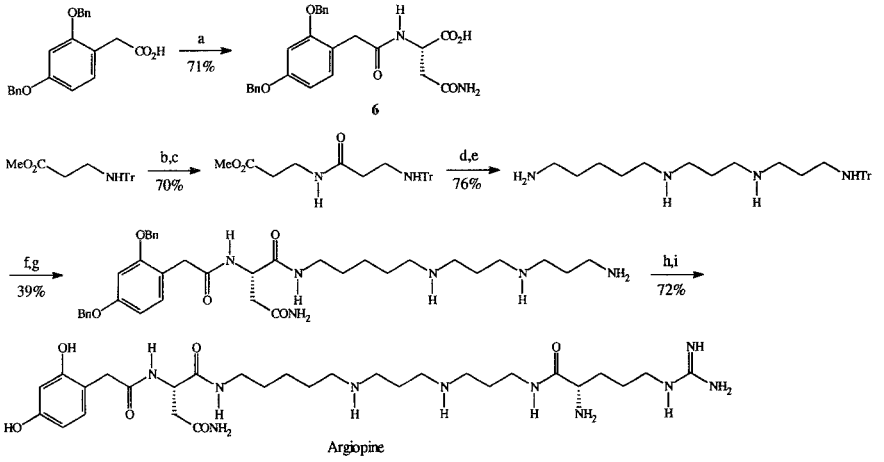
Clavamine

SCHEME. 12. Teshima, 1990 (ref. 209). a. BOC-Ala-OSu, TEA, DMF; b. TFA; c. BOC-Gly-OSu, TEA, DMF; d. Z-Arg(Z_2)-OH, THF, *i*-Bu-OCOCl, TEA; e. CF_3SO_3H, TFA, *m*-cresol, thioanisole.

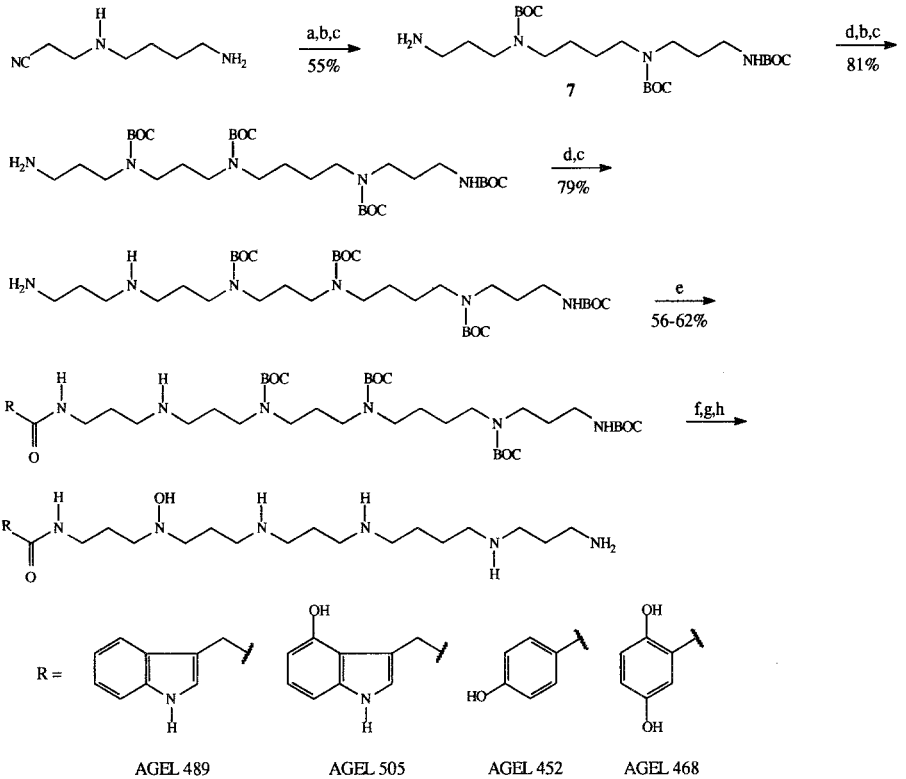


NSTX-3

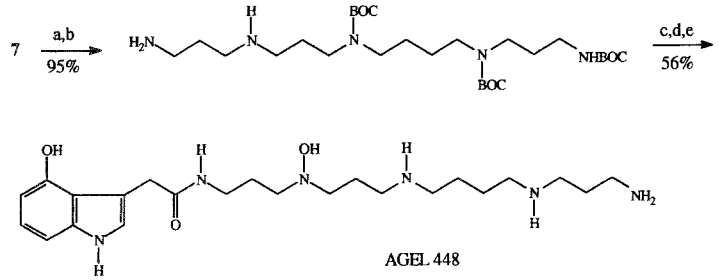
SCHEME. 13. Teshima, 1991 (refs. 202, 204, 205). a. BnBr, NaH, DMF; b. NaOH, dioxane; c. $(COCl)_2$, pyr, PhH; CH_2N_2, THF; d. $PhCO_2Ag$, HONSu, DMF; e. $PhthNCO_2Et$, TEA, THF; f. HCl, acetone; g. β-Ala, $NaBH_3CN$, MeOH, HOAc; h. Z-Cl, $NaHCO_3$; i. $BOCHN(CH_2)_5NH_2$, WSCl, HOBt; j. H_2NNH_2, EtOH; k. Troc-ONSu, TEA, EtOAc; l. TFA; m. BOC-Asn-O*p*NP, TEA, DMF; n. 5, TEA, DMF; o. Zn, HOAc; p. Z-Arg(Z_2)-OH, *i*-Bu-OCOCl, TEA; q. H_2, Pd-C.



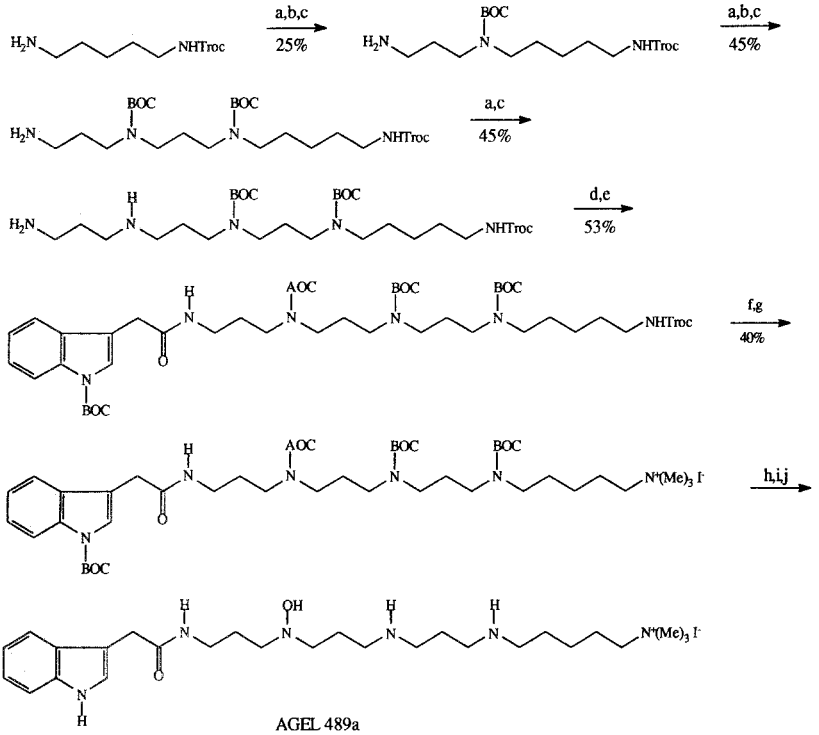
SCHEME. 14. Yelin, 1988 (ref. 212). a. DCC, HO-NSu, L-Asp; b. aq NaOH; c. DCC, HO-NSu, β-Ala; d. $H_2N(CH_2)_5NH_2$; e. $LiAlH_4$, THF, Δ; f. 6, DCC, HOBt; g. TFA; h. Z-Arg(Z_2)-ONSu; i. H_2, Pd-C, MeOH, 0.1% TFA.



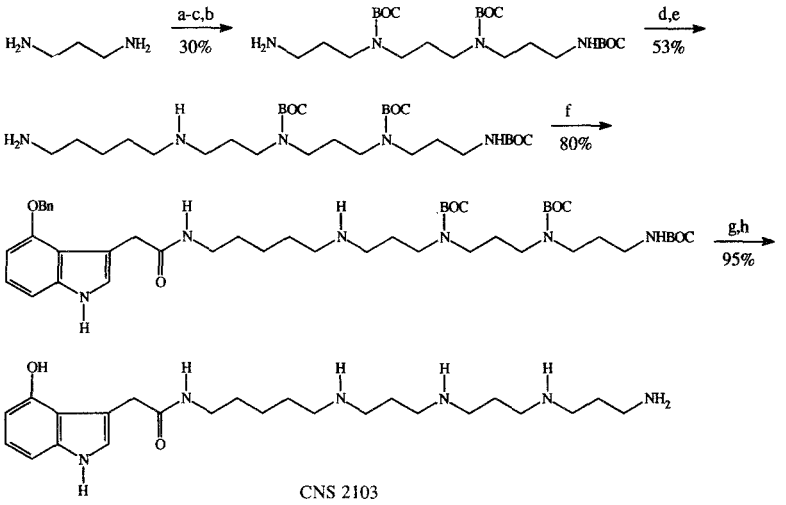
SCHEME. 15. Jasys, 1990a (ref. 170). a. $BOCHN(CH_2)_3Br$, KF-celite, CH_3CN, 70°C; b. $(BOC)_2O$, CH_2Cl_2; c. H_2 (50 psi), $Pd(OH)_2$-C, HOAc; d. CH_2:CHCN; e. R-CO_2H, DCC, NHS, CH_2Cl_2; f. MCPBA (or dioxirane or Davis reagent); g. $NaCNBH_3$; h. TFA or HCl, dioxane.



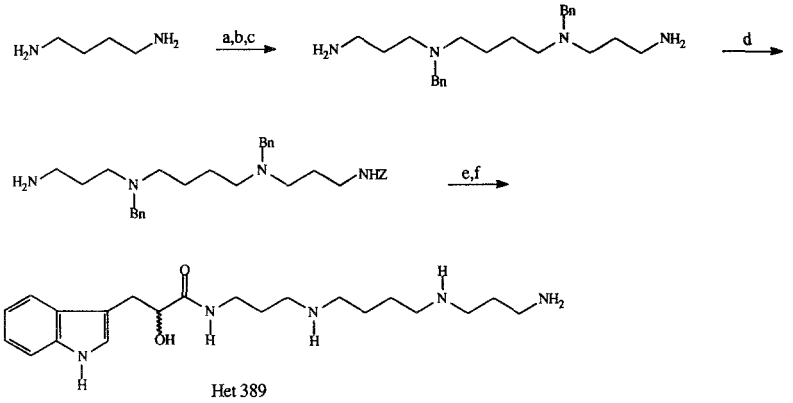
Scheme. 16. Jasys, 1990 (ref. 170). a. CH_2:CHCN; b. H_2 (50 psi), $Pd(OH)_2$-C, HOAc; c. N-BOC-4-MOMO-indole-3-acetic acid, DCC, NHS, CH_2Cl_2; d. MCPBA (or dioxirane or Davis reagent); e. HCl, dioxane.



Scheme. 17. Jasys, 1992 (ref. 176). a. $PhthN(CH_2)_3Br$, KF-celite, 40°C; b. $(BOC)_2O$, CH_2Cl_2; c. H_2NNH_2, MeOH, 50°C; d. BOC-indole-3-acetic acid, DCC, NHS, CH_2Cl_2; e. CH_2:$CHCH_2OCOCl$, DMAP; f. Zn, NH_4OAc , THF; g. MeI, KF-celite; h. $(Ph_3P)_4Pd$, PPh_3, HOAc; i. 2-(phenylsulfonyl)-3-phenloxaziridine; j. TFA.



SCHEME. 18. McCormick, 1993 (ref. 6). a. CH_2:CHCN, MeOH; b. $(BOC)_2O$, CH_2Cl_2; c. $LiAlH_4$, Et_2O; d. $PhthN(CH_2)_5Br$, KF-celite, CH_3CN, Δ; e. H_2NNH_2, MeOH; f. 4-BnO-indole-3-acetyl-O*p*NP, DMF; g. H_2, $Pd(OH)_2$-C, MeOH, H_2O; h. TFA or HCl, MeOH.



SCHEME. 19. Skinner, 1990 (ref. 32). a. CH_2:CHCN; b. BnI; c. $LiAlH_4$, $AlCl_3$; d. Z-Cl, KOH, DMSO; e. (±)-indole-3-lactic acid, DCC, HOBt, DMF; f. H_2, Pd-C, HOAc, H_2O.

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SOIL TRANSFORMATION OF WHEAT AND CORN METABOLITES MBOA AND DIM_2BOA INTO AMINOPHENOXAZINONES

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Abstract—The defensive cyclic hydroxamates 7-methoxy-2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIMBOA) and 7,8-dimethoxy-2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIM_2BOA) of wheat and corn are transformed in nonsterile soil, via 6-methoxy-2(3H)-benzoxazolone (MBOA) and 6,7-dimethoxy-2(3H)-benzoxazolone (M_2BOA) respectively, into 2-amino-7-methoxy-3H-phenoxazin-3-one and 2-amino-4,6,7-trimethoxy-3H-phenoxazin-3-one. The soil transformation is similar of that undergone by the rye metabolite 2(3H)-benzoxazolone (BOA) into 2-amino-3H-phenoxazin-3-one. The transformations to phenoxazinones are not observed in sterile soil. The 2-amino-3H-phenoxazin-3-one inhibits barnyard grass radicle elongation, but the methoxylated aminophenoxazinones are not significantly inhibitory.

Key Words—DIMBOA, MBOA, DIM_2BOA, M_2BOA, 2-amino-7-methoxy-3H-phenoxazin-3-one, 2-amino-4,6,7-trimethoxy-3H-phenoxazin-3-one, corn, wheat, soil microbial transformation, phytotoxicity.

INTRODUCTION

Rye, wheat, and corn produce cyclic hydroxamic acids that have antifungal, antibacterial, and antiinsect activity important to the defense of these plants (Niemeyer, 1988; Xie et al., 1992). Rye contains only DIBOA (Figure 1, **1a**), while wheat contains the methoxylated DIMBOA **(1b)** and corn contains both DIMBOA and the dimethoxylated DIM_2BOA **(1c).** These defensive hydroxa-

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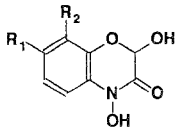
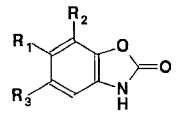
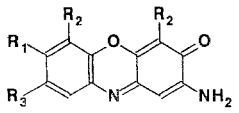


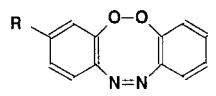
1a, DIBOA: $R_1 = R_2 = H$
1b, DIMBOA: $R_1 = CH_3O$, $R_2 = H$
1c, DIM_2BOA: $R_1 = R_2 = CH_3O$



2a, BOA: $R_1 = R_2 = R_3 = H$
2b, MBOA: $R_1 = CH_3O$, $R_2 = R_3 = H$
2c, M_2BOA: $R_1 = R_2 = CH_3O$, $R_3 = H$
2d: $R_1 = R_2 = H$, $R_3 = Cl$



3a: $R_1 = R_2 = R_3 = H$
3b: $R_1 = CH_3O$, $R_2 = R_3 = H$
3c: $R_1 = R_2 = CH_3O$, $R_3 = H$
3d: $R_1 = R_2 = H$, $R_3 = Cl$



4a: R = H
4b: $R = CH_3O$

FIG. 1. Structures of cyclic hydroxamic acids and their decomposition products.

mates are stored as relatively nontoxic, stable glucosides that are released by a glucosidase when tissue is damaged. The toxic hydroxamates **1a–1c** are unstable, with a half-life of one day or less in aqueous solution (pH 5–7) at room temperature (Bredenberg et al., 1962; Woodward et al., 1978; Atkinson et al., 1991). The major decomposition products are the corresponding benzoxazolones BOA **(2a)**, MBOA **(2b)**, and M_2BOA **(2c)**, accompanied by unidentified pigments. The benzoxazolones are stable, but are an order of magnitude less toxic to plants, insects, fungi, and bacteria than the hydroxamic acids. However, BOA has weak phytotoxicity (Wolf et al., 1985) and may be involved in weed suppression by rye cover crops (Barnes and Putnam, 1987).

It has been shown that BOA is converted in the soil by microorganisms into a more phytotoxic red pigment that may also play a role in rye allelopathy (Nair et al., 1990). We have recently demonstrated that nonsterile North Carolina soil converts BOA into the red pigment 2-amino-3H-phenoxazin-3-one **(3a)** (Gagliardo and Chilton, 1992), not the isomeric 2,2′-oxo-1,1′-azobenzene **(4a)** reported from Michigan soil (Nair et al., 1990). We now report that the methoxylated MBOA and M_2BOA of wheat and corn are also converted into methoxylated aminophenoxazinones in nonsterile soil.

METHODS AND MATERIALS

General. Analytical TLC was performed on E. Merck DC-Alufolien, Kieselgel 60 F-254 (0.2 mm). Preparative TLC was performed on 0.5-mm plates. UV spectra were obtained on a Perkin-Elmer Lambda 48 UV/VIS spectrophotometer. NMR was measured on a 300-mHz GE Omega spectrometer. Mass spectra were obtained on a Hewlett-Packard 5985-B mass spectrometer.

Preparation of Intermediates. 5-Methoxy-2-nitrosophenol was prepared by nitrosation of 3-methoxyphenol (Hodgson and Clay, 1929). 2,3-Dimethoxy-6-nitrophenol, prepared by nitration of 2,3-dimethoxyphenol (Baker and Smith, 1931), was used to synthesize DIM_2BOA as previously reported (Atkinson et al., 1991).

5-Chloro-2(3H)-benoxazolone ***(2d).*** A solution of 30 mmol 4-chloro-2-aminophenol in 100 ml anhydrous tetrahydrofuran was mixed with 45 mmol 1,1-carbonyldiimidazol. The solution was heated at reflux 4 hr. Solvent was removed in vacuo, and the residual solid was partitioned between 2 N hydrochloric acid and ethyl acetate. Evaporation of the dried ethyl acetate gave a solid that was crystallized from ethyl acetate–petroleum ether to give colorless needles, mp 191–192, lit mp 188 (Grafe and Liebenow, 1973). UV maxima in methanol: 283 nm (6000), 207 (26,000); ^1H NMR in $CDCl_3$: 6.92 ppm (H-6, dd, $J = 8$, 2), 6.94 (H-4, d, $J = 2$), 6.97 (H-7, d, $J = 8$), 11.2 (NH_2, broad). EI-MS *m/z*: 171/169 (100%, M^+), 115/113 (39), 78 (31).

Synthesis of Substituted 2-Amino-3H-phenoxazin-3-ones. Substituted nitrophenols were first reduced to aminophenols with sodium borohydride under nitrogen in the presence of 5% palladium on charcoal. Sodium dithionite was used to reduce 5-methoxy-2-nitrosophenol to 5-methoxy-2-aminophenol (Allen and Laird, 1971). The aminophenols were not isolated, but were immediately allowed to oxidize to aminophenoxazinones in the presence of air and palladium catalyst.

Palladium on charcoal (100–170 mg) was suspended in 14 ml water, and 0.02 mol sodium borohydride in 14 ml water was added. A stream of nitrogen was bubbled through the mixture, and a solution of 0.01 mol 5,6-dimethoxy-nitrophenol, *o*-nitrophenol, or 4-chloro-2-aminophenol in 40 ml methanol was added dropwise during 5 min. Nitrogen bubbling was stopped, and the mixture was stirred in the presence of air for 30 min for preparation of the methoxylated aminophenoxazinones or for 24 hr for the more slowly oxidized *o*-aminophenol and 4-chloro-2-aminophenol. The dark reaction mixture was filtered through celite. The celite was rinsed with fresh methanol. The pooled aqueous–methanolic solution was concentrated to remove most methanol, and the aqueous residue was extracted six times with ethyl acetate. The ethyl acetate was back-extracted with water, dried over sodium sulfate, and evaporated to yield a crude solid. Most of the crude, low-solubility solid was dissolved by repeated extrac-

tion with boiling acetone–ethyl acetate–methanol 7:2:1. Solvent was evaporated, and impurities in the crude aminophenoxazinones were removed by extraction with a limited amount of the same mixed solvent. The yields and melting points of the substituted 2-amino-3H-phenoxazin-3-ones were: **3b:** 29%, mp 268–270 (dec.), lit. mp 268–270 (dec.) (Buckley et al., 1982); **3c:** 32%, mp 217–218; **3d:** 24%, mp 280 (dec.). NMR and MS of these aminophenoxazinones are given in Tables 1 and 2.

Soil Transformation of MBOA, DIM_2BOA, and 5-Chlorobenzoxazolone. The same Cecil and Appling gravelly sandy loam was used for soil transformations as in the preceding study (Gagliardo and Chilton, 1992). MBOA (100 mg), DIM_2BOA (20 mg), or 5-chlorobenzoxazolone (100 mg) was mixed with 50–100 g nonsterile soil and 30 ml water. The mixture was incubated in the dark at room temperature. After 15 days or more the soil was extracted by stirring for several hours with two 100-ml portions of methanol. The residue of the pooled methanolic fraction was analyzed for the presence of starting materials and transformation products.

Radicle Elongation Assays. A filter paper circle (Whatman No. 1, 6 cm)

TABLE 1. PROTON NMR SPECTRA OF AMINOPHENOXAZINONES IN DMSO-d_6

	Aminophenoxazinone			
H#	3a	3b	3c	3d
1	6.34, s[a]	6.30, s[a]	6.24	6.34, s[a]
4	6.35, s[a]	6.32, s[a]		6.38, s[a]
6	7.55, m[b]	7.09, d, J = 2 Hz		7.54, d, J = 9 Hz
7	7.55, m[b]			7.46, dd, J = 9, 2 Hz
8	7.55, m[b]	6.99 dd, J = 8, 2 Hz	7.12, d, J = 9 Hz[a]	
9	7.75, d, J = 8 Hz[b]	7.62, d, J = 8 Hz	7.43, d, J = 9 Hz[a]	7.75, d, J = 2
NH_2	6.78, br s	6.55, br s	6.57, br s	7.03, v. broad
MeO		3.83	3.88, 3.89, 3.90	

[a,b] Assignments of proton resonances with the same letter are arbitrary.

TABLE 2. ELECTRON IMPACT MASS SPECTRA OF AMINOPHENOXAZINONES

Aminophenoxazinone	M^+	$[M-CH3]^+$	$[M-HCN]^+$	$[M-HCN, H]^+$
3a	212(100%)		185(50)	184(21)
3b	242(100)	227(78)	215(10)	214(2)
3c	302(100)	287(75)		
3d	248/246(100)		221/219(53)	220/218(27)

was wet evenly with methanol in which 15, 60, or 240 μg of test compound was dissolved. Filter paper wet with methanol was used as a control. The dried papers were placed in 60-mm Petri dishes, wet with 1.5 ml water, and 10 seeds of barnyard grass (*Echinocloa crus-galli* L.) were added. Radicle lengths were measured after incubation in the dark for 72 hr at ambient temperature. Radicle lengths were averaged for each Petri dish. Three replicate dishes were used for each compound at each concentration. The entire experiment was repeated four times (August, September, January, and April). The data were analyzed using SAS (1988) PROC GLM and an α value of 0.05.

RESULTS

Isolation and Identification of Soil Transformation Products: MBOA. Nonsterile soil containing 100 mg MBOA was incubated at room temperature for 19 days before extraction with methanol. Removal of solvent gave 34 mg of solid, which contained unreacted MBOA (R_f = 0.40, chloroform–methanol 95:5) and an orange pigment (R_f = 0.48). Preparative TLC on silica gel developed with chloroform methanol 95:5 gave 6 mg of amorphous, orange 2-amino-7-methoxy-3H-phenoxazin-3-one **(3b)** having NMR and mass spectra identical to those of synthetic **3b** (Table 1 and 2). The only other major product present was unreacted MBOA. Only MBOA and no phenoxazinone was recovered after incubation for 19 days in sterile soil.

Soil Transformation of DIM_2BOA. Incubation of 20 mg DIM_2BOA in nonsterile soil and another 20 mg in sterile soil was terminated at 28 days. The small amount of solid obtained on evaporation of methanol was examined by silica gel TLC. The R_fs of relevant standards in the solvent systems hexane–acetone 1:1 and toluene–ethylacetate 5:4 were: DIM_2BOA, 0.25, 0.09; M_2BOA, 0.55, 0.45; and 4,6,7-trimethoxy-2-amino-3H-phenoxazin-3-one, 0.49, 0.28. The major substance present in the extract of nonsterile soil comigrated with 4,6,7-trimethoxy-2-amino-3H-phenoxazin-3-one; a small amount of M_2BOA was also present. M_2BOA, but no aminotrimethoxyphenoxazinone was present in the extract of sterile soil. No DIM_2BOA was present in the extract of either sterile or nonsterile soil.

Soil Transformation of 5-Chlorobenzoxazolone ***(2d).*** Nonsterile soil containing 100 mg chlorobenzoxazolone was incubated 15 days before extraction with methanol. Removal of solvent gave 74 mg of dark orange solid still containing 5-chlorobenzoxazolone. The pigment in the solid was further purified by preparative TLC (hexane–ethyl acetate 7:3) followed by crystallization from methanol to give red needles of 2-amino-8-chloro-3H-phenoxazin-3-one **(3d).** The NMR and mass spectra of the soil transformation product were identical to those of synthetic **3d** (Tables 1 and 2). Only 5-chlorobenzoxazolone, and no **3d,** was recovered when the experiment was repeated with sterile soil.

Phytotoxicity. The effects of the four aminophenoxazinones **3a–3d** on radicle length of barnyard grass were measured at ambient temperature on four different dates (Figure 2). Differences in radicle length of controls on the four test dates were probably due largely to different ambient temperatures (21–25°C). The inhibitory effect of dose of 2-amino-3H-phenoxazin-3-one, **3a,** on radicle length was statistically significant on all four dates. The data were better fit by a quadratic than by a linear equation; however, the upturn of the curve cannot be considered meaningful without measurements at additional concentrations. Significant regressions are given in Table 3. No statistically significant effects were observed for the methoxy-and chlorosubstituted compounds **3b, 3c,** and **3d.**

DISCUSSION

We have recently shown that the chemical structure of the red soil transformation product of the rye metabolite BOA **(2a)** is 2-amino-3H-phenoxazin-3-one **(3a)** (Gagliardo and Chilton, 1992). None of the isomeric 2,2′-oxo-1,1′-

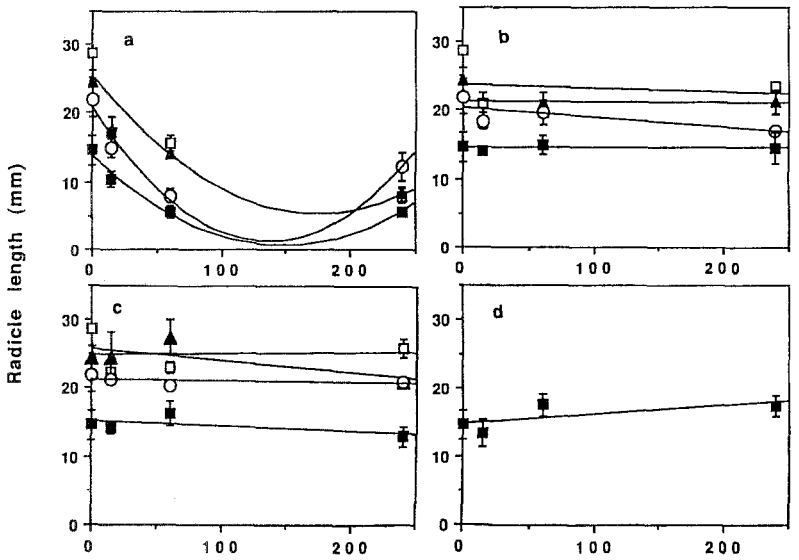


FIG. 2. Effect of aminophenoxazinones on radicle length of barnyard grass at ambient temperature on four different dates. Each datum is an average of three Petri dishes (10 seeds/dish). Standard error bars are shown. (a) 2-Amino-3H-phenoxazin-3-one **(3a)** fit to quadratic equations; quadratics for dates 1 (□) and 2 (△) coincide. (b) 2-Amino-7-methoxy-3H-phenoxazin-3-one **(3b).** (c) 2-Amino-4,6,7-trimethoxy-3H-phenoxazin-3-one **(3c).** (d) 2-Amino-8-chloro-3H-phenoxazin-3-one **(3d).** Data in panels b–d were fit by linear equations. Significant regressions are presented in Table 3.

TABLE 3. PARTIAL REGRESSION COEFFICIENTS AND P AND R^2 VALUES FOR SIGNIFICANT EFFECTS OF 2-AMINO-3H-PHENOXAZIN-3-ONE ON BARNYARD GRASS RADICLE LENGTH

Date	Intercept	Linear	Quadratic	P	R^2
1	25.27[a]	−0.2275	0.0007	0.0011	0.7786
2	22.63	−0.1908	0.0005	0.0001	0.8642
3	20.73	−0.2842	0.0010	0.0021	0.7465
4	13.89	−0.1802	0.0006	0.0011	0.7779

[a]Dependent variable in millimeters; independent variable in micrograms per 1.5 ml H_2O.

azobenzene **(4a)** previously reported from Michigan soil (Nair et al., 1990) was found in the North Carolina soil. Nair et al. extended their study to the analogous corn metabolite MBOA **(2b)** and found that nonsterile soil also transformed it into a red pigment having a mass spectral molecular weight of 242, to which the structure 4-methoxy-2,2′-oxo-1,1′-azobenzene **(4b)** was assigned in analogy to structure **4a** assigned to the transformation product of BOA isolated from Michigan soil. The proposed structure **4b** is essentially an oxidative dimer of 2-amino-5-methoxyphenol derived from MBOA, but requires the selective loss of one of two methoxyls. A microbial demethoxylation step was proposed to occur before dimerization.

We have prepared a red soil transformation product of MBOA and found it to be identical by melting point, TLC behavior, ultraviolet, NMR, and mass spectra to synthetic 7-methoxy-2-amino-3H-phenoxazin-3-one, **3b.** The reason for the selective loss of one of the two methoxyls in the oxidative dimerization is obvious. Loss of a substituent at the 5 or 6 position in the oxidative dimerization of substituted *o*-aminophenols is well-documented when the substituent is a good anionic leaving group such as a halide or methoxyl (von Auwers et al., 1924). A poor leaving group such as alkyl is known to block completion of aromatization of the oxidative dimer (Barry et al., 1989).

While only DIMBOA is found in wheat, the more methoxylated DIM_2BOA is found in appreciable quantities in some corn varieties and even equals DIMBOA in a few cases. We have shown that DIM_2BOA is transformed into the corresponding 2-amino-4,6,7-trimethoxy-3H-phenoxazin-3-one **(3c)** in soil. The cyclic hydroxamates DIBOA, DIMBOA, and DIM_2BOA all decompose spontaneously to benzoxazolones with half-lives of about one day at pH 5.6 and less at higher pH (Bredenberg et al., 1962; Woodward et al., 1978; Atkinson et al., 1991). It is probable that DIM_2BOA is converted into a phenoxazinone in the soil via M_2BOA. In sterile soil DIM_2BOA was converted to M_2BOA, but no phenoxazinone was found, indicating that at least one step in the conversion of benzoxazolones to phenoxazinones requires the presence of a microorganism.

It is likely that soil microorganisms are capable of hydrolyzing many other benzoxazolones to substituted aminophenols. The resulting substituted aminophenols are known to be oxidized in nonsterile soil to give phenoxazinones (Briggs and Walker, 1973). Not surprisingly, we find that the nonnatural 5-chlorobenzoxazolone **(2d)** is converted into 2-amino-8-chloro-3H-phenoxazin-3-one **(3d)** in nonsterile soil. Similarly, the 6-chlorobenzoxazolone-containing insecticide phosalone has been found to be converted in soil into the isomeric 2-amino-7-chloro-3H-phenoxazin-3-one (Ambrosi et al., 1977; Golovleva et al., 1983).

Potential phytotoxicity of the three aminophenoxazinones **(3a–3c)** derivable from the major cyclic hydroxamic acids of corn, wheat and rye was evaluated using a standard method for measuring inhibition of radicle length (Nair et al., 1990; Gagliardo and Chilton, 1992). Unsubstituted aminophenoxazinone significantly inhibited barnyard grass radicle length as previously reported. However, the two methoxylated aminophenoxazinones derived from DIMBOA and DIM_2BOA have no effect on barnyard grass radicle length over the concentration range measured. Microscopic examination of the Petri dishes in which the assays were performed revealed the presence of very tiny red crystals within the matrix of the wet filter paper for all four aminophenoxazinones at all but the lowest concentration tested. This indicates that the higher amounts added (60 and 240 μg) exceed the solubility of these compounds in water. Thus, solubility in water limits the concentrations that a seedling will experience. The actual concentration in the soil is likely to be further lowered by partitioning between water and soil-adsorbed phases. It is doubtful that the soil transformation products **3b** and **3c** of the corn and wheat hydroxamic acids DIMBOA and DIM_2BOA play any major allelopathic role against barnyard grass in the soil.

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DETERRENT AND INSECTICIDAL EFFECTS OF EXTRACTS OF PITHRAJ, *Aphanamixis polystachya* (MELIACEAE), AGAINST *Tribolium castaneum* IN STORAGE

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Abstract—Laboratory experiments were carried out to investigate the efficacy of seed extracts of pithraj, *Aphanamixis polystachya* (Wall & Parker), a locally grown plant in Bangladesh, against the red flour beetle, *Tribolium castaneum* (Herbst.). Results of three different bioassays showed that crude extracts of pithraj seeds have strong repellent effects and moderate feeding deterrent and insecticidal (direct-contact) effects on adult *Tribolium castaneum*.

Key Words—Pithraj, *Aphanamixis polystachya*, Meliaceae, repellent, feeding deterrent, *Tribolium castaneum*, red flour beetle, Coleoptera, Tenebrionidae.

INTRODUCTION

To alleviate insect pest problems in storage, synthetic pesticides are generally recommended. These may have drawbacks, including toxicity to nontarget organisms, development of pest resistance, and environmental pollution. Hence, there is a worldwide interest in the development of alternative strategies, including the use of new types of insecticides derived from a reevaluation of age-old, traditional botanical pest control agents (Heyde et al., 1984). In the rural areas of South Asia, including Bangladesh, farmers traditionally mix leaves, bark, seeds, roots, or oils of certain plants with stored grains to keep them free from insect attacks. Such techniques have been inherited as part of the traditional culture (Saxena et al., 1988). However, few studies have been conducted in Bangladesh on the traditional use of botanicals against stored products pests

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(Anonymous, 1985; Islam, 1984, 1987; Islam, 1985; Khanam et al., 1990, 1991). The present study was undertaken with a locally grown plant, pithraj, *Aphanamixis polystachya* Wall & Parker (synonym *Amoora ruhituka* Wright & Arn.), used traditionally by farmers to protect stored products from insect attacks. We tested pithraj against the red flour beetle, *Tribolium castaneum* (Herbst), a major stored products pest in Bangladesh, to determine the repellent, feeding deterrent, and insecticidal action of the seed extracts.

METHODS AND MATERIALS

The seeds of pithraj, *Aphanamixis polystachya* (Meliaceae), used in this study, were collected from Bangladesh, and the red flour beetle, *Tribolium castaneum*, was obtained from the Blades Biological Company, Kent, England. The beetle was reared in glass jars (14 × 10.5 × 30 cm) on a diet of wholemeal wheat flour mixed with brewer's yeast in a 19:1 ratio. The cultures were started with 100–120 adult beetles of each sex. Five pairs of adults about 5–6 days old were placed in 13.9 × 2.8-cm Petri dishes containing the rearing media. The dishes were sealed and a maximum of seven days were allowed for mating and oviposition. Then the parent stocks were removed and the egg-containing media were transferred to media in the breeding containers. The jars were covered with pieces of cloth fastened with rubber bands to prevent contamination and the escape of insects. The experiments were conducted in the laboratory of the Department of Biology, University of Southampton, England. The rearing and experimental conditions were 12-hr light and dark periods at 27 ± 2°C and 70 ± 1% relative humidity.

Extraction

Seeds of *Aphanamixis polystachya* were air-dried, ground, and then extracted in a Soxhlet apparatus separately with petroleum ether (at 55°C), acetone (at 65°C), and 95% ethanol (at 80°C), for 6 hr in each case. The petroleum ether and acetone solvents were evaporated in a rotary vacuum evaporator at 40°C under reduced pressure yielding the petroleum ether (PSE) and acetone (ASE) extracts. The ethanol solvent was completely evaporated, and the extract was redissolved in petroleum ether solution, stirred for 20 min and then filtered. From the filtered solution, solvent was evaporated to obtain the ethanol extract (ESE).

Insect Responses to Plant Extracts

Repellency Tests Using Filter Papers. Repellency was tested by Standard Method Number 3 described by McDonald et al. (1970), with some modifications. Substrata were prepared by cutting in half Whatman No. 40 filter-paper disks (8 cm diam.), dipping them in the 0.5% and 1.0% solutions of pithraj

extracts for 1 min, and then air-drying them overnight at room temperature. Each treated disk half was then attached lengthwise, edge-to-edge, to an untreated disk half with cellulose tape and placed in a Petri dish. The orientation of the seam was changed in each replicate to avoid the effects of any external directioned stimulus affecting distribution of the test insects. Ten adults (6–7 days old) were released in the middle of each filter-paper circle and a cover was placed on the Petri dish. For each concentration of plant extract, four replications were made. Individuals that settled on each filter paper disk half were counted at hourly intervals for 5 hr. The average of the counts was converted to express the percentage repulsion (R) as follows:

$$R = 2 \times (C - 50)$$

where C is the percentage of insects on the untreated half. Positive values expressed repellency and negative values attractancy. Data were analyzed using analysis of variance after correction of the percentage repulsion by the use of Abbott's (1987) formula and transformation into arcsin $\sqrt{}$percentage values. The averages were then assigned to different classes using the following scale:

Class	Percent repulsion
0	>0.01 to <0.1
I	0.1–20
II	20.1–40
III	40.1–60
IV	60.1–80
V	80.1–100

Feeding Deterrence Test. The potency of the feeding deterrence effect of pithraj seed extracts against *Tribolium castaneum* was determined by using the wheat wafer disk bioassay described by Nawrot et al. (1986). Wheat wafer disks (4.1 cm diam.) obtained from the Poor St. Claire's Convent, Southampton, England, were used as the test food. The disks were saturated by dipping in either solvent only (control disks K) or in 1.0% solution of PSE, ASE, or ESE (treated disks E). The disks were then air-dried for 30 min and weighed before presenting them to the 10 adults (6–7 days old) over the following five-day period, during which they were the sole food source.

Feeding of the insects was recorded under three conditions: (1) on pure food, composed of two control (KK) disks, (2) on food with a choice between one treated disk (E) and one control disk (K) (choice test), and (3) on food with two treated disks (EE) (no-choice test). Each treatment was replicated five times. After the completion of the experiment, the disks were reweighed and, based

on the amount of food consumed in control (KK), choice (KE), and no-choice (EE) test, three coefficients for feeding deterrent activity were calculated as follows:

1. Absolute deterrence coefficient:

$$A = (KK - EE/KK + EE) \times 100$$

2. Relative deterrence coefficient:

$$R = (K - E/K + E) \times 100$$

3. Total deterrence coefficient:

$$T = A + R$$

Values of the total deterrence coefficient served as an index of feeding deterrence activity expressed on a scale between 0 and 200. The index zero (0) was an inert compound and 200 a highly deterrent compound. Feeding deterrents having an index of 150–200 were marked ++++; 100–150, +++; 50–100, ++, and 0–50 +. Data were analyzed by analysis of variance.

Topical Application. Contact toxicity of pithraj extracts was tested by topical treatment, using Standard Method Number 1 described by McDonald et al. (1970) with slight modifications. Stock solutions were prepared by dissolving 100 mg of PSE, ASE, or ESE extract in 1 ml of either petroleum ether or acetone solvent. Lower concentrations (60, 40, 20, and 10 mg/ml) were obtained by dilution of the stock solution with the solvent. Insects were chilled for a period of 10 min, and then the immobilized insects were picked up individually with a small suction tube. Using a capillary tube, 1 μl of the extract solution (100, 60, 40, 20, or 10 μg per insect) was applied to the dorsum of each insect. Fifty unsexed insects, in five replicates of 10 each, were treated at each dose. Control insects were treated with solvent. Treated and control insects were transferred to Petri dishes (10 insects/dish). The insects were examined daily, and those that did not move or respond to gentle touch were considered dead. Dead insects were recorded at 24, 48, and 72 hr after treatment and corrected mortality rates were calculated using the formula of Abbott (1987). Concentration–mortality data were analyzed by probit analysis (Finney, 1971).

RESULTS

Repellency Effects

All three extracts of pithraj strongly repelled the red flour beetle (Table 1). ASE was the most repellent (88 and 93% repellency at 0.5% and 1.0% concentration, respectively) to the beetle, although the repulsion differences were

not statistically significant ($P < 0.05$). Although repellency varied with concentration, all extracts belonged to class V repellency. In all cases, repellency decreased with time. The rapid drop in repellency could be attributed to the closed condition of the test arena in the Petri dishes, leading to saturation of the test arena with volatiles from the pithraj seed extracts.

Feeding Deterrence Effects

All three pithraj extracts inhibited the feeding activity of *T. castaneum* (Table 2); ASE was the most deterrent, while ESE was the least effective. There were no significant differences among feeding deterrent activities of the PSE,

TABLE 1. AVERAGE REPELLENCY OF PITHRAJ SEED EXTRACT TO *T. castaneum* ADULTS USING TREATED FILTER PAPER TEST[a]

Extract	Conc of extract	Average repellency (%) at hours after treatment					Mean	Repellency class
		1	2	3	4	5		
Petroleum ether	0.5%	95	95	95	75	60	84	V
	1.0%	95	85	95	70	60	81	V
Acetone	0.5%	100	85	95	85	75	88	V
	1.0%	85	95	100	95	90	93	V
Ethanol	0.5%	95	95	90	80	70	86	V
	1.0%	80	80	90	85	90	85	V
F value		NS[b]	NS	NS	NS	NS	NS	

[a]Original data were transformed into arcs in $\sqrt{}$ percentage values during ANOVA test.
[b]NS = not significant.

TABLE 2. FEEDING DETERRENT ACTIVITY COEFFICIENTS OF PITHRAJ SEED EXTRACTS AGAINST *T. castaneum* ADULTS[a]

Extract	Coefficient of deterrency			Efficacy of Extract
	Absolute	Relative	Total	
Petroleum ether	−9.98	73.10	63.12	+ +
Acetone	16.92	71.07	87.99	+ +
Ethanol	−12.91	61.73	48.82	+
F value	NS[b]	NS	NS	

[a]Original data were transformed into arcs in $\sqrt{}$ percentage values during ANOVA test.
[b]NS = not significant.

ASE, and ESE extracts, but PSE and ASE were ranked more repellent (++) than ESE (+) by the method used.

Topical Application

The pithraj seed extracts were moderately toxic to *T. castaneum*; 42–55% adults died within 72 hr of treatment at a dose of 100 μg/insect (Table 3). ESE applied topically was more toxic than ASE or PSE. At lower doses, the extracts showed no direct contact toxicity. Probit analysis showed that ESE was most toxic to *T. castaneum*, whose LD_{50} was 90 μg/insect, whereas it was 234 μg/insect in the case of ASE (Table 4).

Figure 1 gives the regression lines $y = 2.38 + 1.28x$ for PSE, $y = 1.92 + 1.30x$ for ASE, and $y = 0.95 + 2.07x$ for ESE, which were calculated by probit analysis (Finney, 1971). The steepness of the slopes indicated that *Tribolium castaneum* adults are moderately susceptible to pithraj seed extracts.

TABLE 3. TOXICITY OF PITHRAJ SEED EXTRACTS APPLIED TOPICALLY TO *T. castaneum* ADULTS[a]

Extract	Dose (μg/insect)	% average mortality at hours after treatment ± SE[b]		
		24	48	72
Petroleum ether	10	6 ± 0.8 b[c]	7 ± 1.1 b	11 ± 1.1 b
	20	14 ± 1.3 ab	12 ± 1.3 b	14 ± 1.9 b
	40	14 ± 1.3 ab	23 ± 2.1 ab	29 ± 2.0 ab
	60	18 ± 1.8 ab	25 ± 2.2 ab	35 ± 1.6 ab
	100	34 ± 3.7 a	45 ± 4.3 a	49 ± 3.5 a
Acetone	10	4 ± 0.8 b	6 ± 0.8 b	7 ± 1.1 b
	20	4 ± 0.8 b	6 ± 1.3 b	9 ± 0.9 b
	40	6 ± 1.3 b	8 ± 1.8 b	11 ± 1.5 b
	60	10 ± 1.7 b	10 ± 1.7 b	13 ± 1.6 b
	100	38 ± 2.7 a	40 ± 2.8 a	43 ± 3.3 a
Ethanol	10	4 ± 0.8 c	4 ± 0.8 c	5 ± 0.6 c
	20	6 ± 0.8 c	8 ± 0.6 c	9 ± 0.9 c
	40	6 ± 0.8 c	10 ± 1.0 c	10 ± 0.6 c
	60	34 ± 1.3 ab	42 ± 1.2 ab	50 ± 0.8 ab
	100	42 ± 3.1 a	52 ± 3.7 a	55 ± 3.6 a

[a] Original data corrected by Abbott's (1987) formula and then transformed into arcs in $\sqrt{}$ percentage values before ANOVA and DMRT (Duncan, 1951) test.
[b] SE = standard error of mean.
[c] Values followed by the same letter within a column are not significantly different at the 0.05 level by DMRT (Duncan, 1951).

TABLE 4. PROBIT ANALYSIS FOR CONTACT TOXICITY AT 72 H AFTER TOPICAL APPLICATION OF PITHRAJ EXTRACTS TO *T. castaneum* ADULTS[a]

Extract	Insects (*N*)	LD_{50} (μg/insect)	95% fiducial limit	Slope ± SE[b]
Petroleum ether	250	113.0	67.0 – 190.0	1.28 ± 0.27
Acetone	250	234.0	115.0 – 472.0	1.30 ± 0.32
Ethanol	250	90.0	42.0 – 192.0	2.07 ± 0.33

[a]Values were based on five replicates of 10 insects each.
[b]SE = standard error.

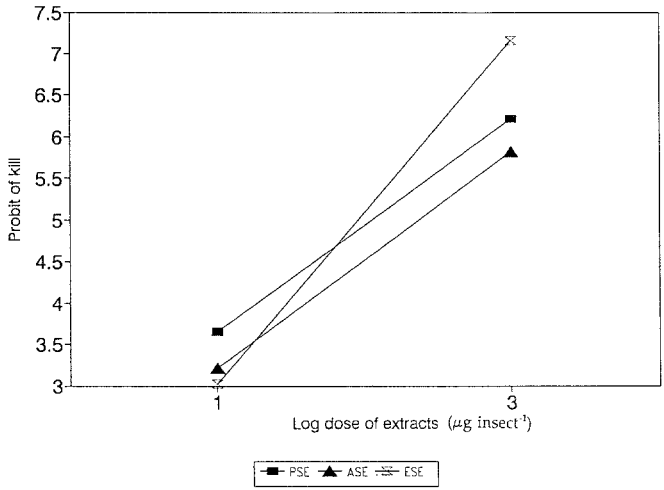

FIG. 1. Relationship of probit kill of *T. castaneum* adults to log concentration of pithraj seed extracts. At least 50 insects were tested for each concentration.

DISCUSSION

Repellents, feeding deterrents, and insecticides of natural origin are rational alternatives to synthetic insecticides. These compounds are naturally produced in many plants and play a vital role as resistance factors in plant defense systems. Crude extracts of pithraj seeds have been shown here to have strong repellency, moderate feeding deterrence, and some measure of contact toxicity to *Tribolium castaneum*. Nawrot et al. (1986) reported that among the stored grain pests, *Tribolium* spp. react more strongly to antifeedants and the adults were more sensitive than the larvae. Islam (1984) reported weak feeding deterrent effects of *A. polystachya* (referred to as *Amoora ruhituka* Wright & Arn.) on *Dicladispa*

armigera and repellent effects on *Sitotroga cerealella* and *Spilosoma obliqua*. Islam (1985) found that seed extracts of pithraj (*Amoora ruhituka* Wright & Arn.) also deterred feeding by *Epilachna 12-punctata*. Islam (1987) gives data on the mortality effects of seed extracts of *A. polystachya* (referred to as *Amoora ruhituka*) on the larvae of *Callosobruchus chinensis*. Khanam et al. (1990, 1991) reported that seed coat extracts of pithraj (*A. polystachya*) had a deleterious effect on the growth and development of *Tribolium confusum* and, in another experiment, they found that pithraj seed extracts were toxic to the adults of *Sitophilus oryzae*. In the present experiment, the rapid decrease of repellency with time may be explained by the findings of Jilani and Saxena (1990), who found that the repellency of compounds with low molecular weights and high volatility decreased rapidly over time. The present results also demonstrate the lower extraction capability of petroleum ether, acetone, and ethanol from pithraj seeds. The present study has shown that pithraj seed extracts have strong repellent but moderate feeding deterrent and direct-contact effects on *T. castaneum* adults. The study also confirms the validity of traditional use of pithraj against stored-grain pests.

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TOXICITY OF LINEAR FURANOCOUMARINS TO *Spodoptera exigua*: EVIDENCE FOR ANTAGONISTIC INTERACTIONS

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Abstract—The linear furanocoumarins psoralen, bergapten, and xanthotoxin were tested for toxicity to the beet armyworm *Spodoptera exigua* (Hübner) under short ultraviolet (UVB) radiation. Increased dietary concentrations of each furanocoumarin significantly decreased insect larval weight, extended generation time, and induced higher mortality. Xanthotoxin was the most toxic, followed by psoralen and bergapten. Combining psoralen with bergapten, xanthotoxin, or both resulted in significantly antagonistic effects on insect mortality. The combination of bergapten and xanthotoxin, however, produced additive effects. The implications of these observations for *S. exigua* resistance in the wild plant accession of *Apium prostratum* and the enigma the findings represent for plant-insect relationships are discussed.

Key Words—*Spodoptera exigua*, Lepidoptera, Noctuidae, psoralen, 5-methoxypsoralen, 8-methoxypsoralen, furanocoumarins, antagonistic toxicity, plant–insect interactions, *Apium prostratum*.

INTRODUCTION

The linear furanocoumarins are plant secondary compounds that have been isolated from members in a number of plant families including Rutaceae, Apiaceae, Compositae, Leguminosae, Moraceae, Pittosporaceae, Solanaceae, and Thy-

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meaceae (Scott et al., 1976; Murray et al., 1982). These compounds are photosensitizers (Igali et al., 1970; Zangerl and Berenbaum, 1987) and have shown toxicity against a broad spectrum of animals (Berenbaum, 1978; Berenbaum and Neal, 1985; Trumble et al., 1991). Because of this toxicity, it has been suggested that the linear furanocoumarins can play an important role in plant–herbivore interactions (Berenbaum, 1978; Berenbaum and Feeny, 1981; Trumble et al., 1991).

The major linear furanocoumarins isolated from *Apium* species are psoralen, bergapten (5-methoxypsoralen), and xanthotoxin (8-methoxypsoralen) (Trumble et al., 1990). These compounds were toxic to the beet armyworm *Spodoptera exigua* (Hübner) when combined in artificial diet (psoralen 15 μg/ml diet + bergapten 38 μg/ml diet + xanthotoxin 82 μg/ml diet) during assays exposed to 350 $\mu W/cm^2$ UVB radiation (Trumble et al., 1991). Diawara et al. (1992) reported higher concentrations of these furanocoumarins in the *S. exigua*-resistant wild *Apium prostratum* ssp. *prostratum* than the susceptible celery *A. graveolens*. However, *A. prostratum* resistance was primarily nonpreference based, and additional studies designed to test furanocoumarin-free plant extracts for insect preference suggested that the resistance was not only furanocoumarin-induced. Subsequent studies found no significant correlation between *S. exigua* resistance in celery breeding lines and linear furanocoumarin concentrations (Diawara et al., 1993). Therefore, the potential role of these linear furanocoumarins in the *A. prostratum–S. exigua* relationship is not clearly defined. We initiated this study to compare three linear furanocoumarins (psoralen, bergapten, and xanthotoxin) for toxicity against *S. exigua* and to determine the additive, synergistic, or antagonistic effect of their combinations. Information on the relative toxicity of these different linear furanocoumarins to herbivores, when ingested alone or in combination, will improve our understanding of the role of these compounds in plant–herbivore interactions.

METHODS AND MATERIALS

The three linear furanocoumarins psoralen, bergapten, and xanthotoxin were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). *S. exigua* larvae were from a colony less than 1 year old maintained on artificial diet (Shorey and Hale, 1965) at 27 $\pm$ 2°C and 16:8 hr light–dark photoperiod. Activity of the three linear furanocoumarins against *S. exigua* was determined in a two-phase experiment conducted between April 1991 and May 1992.

Experiment I: Toxicity of Individual Furanocoumarins. Seven concentrations of each of the three linear furanocoumarins were tested: 0 (control), 62.5, 125, 250, 375, 500, and 750 μg/g diet. This range, which was chosen following pilot studies of toxicity, includes the total concentration range of 0 to 406

μg/g fresh leaf found in *Apium* accessions for the three furanocoumarins (Trumble et al., 1990; Diawara et al., 1992). Procedures for incorporating the chemicals in insect diet were modified from Chan et al. (1978). For each concentration, the furanocoumarin was dissolved in 10 g of acetone, which was removed by vacuum after adsorbtion of the furanocoumarin onto 3 g of alphacel. Approximately 82 g of diet medium was then added to the alphacel, which had been diluted in 15 g of warm dH_2O, to bring the total weight to 100 g (alphacel constituted 3% of the diet medium). The mixture was blended for 5 min and then dispensed into 30-ml clear plastic cups.

Diets were allowed to cool at ambient temperature and one neonate *S. exigua* was placed in each cup. Cups were then covered with Teflon FEP Fluorocarbon film (E.I. DuPont de Nemours & Co., Wilmington, Delaware). A total of 15 larvae per chemical per concentration were evaluated. Cups were arranged in a randomized complete block design in an environmental chamber at 27 $\pm$ 2°C, 75% relative humidity, and 16:8 hr light–dark photoperiod. A 300 $\mu W/cm^2$ UVB radiation, produced by fluorescent lamps (40-W Sylvania 350 Black Light, burn in >50 hr, Inland Lighting Supplies, Riverside, California), was maintained beneath the Teflon film by adjusting the height of the lamps. This value was chosen on the basis of UV radiation readings taken during February 1991 under canopy of celery grown at the University of California Agricultural Operations fields at Riverside, California, using a System 371 Optical Power Meter equipped with a model 268 detector head (United Detector Technology, Hawthorne, California). To approximate daylight conditions, UV radiation was maintained for 6 hr/day from 1000 hr to 1600 hr within the daytime of the 16:8 hr light–dark photoperiod of the environmental chamber. For each diet treatment, weight of larvae at nine days, weight of pupae, generation time from egg to pupa and from egg to adult, and survival to pupa and to adult were recorded. The experiment was replicated four times.

For statistical analysis, data for larval and pupal weight, generation time, and larval stage mortality were analyzed using ANOVA and overall mortality data were analyzed using the Proc Probit procedures (SAS Institute, 1990). Control mortality was <2.3% and Abbott's (1925) formula was used to correct overall survival data for control mortality whenever less than 100% survival was recorded on the control treatment.

Experiment II: Toxicity of Furanocoumarin Combinations. Joint effects were determined by testing all combinations of LC_{25}s (estimated from the probit lines generated in experiment I) of the three compounds for toxicity to *S. exigua*. The treatments were control, psoralen, bergapten, xanthotoxin, psoralen + bergapten, psoralen + xanthotoxin, bergapten + xanthotoxin, and psoralen + bergapten + xanthotoxin. Dietary treatment preparations and bioassay procedures as well as insect growth and/or survival variables recorded were the same as for the phase I experiment. This experiment also was replicated four times.

For statistical analysis, a χ^2 test (Gomez and Gomez, 1984) was used to analyze the joint effects of 1 : 1 (LC_{25} : LC_{25}) combinations of the three chemicals. Expected mortality was determined using the formula $E = O_a + O_b(1 - O_a)$, where E is the expected percent mortality, O_a is the expected percent mortality due to chemical a alone, and O_b is the expected percent mortality due to chemical b alone (Finney, 1971; Salama et al., 1984). Finney's method (1971) was modified as reported by Salama et al. (1984) and Moar et al. (1990) to test the joints effects of 1 : 1 : 1 (LC_{25} : LC_{25} : LC_{25}) combinations of the three chemicals. The formula used to determine expected mortality was $E = O_a + O_b(1 - O_a) + O_c(1 - O_a)(1 - O_b)$, where E is the expected percent mortality, O_a is the expected percent mortality due to chemical a alone, O_b is the expected percent mortality due to chemical b alone, and O_c is the expected percent mortality due to chemical c alone. Calculated χ^2 values were compared with χ^2 tabular values ($df = 3$, $P < 0.05$). Depending on whether the calculated χ^2 value was significantly greater or smaller than the tabular value, we concluded that there were additive, synergistic, or antagonistic reactions among the different chemicals involved.

RESULTS AND DISCUSSION

Experiment I: Toxicity of Individual Furanocoumarins. Overall, increased dietary concentrations of all three furanocoumarins significantly decreased *S. exigua* larval weight as compared with the control (for psoralen $P < 0.001$, $F = 92.55$, $df = 6, 284$; for bergapten $P < 0.001$, $F = 16.26$, $df = 6, 305$; for xanthotoxin $P < 0.001$, $F = 48.26$, $df = 6, 290$) (Figure 1a). However, no significant differences were found between concentrations exceeding 125 μg/g diet for any of the individual test chemicals. Significant differences were seen among the three furanocoumarins for larval weight ($P < 0.001$, $F = 16.26$, $df = 2, 879$). This differential larval growth, however, varied within concentration, as significant furanocoumarin × concentration interactions occurred ($P < 0.001$, $F = 9.15$, $df = 12, 879$). At the lowest rate of 62.5 μg/g of furanocoumarin in diet, larvae ingesting bergapten had the lowest weight (Figure 1a). However, at rates of 250 μg/g diet and above, a trend of decreasing larval size was evident where psoralen > xanthotoxin > bergapten.

Pupal weights also were significantly reduced as psoralen ($P = 0.0001$, $F = 13.94$, $df = 6, 225$) or xanthotoxin ($P < 0.001$, $F = 6.57$, $df = 6, 222$) concentrations increased in the diet (Figure 1b). However, increasing concentrations of bergapten in the diet had no effects on insect pupal weight ($P = 0.231$, $F = 1.36$, $df = 6, 287$). The three compounds differed in their influence on pupal weight ($P < 0.001$, $F = 9.93$, $df = 2, 766$), but no consistent patterns were observed in the ranking of the three chemicals for toxicity as their dietary

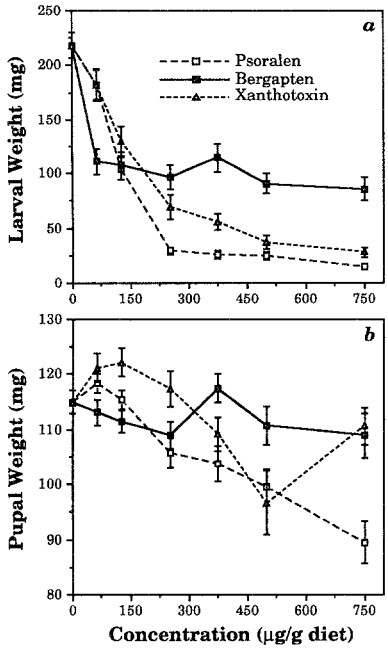

FIG. 1. Differential influence of increased concentrations of psoralen, bergapten, and xanthotoxin on *Spodoptera exigua* larval weight (a) and pupal weight (b). Each data point represents at least 50 observations. Bars represent standard errors.

concentrations increased (Figure 1b) due to furanocoumarin by concentration interactions, i.e., differential response to furanocoumarins ($P = 0.0001$, $F = 4.46$, $df = 12, 766$).

Insect generation time from egg to pupa was significantly extended as dietary furanocoumarin concentrations increased (for psoralen $P < 0.001$, $F = 58.47$, $df = 6, 254$; for bergapten $P < 0.001$, $F = 11.47$, $df = 6, 288$; for xanthotoxin $P < 0.001$, $F = 23.37$, $df = 6, 225$) (Figure 2a). Generation time from egg to adult emergence also significantly increased as dietary individual furanocoumarin concentrations increased (for psoralen $P = 0.001$, $F = 22.72$, $df = 6, 192$; for bergapten $P = 0.0001$, $F = 5.65$, $df = 6, 212$; for xanthotoxin $P = 0.0001$, $F = 15.11$, $df = 6, 167$) (Figure 2b). However, similar to trends found with larval weight, concentrations of bergapten above 62.5 μg/g diet or concentrations of psoralen or xanthotoxin above 125 μg/g diet did not produce significantly different generation times. Overall, larvae reared on diets containing psoralen took significantly longer to pupate ($P < 0.001$, $F = 42.14$, $df = 2, 769$) and to emerge as adult ($P < 0.001$, $F = 22.31$, $df = 2, 573$) compared with larvae exposed to xanthotoxin or bergapten (Figure 2). Like the trend seen

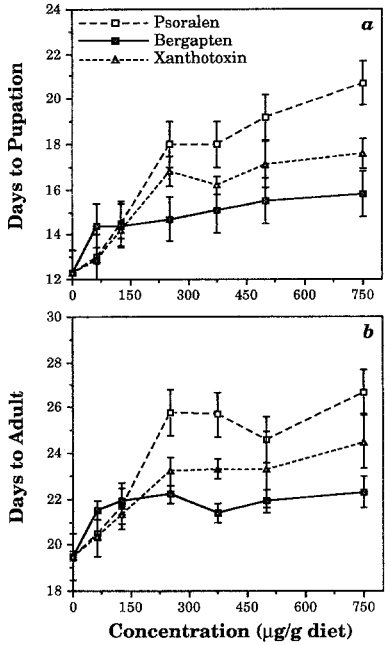

FIG. 2. Differential influence of increased concentrations of psoralen, bergapten, and xanthotoxin on *Spodoptera exigua* generation time from egg to pupa (a) and from egg to adult (b). Each data point represents 5–50 observations. Bars represent standard errors.

for the growth variables of larval and pupal weight, this differential developmental time was not consistent across concentrations as evidenced by significant furanocoumarin by concentration interactions for both days to pupation ($P < 0.001$, $F = 9.02$, $df = 12, 769$) and days to adults ($P < 0.001$, $F = 5.44$, $df = 12, 573$). Feeding on psoralen and xanthotoxin significantly extended insect generation times at high concentrations, but not at concentrations lower than 150 μg/g diet (Figure 2).

Higher rates of three furanocoumarins significantly increased larval stage mortality ($P = 0.001$, $F = 4.67$, $df = 6, 60$) (Figure 3). No furanocoumarin × concentration interactions occurred for this variable ($P = 0.250$, $F = 1.31$, $df = 12, 60$); xanthotoxin induced a significantly higher larval mortality than the other compounds ($P = 0.0037$, $F = 6.47$, $df = 2, 60$). Although psoralen numerically caused more mortality than bergapten at most rates, these furancoumarins were not significantly different in their toxicity (Figure 3).

Based on LC values, xanthotoxin was the most toxic of all three linear furanocoumarins to *S. exigua*; the LC_{50}s for xanthotoxin, psoralen, and bergapten were 245.89, 385.16, and 449.09 μg/g diet, respectively (Table 1). In

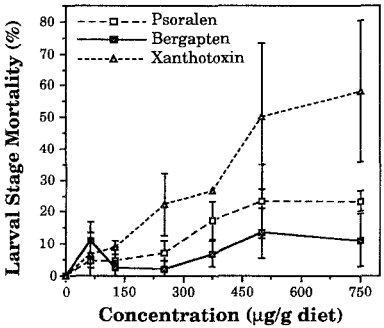

FIG. 3. Differential influence of increased concentrations of psoralen, bergapten, and xanthotoxin on *Spodoptera exigua* mortality during the larval stage (from egg hatch to pupation). Each data point represents 5–50 observations. Bars represent standard errors.

TABLE 1. TOXICITY OF PSORALEN, BERGAPTEN, AND XANTHOTOXIN AGAINST *Spodoptera exigua* WHEN INCORPORATED INTO ARTIFICIAL DIET

Treatment	N^a	Slope ± SEM[b]	LC_{25} (95% FL)[b]	LC_{50} (95% FL)[b]
Psoralen	60	1.671 ± 0.263	152.1 (104.4–196.3)	385.2 (303.5–526.5)
Bergapten	60	1.423 ± 0.363	137.8 (86.8–250.9)	449.9 (310.2–679.0)
Xanthotoxin	60	1.857 ± 0.247	106.6 (72.4–138.4)	245.9 (197.5–305.1)

[a] Number of larvae assayed.
[b] Micrograms furanocoumarin per gram diet. FL = Fiducial limit.

general, larvae were more adversely affected by increased dietary concentrations of xanthotoxin and psoralen than bergapten. Psoralen was more detrimental to insect growth (Table 3 below). Xanthotoxin has also been reported to be toxic to other *Spodoptera* species including the southern armyworm *S. eridania* (Cramer) (Berenbaum, 1978) and the fall armyworm *S. frugiperda* (J.E. Smith) (Ivie et al., 1983).

Experiment II: Joint Action Using LC_{25}s of Psoralen, Bergapten, and Xanthotoxin. Combining psoralen with either bergapten, xanthotoxin, or both resulted in a significantly antagonistic effect on insect mortality (Table 2). The combination of bergapten and xanthotoxin, however, produced an additive effect. Developmental data presented in Table 3 provide an insight into the potential mechanisms of these antagonistic effects. As suggested by the heavier 9-day weight, we suspect the larvae ate at a significantly faster rate when reared on the control diet or LC_{25}s of the three furanocoumarins alone than when reared on chemical combinations. As a result they ingested more allelochemicals than

TABLE 2. COMBINED TOXICITY OF PSORALEN, BERGAPTEN, AND XANTHOTOXIN AGAINST *Spodoptera exigua* WHEN INCORPORATED INTO ARTIFICIAL DIET

Mortality (%) using LC_{25}[a]			Expected % mortality	Observed % mortality	χ^2 value	Effect interpretation
Psoralen	Bergapten	Xanthotoxin				
23.9	11.8		32.9	23.4	36.524	Antagonism[ab]
23.9		22.4	40.9	13.5	77.237	Antagonism[a]
	11.8	22.4	31.5	28.2	1.241	Additivity[b]
23.9	11.8	22.4	47.9	37.9	29.662	Antagonism[a]

[a] Actual percent mortality observed using estimated lethal concentrations needed to kill 25% of the test population at adult emergence.
[b a] $\alpha = 0.001$, $df = 3$; [b] $\alpha = 0.1$, $df = 3$.

TABLE 3. DEVELOPMENTAL VARIABLES OF *Spodoptera exigua* REARED ON DIETS CONTAINING COMBINATIONS OF PSORALEN, BERGAPTEN, AND XANTHOTOXIN

Treatment	Weight (mg)		Survival to pupation (%)[c]	Developmental time (days)	
	9-day larvae[a]	Pupae[b]		Egg–pupa[d]	Egg–adult[e]
Control	261.3 a[f]	114.6 a	100.0 a	12.9 e	19.3 d
Psoralen	148.4 c	115.2 a	93.4 a	16.1 c	23.2 b
Bergapten	203.5 b	118.6 a	93.3 a	14.2 d	20.7 c
Xanthotoxin	226.0 b	115.4 a	94.8 a	13.8 de	20.6 c
Psoralen + bergapten	40.1 d	116.6 a	91.7 a	17.2 b	24.2 a
Psoralen + xanthotoxin	52.2 d	135.7 a	95.0 a	17.4 b	24.4 a
Bergapten + xanthotoxin	104.1 c	118.8 a	86.7 a	15.8 c	22.4 b
Psoralen + bergapten + xanthotoxin	26.4 d	117.0 a	86.7 a	18.6 a	24.6 a

[a] $P = 0.0001$; $F = 125.33$; $df = 7,468$
[b] $P = 0.3978$; $F = 1.05$; $df = 7,441$
[c] $P = 0.3852$; $F = 1.12$; $df = 7,31$
[d] $P = 0.0001$; $F = 74.22$; $df = 7,440$
[e] $P = 0.0001$; $F = 80.48$; $df = 7,364$
[f] Means within a column followed by the same letter are not significantly different at the 5% level (SAS, 1990).

could be metabolized; these larvae developed quickly to the pupal stage, but many could not survive to adulthood. On the other hand, combining the different furanocoumarins could reduce rate of food intake by larvae through increased feeding deterrency. Antifeedant activity against insects has been reported for bergapten (Muckensturm et al., 1981) and xanthotoxin (Yajima and Munakata,

1979). Combining these chemicals with psoralen increased furanocoumarin concentrations in the diet and apparently reduced the feeding rate (larvae did not starve themselves because fecal pellets were observed in the test cups during the experiment). A reduced feeding rate might allow larvae to metabolize the furanocoumarins better by either detoxifying them in the midgut prior to absorption (Ivie et al., 1983; Bull et al., 1984; Nitao, 1990) or by excreting them efficiently (Nitao, 1990). Larvae in our tests reared on chemical combinations usually took significantly longer to pupate, but were able to survive to the adult stage at a higher percentage than insects reared on the single compounds (Tables 2 and 3).

A second possible mechanism would occur if combining the linear furanocoumarins increased mixed function oxidase (MFO) activities in *S. exigua* larvae resulting in less biological effect with higher concentrations of combinations than lower concentrations of individual compounds. Increased enzymatic activity following ingestion of xanthotoxin has been reported in the Lepidoptera larvae *Trichoplusia ni* (Hübner) (Lee and Berenbaum, 1989) and *Depressaria pastinacella* (Duponchel) (Nitao, 1989). Although there is limited literature available on enzymatic induction in *S. exigua*, secondary compounds have been shown to increase MFOs in larvae of *S. eridania* (Brattsten et al., 1977). Bull et al. (1984) reported that both the xanthotoxin-tolerant *Papilio polyxenes* (Stoll) and the xanthotoxin-susceptible *S. frugiperda* metabolized this chemical by oxidative cleavage of the furan ring, but the rate of the metabolism is much higher in *P. polyxenes*.

At the LC_{25} concentrations used in our study, the furanocoumarins acted on insect growth primarily during the pupal stage; percentages of larvae surviving to pupation were not significantly different and were relatively high for all treatments (Table 3). Although insects reared on diets containing chemical combinations took significantly longer to emerge as adults compared with larvae feeding on single compounds, these differences resulted only from the extended feeding time during the larval stage (from egg to pupa) because the average number of days from pupa to adult was comparable for all treatments, varying between six and seven days (Table 3). Therefore, the different dietary treatments did not differ much in their effect on insect developmental time during the pupal stage.

These findings have implications for *S. exigua* resistance in the wild plant accession *Apium prostratum* (Diawara et al., 1992). Diawara et al. (1992) reported 100% *S. exigua* larval stage mortality on diet containing *A. prostratum* with a total concentration of linear furanocoumarins less than 250 μg/g diet. However, in the study reported here, furanocoumarins singly or in combination with one or two others did not induce $>60\%$ larval mortality even at dietary concentrations as high as 750 μg/g diet. Overall mortality was $<50\%$ when LC_{25}s of all three chemicals were mixed in the diet. Therefore, in spite of the

fact that these furanocoumarins may be deterrent or toxic to larvae, the findings reported here support suggestions by Diawara et al. (1992) that other factors may be involved in the strong *S. exigua* nonpreference feeding resistance observed in *A. prostratum*.

Finally, this study raises broader questions. If generalists are usually affected most strongly by the presence or absence of deterrents, and specialists by the presence of attractants (Jermy and Szentesi, 1978; Berenbaum, 1981a,b), why would the generalist *S. exigua* feed readily on a diet with high concentrations of potentially feeding-deterrent furanocoumarins? Further, why would plants produce a complex of furanocoumarins when feeding on individual furanocoumarins causes greater mortality for *S. exigua*? Perhaps the most conservative hypothesis is that evolution of this defense occurred under selection pressure from other mortality factors including pathogens (Karasawa et al., 1990; McCloud et al., 1992), other herbivores (Berenbaum, 1981a,b; Trumble et al., 1990), or as an allelopathic protection against other plants (Friedman et al., 1982). Thus, combinations of chemicals may have greater effects on these "driving" mortality components, and any negative impact on *S. exigua* would be fortuitously beneficial. Certainly, the extended developmental time observed for *S. exigua* larvae feeding on combinations of furanocoumarins could help protect the plant by enhancing potential mortality from natural enemies or environmental factors. Although some of these latter, specific questions may be addressed rapidly by additional experimentation, the broader questions of why and how the generalist *S. exigua* evolved an effective detoxification system for the furanocoumarins will require a much better knowledge of plant–insect associational history than is currently available.

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INTERACTIONS AMONG *Heliothis virescens* LARVAE, COTTON CONDENSED TANNIN AND THE CryIA(c) δ-ENDOTOXIN OF *Bacillus thuringiensis*

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Abstract—The potential interactions among a plant-produced allelochemical, a phytophagous insect, and an endotoxin produced by *Bacillus thuringiensis* were investigated using purified cotton condensed tannins, the CryIA(c) δ-endotoxin from *B. thuringiensis* subsp. *kurstaki* strain HD-73, and larvae of *Heliothis virescens*. Purified condensed tannin from cotton fed to neonate *H. virescens* reduced feeding and mortality caused by insecticidal crystals of *B. thuringiensis*. In fifth instars, tannin reduced relative growth rate (RGR), relative consumption rate (RCR), but antagonized the effects of the crystal δ-endotoxin. Tannin did not deter feeding of fifth instars in choice tests with cellulose-ester disks. Masking tannin from interacting with the dietary ingredients of artificial diets and crystal protein by encapsulation in alginate gel suggested that tannin adversely affected feeding after ingestion. These results suggest that insect control tactics that employ δ-endotoxins in microbial insecticides and transgenic cotton plants may not be compatible when used in conjunction with plants containing high tannin concentrations.

Key Words—*Bacillus thuringiensis* subsp. *kurstaki* strain HD-73, insecticidal proteins, cotton condensed tannin, *Heliothis virescens*, Lepidoptera, Noctuidae, relative growth rate, relative consumption rate, bioassays, *Gossypium hirsutum*, tritrophic interactions.

INTRODUCTION

Pressure to reduce or eliminate synthetic chemical insecticides from insect control programs is leading to more extensive use of the insecticidal bacterium,

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Bacillus thuringiensis (Bt) in insect control programs. This bacterium kills insects primarily through the action of δ-endotoxins, insecticidal proteins produced during sporulation that, after ingestion, destroy the midgut epithelium of sensitive insects (see Hofte and Whiteley, 1989; Gill et al., 1992 for reviews). At present, Bt is used only as a microbial insecticide in which preparations of sporulated cells containing the δ-endotoxins are applied to crops much as are most conventional chemical insecticides. However, several species of transgenic plants including cotton, potatoes, and tomatoes have now been developed that produce Bt δ-endotoxins as systemic insecticides (Fischhoff et al., 1987; Perlak et al., 1990). The advent of recombinant DNA technology makes it likely that the use of these proteins for insect control will expand in the future.

Although it is now generally recognized that important interactions can occur among herbivores, microbes, and plant materials (Berenbaum, 1988; Barbosa et al., 1991), there have been few studies of the interactions among Bt, the plants on which it is applied, and the target insect (Reichelderfer, 1991). Moreover, even where such interactions have been examined, very little is known about how specific compounds in these systems affect the physiology and development of herbivores and/or the effectiveness of Bt insecticidal proteins.

Some of the first studies carried out to evaluate tritrophic interactions involving Bt insecticides in vitro suggested that plants and pathogens were incompatible, i.e., the efficacy of Bt was reduced when it was mixed with plant extracts (Smirnoff and Hutchinson, 1965; Morris, 1974). In other studies where the interactions of Bt with specific phytochemicals such as L-canavanine (Felton and Dahlman, 1984), rutin or nicotine (Krischik et al., 1988), and chlorogenic acid and phenoloxidase (Ludlum et al., 1991) were examined, the results were variable, in some cases showing enhancement (Felton and Dahlman, 1984; Ludlum et al., 1991) or showing varying levels of reduction in Bt efficacy (Krischik et al., 1988).

Tannins are a class of plant allelochemicals that affect different insect species differently, ranging from no apparent effect to retarded growth and consumption to death (Schultz, 1989); thus, no overall generalization on the effects of tannins on herbivores can yet be made (Bernays et al., 1989). In previous studies on two important lepidopteran pests of cotton, *Gossypium hirsutum* (L.), tannins in cotton caused a nonlethal reduction in food intake of *Heliothis virescens* (F.) (Noctuidae) (Chan et al., 1978; Hedin et al., 1983). Similarly, cotton condensed tannins inhibited the growth of *Helicoverpa* (= *Heliothis*) *zea* in a dosage-dependent manner when incorporated into artificial diets (Klocke and Chan, 1982). Due largely to these "antibiosis" traits, condensed tannin is one of the major phytochemicals that is selected in cotton breeding programs (White et al., 1982).

Given the increased interest in using Bt δ-endotoxins in a wider range of

microbial insecticides and transgenic plants, and plant breeding programs aimed at increasing the levels of tannins in cotton, we examined the tritrophic interactions among cotton condensed tannin, the CryIA(c) δ-endotoxin of Bt, and larvae of *H. virescens*. We emphasized the determination of how the interaction between tannin and δ-endotoxin in the insect midgut affected larval growth and development. Key parameters measured were the nutritional indices of relative consumption rate (RCR) and relative growth rate (RGR). Information on this tritrophic interaction would provide an indication of the compatibility of δ-endotoxins and tannins for suppression of *H. virescens*, both when Bt is applied as a bacterial insecticide as well as when the insecticidal δ-endotoxin is produced by transgenic plants.

METHODS AND MATERIALS

Insects. Heliothis virescens was reared in an incubator at 28 ± 1°C under a 16:8 hr photo/scotophase essentially as described by Shorey and Hale (1965). Eggs were surface-sterilized with a 10% aqueous formaldehyde solution, and the larvae were reared on a lima bean–wheat germ diet.

Isolation of CryIA(c) δ-Endotoxin Crystals. A culture from a lyophilized powder of the HD-73 isolate of *Bacillus thuringiensis* subspecies *kurstaki* (H 3a3b), in which the parasporal crystal is composed only of the CryIA(c) δ-endotoxin (Adang et al., 1985), was first grown on nutrient agar and then, for production of crystals, on a peptonized milk medium at 28°C for 72 hr until sporulation and lysis were complete (Ibarra and Federici, 1986). This δ-endotoxin was selected for the assays because it is highly toxic (LC_{50} = 0.14 μg/g diet) to *H. virescens*, and 2.5-fold more toxic than the HD-1 strain of *B. thuringiensis* subsp. *kurstaki* (LC_{50} = 0.35 μg/g diet), the isolate recommended for control of this pest (Navon, unpublished data).

The crystals were separated from the spores using a separatory funnel and a biphasic liquid system containing sodium dextran sulfate and polyethylene glycol (Goodman et al., 1967). After mixing the spore–crystal complex with the solutions, and shaking the suspension, the crystals accumulated in the sodium dextran sulfate at the lower end of the funnel. To increase the concentration and purity of the crystals, the sodium dextran sulfate fraction was extracted four times at 4°C with polyethylene glycol. The sodium dextran sulfate was removed from the crystal fraction by washing with double-distilled water, after which the crystals were freeze-dried. Crystal purity was >99% as assessed by phase microscopy.

Isolation and Purification of Tannin. The crude tannin powder was obtained from Dr. P.A. Hedin (Boll Weevil Research Laboratory, Stoneville, Mississippi). This powder was obtained by extracting leaf powder from high-tannin

cotton cultivars with chloroform–ethanol (1 : 1) and then separating the polar and nonpolar phases by adding distilled water. The polar phase, containing tannins, was evaporated to dryness at room temperature, leaving a brown powder. We dissolved this powder in 95% ethanol, then purified on a Sephadex LH-20 column according to the procedure of Hagerman and Butler (1978, see also Lane and Schuster 1981). A pressure of 10 psi was used in the column to increase the rate of tannin extraction. The tannin was washed from the Sephadex with aqueous 50% acetone. The acetone was then removed by rotary evaporation at room temperature. The resulting aqueous tannin mixture was then lyophilized, yielding a brown powder.

The concentration of tannin in this powder was determined by the protein-binding colorimetric assay of Hagerman and Butler (1978) and standardized against tannic acid (Sigma Chemical Co.) that was also purified on a Sephadex column, at concentrations of 0.2–1.0 mg/ml. Purified condensed tannin and purified tannic acid showed similar dose responses in this assay. The slope of the regression line between absorbance and concentration of condensed tannin was 0.75 ($r = 0.98$, intercept = 0.02) and that for tannic acid was 0.82 ($r = 0.98$, intercept 0.08).

Disk Bioassays. To determine whether the presence of tannin affected larval feeding choice, a choice bioassay for cotton condensed tannin was conducted. An ultrafiltration disk (24 mm diam., 0.5 μm pore size) composed of cellulose nitrate and acetate (Micron Separation Inc., Westboro, Massachusetts) was used as a feeding substrate for the assay. Cotton condensed tannins were dissolved in 50% aqueous ethyl acetate at concentrations of 10, 20, or 40 mg/ml. Sixty-five microliters of one of the tannin solutions was applied to a disk. Untreated disks weighed 20 mg; therefore, the final concentrations of tannin on the disks were 32.5, 65, or 130 μg/mg of disk substrate. After the disk had dried, 65 μl of a 5% aqueous sucrose solution was applied to the disk. Control disks were treated with the solvent and sucrose only.

Newly ecdysed fifth instars that had been starved for 24 hr at 17°C were weighed and placed in 9-cm Petri dishes, one larva per dish. In each dish, the disk with tannin was placed on one side and the control disk on the other, each fixed in position with a needle. Water absorbed on cotton also was available to the larvae. The bioassay was conducted for 24 hr, and the disks were weighed at the beginning and end of this period. Four replicate bioassays with 10 larvae per tannin concentration treatment were conducted (total of three treatments $\times$ 10 larvae per treatment $\times$ 4 replications = 120 larvae).

Dietary Bioassays. Interactions between the tannin and CryIA(c) toxin were determined through diet bioassays using neonate and fifth-instar larvae (Navon et al., 1990). In the assays against neonate larvae (0–12 hr old), CryIA(c) crystals were added to the diet in aqueous 0.05% polysorbate 80 at five concentrations, ranging from 0.2 to 5 μg endotoxin/ml diet. Twenty-gram quantities

of artificial diet were made to which 1 ml of one of four aqueous tannin solutions ranging from 0 to 64 mg/ml were added to yield final tannin concentrations ranging from 0.0 to 3.2 mg/g fresh weight of diet. This range is within that of the natural concentration of condensed tannin in high-tannin cotton cultivars, e.g., 0.7% in fresh squares of *G. hirsutum* cultivar Texas 254 (Chan et al., 1978). Four replicate groups of 15 larvae were used per treatment combination (total of 5 crystal treatments × 4 tannin treatments × 15 larvae per treatment combination × 4 replicates = 1200 larvae). Larvae were allowed to feed on the diet for 72 hr, after which mortality was determined.

In a separate experiment, the effect of tannin on larval weight gain was recorded after larvae were reared on control diets or on diets containing 0.125, 0.25, 0.50, 1.0, or 2.0 mg of cotton condensed tannin per gram of diet (fresh weight) for seven days. The bioassays were conducted in four groups of 25 larvae per treatment, each group being initiated on a different day. The EC_{50}, i.e., the concentration that caused an inhibition of weight gain in 50% of the larvae, was determined by linear regression (SAS Institute, 1989).

In the growth and consumption studies using the fifth instar (final instar), fourth instars were first collected from the colony just prior to ecdysis. After molting to the fifth instar, they were starved for 24 hr and held at 17°C before being used in the assays. Larvae were fed on the same diet used for neonates except that the concentration of ascorbic acid was 0.4% instead of 0.1%.

To delay or prevent any interactions with the tannin–δ-endotoxin mixture with the diet, a separate experiment was conducted in which the CryIA(c) crystals and tannin were encapsulated in microcapsules of a sodium alginate gel. The tannin and crystals were blended into Keltone LVR, an aqueous 2% sodium alginate solution (Kelco Division, Merck & Co., Inc., New Jersey). After thorough blending, 2-μl droplets of the alginate mixture were pipetted into a setting solution of 5% $CaCl_2$ using an automated pipet (Rainin Instrument Co. Inc., Woburn, Massachusetts). The alginate microcapsules solidified within 5 min, but were left to harden for 30 min, after which they were collected and washed for 5 min in running water. Free water was absorbed from the surface of the capsules using filter paper (Whatman No. 1). About 500 microcapsules were obtained per milliliter of the sodium alginate mixture. After preparation, the microcapsules were mixed homogeneously into portions of diet (10 g/portion) at 45–55°C using a magnetic stirrer. Immediately after mixing, the diet was placed in a refrigerator at 4°C to prevent separation and layering of the capsules within the diet. To ensure that mixing the capsules into the diet did not affect their digestibility, charcoal powder was added to the capsules in a separate control. The charcoal powder was evenly dispersed in the feces of larvae fed these capsules, indicating the alginate had fully disintegrated within the gut.

In these experiments, the fifth instars were reared individually in lidded 50-ml plastic vials. After being weighed, larvae were placed in the vials and

offered a diet cube of about 1 g held above the bottom of the vial with toothpicks. Larvae were reared for 24 hr and then reweighed. The larvae were offered six diet combinations of unencapsulated tannin and CryIA(c) crystals consisting of 0, 0.05, or 10 μg/g crystals and 0 or 2.4 mg/g tannin. In the experiments with encapsulated materials, four combinations of 0 or 10 μg/g CryIA(c) crystals and 0 or 2.4 mg/g tannin were used. Four groups of 10 larvae were reared in each treatment.

The calculation of values for relative consumption rate (RCR) and relative growth rate (RGR) was based on mean dry weights. The equations for these nutritional indices were as follows: RCR = diet consumed/initial larval weight per 24 hr; and RGR = larval weight gain/initial larval weight per 24 hr. The regression line for the conversion of fresh to dry weight for initial larval weight was, $y = 0.26x - 8.6$ ($r = 0.98$). The midgut of newly ecdysed larvae was essentially without food. To obtain an accurate indication of weight gained in 24 hr, larvae were weighed before and after removal of food in the midgut. More specifically, after the end of the feeding period, larvae were weighed, frozen, and dissected on cooled aluminum foil under a dissecting microscope. The frozen gut contents were removed and the larvae were reweighed. They were then lyophilized and weighed again. The regression equation for conversion from fresh to dry weight of larvae without gut contents was, $y = 0.32x - 24.5$ ($r = 0.91$). The dry weight of the diet was 10.4%, and water loss from the diet to evaporation during the experiment was 14.3%.

Consumption and growth rate data were analyzed by a two-factor (crystals treatment $\times$ tannin treatment) analysis of variance (ANOVA) with interaction using the SAS GLM procedure (SAS Institute, 1989). The variation due to the main effects of CryIA(c) concentration and tannin concentration, as well as the variation due to their interaction, were tested over the variation among blocks within (CryIA(c) treatment tannin treatment) combinations.

RESULTS

The incorporation of tannin into the diet of neonate larvae caused a reduction in weight gain, with the degree of reduction being correlated with the concentration of tannin in the diet (Figure 1). At 0.125 mg tannin/g of diet, the reduction in gain was less than 10%, whereas at 2.0 mg the reduction was greater than 90% over a seven-day period. The EC_{50} for tannin in the diet was 0.25 mg/g. Mortality of larvae in these assays due to tannin ranged from 10 to 20% (data not shown).

The choice bioassays with disks showed that neither cotton tannin or tannic acid decreased feeding except at artificially high concentrations (Figure 2). At concentrations of 13% dry weight of tannin, for example, the consumption of disk was 4.2 $\pm$ 1.0 mg (mean $\pm$ SE) versus 13.0 $\pm$ 1.0 mg in the control.

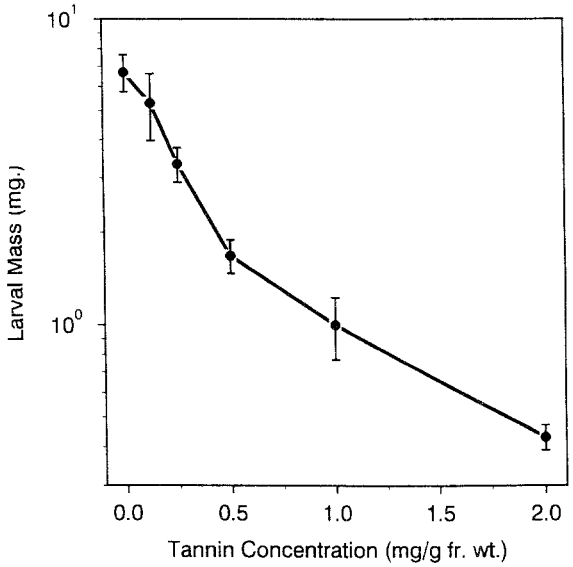

FIG. 1. Mass (mean ± SE) of *Heliothis virescens* larvae after feeding from eclosion for seven days on artificial diets containing purified cotton condensed tannins.

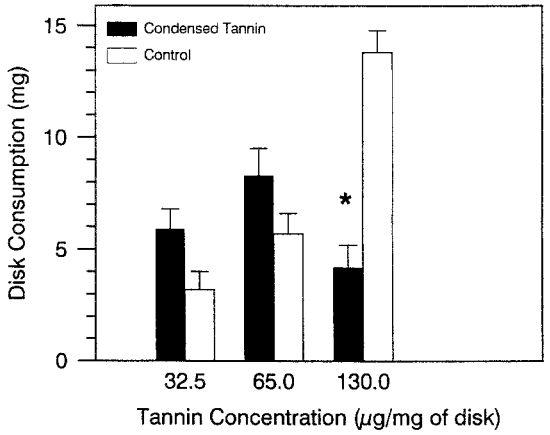

FIG. 2. Consumption (mean ± SE) of cellulose nitrate–cellulose acetate filtration disks by fifth-instar *Heliothis virescens* larvae when treated with purified cotton condensed tannin and sucrose (solid bars) or sucrose alone (open bars) in a paired comparison over 24 hr. Asterisk indicates a statistically significant difference in consumption between treated and control disks (paired Student's *t* test).

When CryIA(c) crystals where fed alone in diet, or with concentrations of cotton condensed tannin of 0.4–0.8 mg/g of diet, the LC_{50}s for the δ-endotoxin were similar and in the range of 30 ng/g of diet, indicating that tannins at these concentrations had little affect on the activity of the toxin (Table 1). However, at a concentration of 3.2 mg/g of diet, the LC_{50} of CryIA(c) almost doubled, suggesting that the allelochemical antagonized δ-endotoxin activity. Tannin alone at concentrations as high as 3.2 mg/g of diet caused no significant mortality in this experiment. The tannin solution added to the diet was deep brown, causing the diet to be brown; no additional diet browning was observed subsequently.

In the experiment where CryIA(c) crystals and tannins were mixed uniformly within diets, relative growth rate (RGR) of fifth instars (Figure 3A) differed significantly due to CryIA(c) treatment ($F_{2,18} = 714.24$, $P < 0.0001$), due to tannin treatment ($F_{1,18} = 116.34$, $P < 0.0001$), and due to the CryIA(c) crystals tannin interaction ($F_{2,18} = 107.58$, $P < 0.0001$). There was no significant variation among blocks within treatment combinations ($F_{18,212} = 1.00$, $P < 0.4658$). RGR declined with increasing CryIA(c) concentrations, but the reduction was more severe in the tannin-free diets than in diets containing tannin. In the absence of CryIA(c) crystals, growth rate was reduced slightly by adding tannin to the diets, but tannins ameliorated the effects of CryIA(c) on growth reduction when both were included in the diet. Apparently, larvae were undergoing some gross physiological breakdown resulting from midgut damage due to the action of the Bt crystals in the absence of tannins, as indicated by negative growth rates, but the combination of tannins and crystals essentially resulted in little to no growth.

TABLE 1. EFFECT OF COTTON CONDENSED TANNIN ON TOXICITY OF CryIA(c) δ-ENDOTOXIN DETERMINED BY 72-HR BIOASSAY AGAINST NEONATE *Heliothis virescens*

Tannin (mg/g)[a]	LC_{50}/CryIA (c) (ng/g ± SE)[b]	Slope (mean ± SE)	Correlation (r)
0	27.0 ± 0.4a	0.87 ± 0.08	0.88
0.4	30.0 ± 0.3a	0.78 ± 0.08	0.89
0.8	34.1 ± 0.5a	0.91 ± 0.10	0.90
3.2	59.1 ± 0.9b	0.69 ± 0.07	0.93

[a] Tannin alone incorporated into the diet as a control at concentations as high as 3.2 mg/g of diet caused no significant mortality.

[b] Means followed by a common letter did not differ ($P < 0.05$) significantly according to ANOVA and Duncan's new multiple-range test.

In the tannin encapsulation experiment (Figure 3B), the presence of tannins again reduced larval growth ($F_{1,12} = 313.14$, $P < 0.0001$), as did CryIA(c) ($F_{1,12} = 3130.18$, $P < 0.0001$). The interaction between CryIA(c) crystals and tannin treatments also was significant ($F_{1,12} = 338.45$, $P < 0.0001$), and there was no significant variation among blocks within treatment combinations ($F_{12,144} = 0.47$, $P = 0.93$). Again, in the absence of the crystals, tannins reduced growth rate, but because CryIA(c) crystals effectively reduced RGR to near zero alone, the addition of tannins to the diet had little if any additional effect (Figure 3B). We did not see any growth rates less than zero due to the effect of CryIA(c) alone and no amelioration of the effect of CryIA(c) by the addition of tannins (Figure 3B).

The effect of tannin encapsulation on RGR was tested explicitly in a three-way ANOVA using the four combinations of treatments [0 and 10 μg/g CryIA(c) and 0 and 2.4 mg/g tannin] common to both experiments. RGR differed significantly due to the encapsulation treatment ($F_{1,24} = 54.35$, $P < 0.001$), due to the presence of tannin ($F_{1,24} = 5.59$, $P = 0.027$), due to the presence of CryIA(c) crystals ($F_{1,24} = 2950.18$, $P < 0.0001$), and also due to all two- and three-way interactions among these main effects (all F values >23.97, all P values <0.0001) (Figure 3A,B). In the absence of tannins, larvae fed diets containing control capsules grew more than larvae fed diets without capsules. When the capsules were filled with tannins, however, growth rates were reduced on the encapsulated tannin diets in the absence of Bt crystals, relative to diets with unencapsulated tannins, but enhanced when diets also contained Bt crystals.

In the experiment with unencapsulated tannin, RCR (Figure 3C) was reduced by the presence of tannins ($F_{1,212} = 332.44$, $P < 0.0001$) and by the presence of CryIA(c) ($F_{2,212} = 1220.23$, $P < 0.0001$), but the magnitude of reductions was heterogeneous over all treatment combinations (interaction: $F_{2,212} = 184.24$, $P < 0.0001$, Figure 3C). Because CryIA(c) crystals alone at 5 μg/g reduced consumption nearly to 0, and consumption rate cannot fall below 0, the addition of tannin had a relatively minor effect in the presence of CryIA(c) compared to the absence of CryIA(c) (Figure 3C). Similar results were obtained when the tannin was encapsulated in the diet (tannin: $F_{1,12} = 343.30$, Bt crystals: $F_{1,12} = 2501.67$, interaction: $F_{2,12} = 195.85$, all Ps < 0.0001, Figure 3D). In the combined analysis of both experiments, RCR varied with encapsulation treatment ($F_{1,24} = 8.14$, $P = 0.0088$), Bt crystals treatment ($F_{1,24} = 3232.40$, $P < 0.001$), tannin treatment ($F_{1,24} = 573.48$, $P < 0.001$), and with all possible two- and three-way interactions (all Fs > 9.13, all Ps < 0.0059). In the absence of Bt crystals, larval consumption rates were slightly higher with diets lacking alginate capsules with or without tannin, but consumption rates were slightly higher in diets with capsules when the CryIA(c) crystals also were present (Figure 3C,D).

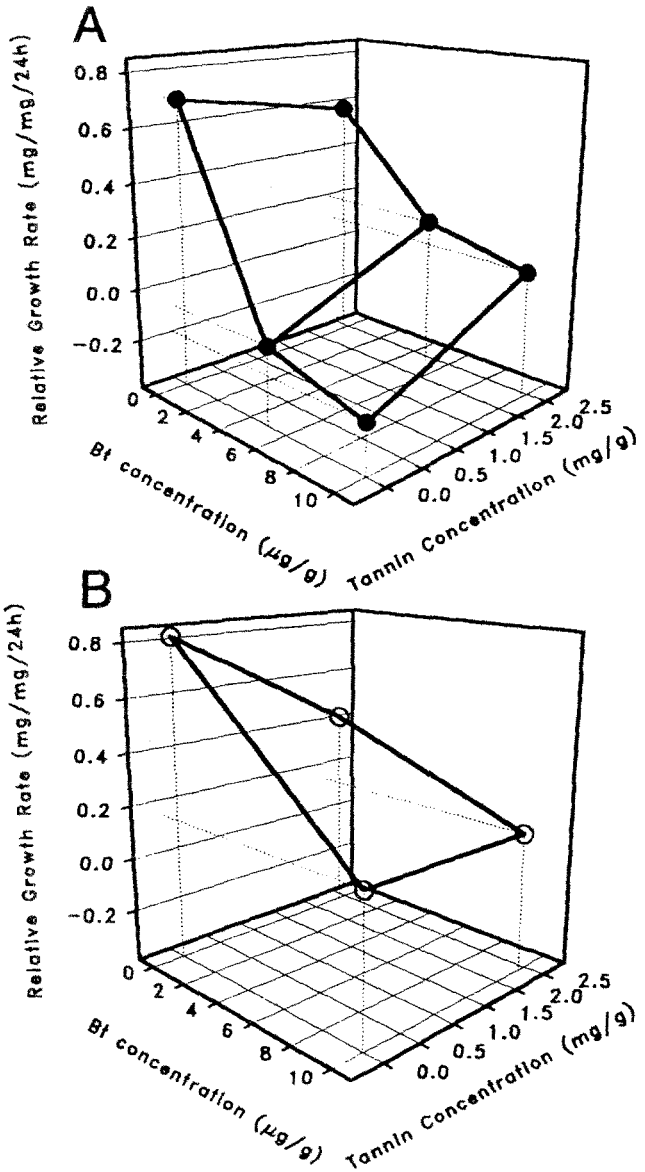

FIG. 3. Mean relative growth rates (A and B) and mean relative consumptions rates (C and D) for fifth-instar *Heliothis virescens* larvae when fed artificial diets containing purified cotton condensed tannins and CryIA(c) δ-endotoxin for 24 hr. Results for diets in which tannins and δ-endotoxin were thoroughly mixed are shown in A and C (filled circles) and results for diets in which tannin was encapsulated are shown in B and D (open circles).

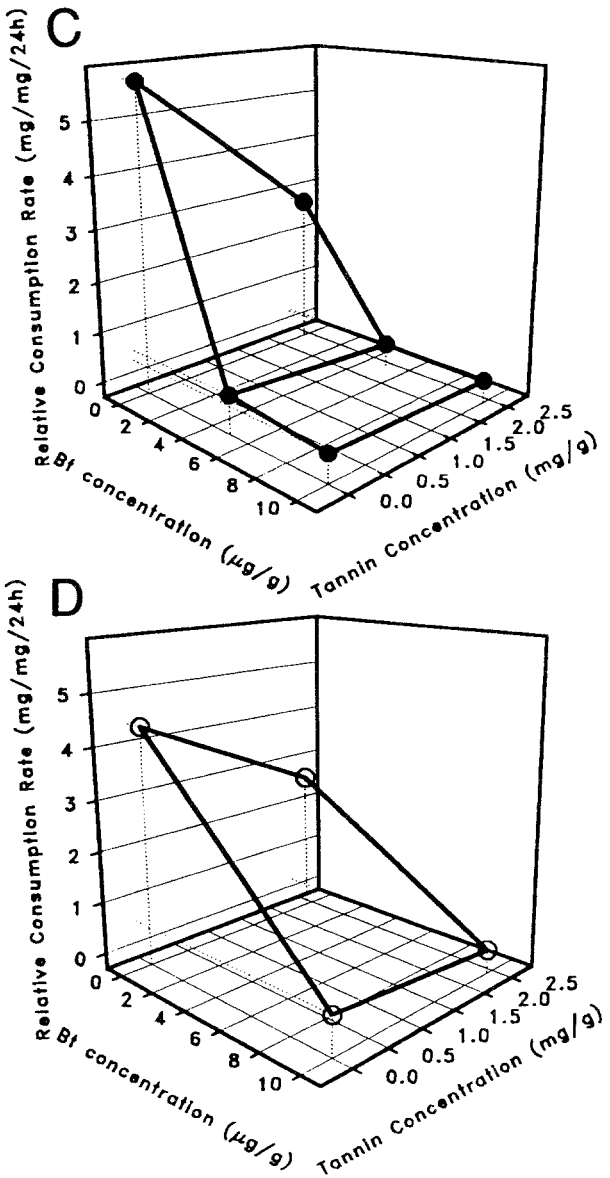

FIG. 3. Continued

DISCUSSION

The sensitivity of *H. virescens* to cotton condensed tannins was demonstrated in all experiments. Results suggest that first instar larvae may be more susceptible than fifth instars, in that the concentration in the diet sufficient to cause a significant reduction in the weight gain of first instars (Figure 1) was only about 10% of that necessary to significantly reduce the growth of fifth instars (Figure 3). Klocke and Chan (1982) also found that the sensitivity of *H. zea* larvae declined with the age of the larvae tested. The two experiments in this study may not be directly comparable, however, because the growth experiment with first instars was seven days in duration, while that with fifth instars was only one day in duration. We might have observed a greater reduction in growth of fifth-instar *H. virescens* fed diets containing 2.4 mg/g of tannin had we allowed the experiment to continue for a longer time.

The combination of dietary tannin and CryIA(c) demonstrated clearly that the tannin antagonized the interaction between the δ-endotoxin and the insect. In the nutritional studies, the negative RGR obtained with unencapsulated tannin and CryIA(c) crystals shows that the rapid cessation of feeding was followed by weight loss. The low amounts of CryIA(c) diet consumed by the larvae was sufficient to cause microbial intoxication, based mostly on midgut effects, because it was shown that mandibular dysfunction was not involved in this feeding arrestment (Navon et al., 1992). The RGR for the unencapsulated CryIA(c) was lower than in the encapsulated form, possibly because in the former, the endotoxin was more available to the larvae. Although the density of alginate capsules in the diet with encapsulated endotoxin (about 50 capsules/g) was sufficient to cause toxicity, the intoxication with the freely dispersed endotoxin in the diet was more effective. Still, RCR was similar for both unencapsulated (Figure 3C) and encapsulated (Figure 3D) tannin.

The low RCR in the unencapsulated tannin is probably not explained simply by feeding deterrence caused by the larvae sensing the allelochemical prior to ingestion. First, the disk choice bioassay showed that tannin was not a preingestive gustatory barrier to the larvae (Figure 2) because larvae did not avoid tannins on a neutral, nonnutritive substrate except at unnaturally high concentrations. Second, a simple feeding deterrence explanation would also not account for the similar reduction in RCR observed with encapsulated tannin. The alginate capsules were ingested with the diet indiscriminately, and the capsules did not interact with the diet but disintegrated in the gut (Navon, personal observation). Because the tannin in the alginate capsule was in the form of a homogeneous complex of tannin chelated by the alginate gel, there was some tannin present on the capsule's surface. Therefore, the larvae may have been able to sense the tannin in the diet prior to ingestion, albeit to a lesser extent than when the tannin was freely dispersed in the diet. Therefore, we believe it most likely that tannin

reduced consumption through postingestive mechanisms. Nevertheless, alginate encapsulation was useful for physically isolating tannins from the CryIA(c) prior to ingestion.

The lower quantities of diets with tannins in addition to CryIA(c) crystals consumed by the larvae meant fewer crystals in the gut. As a result, feeding inhibition caused by the CryIA(c) toxin was also reduced. It seems unlikely that postingestive complexing of dietary proteins with tannin was responsible for the reduced feeding. The quantity of tannin in the bioassays (2.4 mg/g) seemed to be too low to bind a substantial quantity of dietary proteins. Similarly, the antibiotic effect of dietary cotton condensed tannin against *H. virescens* probably cannot be attributed to interactions of tannins with the diet ingredients (Chan et al., 1978). Limited δ-endotoxin inactivation and more extensive toxin inactivation in vitro (Luthy et al., 1985) suggest that tannin binding to CryIA(c) crystals in the diet seems unlikely, but interaction of the tannin with the active toxin cannot be excluded.

Our results indicate that breeding cotton for high-tannin cultivars, although appropriate for some insect pests, may not fit into pest management programs for *H. virescens* if Bt δ-endotoxins are also widely used, regardless of whether these proteins are administered as foliar microbial insecticides or systemically as in transgenic plants. High concentrations of tannin would antagonize the δ-endotoxin activity by reducing larval feeding and possibly by directly inactivating the δ-endotoxin. On the other hand, there is a need to develop better allelochemical defenses in plants, particularly in light of the possible rapid increase in resistance by *H. virescens* to Bt δ-endotoxins (Gould et al., 1992). Thus, increasing natural host plant resistance to *H. virescens* by increasing the levels of deterrent and toxic chemicals, including tannins, may be one of the means to delay the development of resistance to Bt. The development of effective pest-management strategies in which cotton cultivars with high or low levels of tannin are integrated with the use of microbial insecticides such as Bt will obviously require an increased knowledge of their interactions.

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RESPONSES OF MAXILLARY SENSILLA STYLOCONICA IN *Bombyx mori* TO GLUCOSIDES FROM *Osmunda japonica*, A NONHOST PLANT

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Abstract—We isolated glucosides from the royal fern, *Osmunda japonica*, which elicit a deterrent response in larvae of *Bombyx mori*. These compounds were absent in taro (*Colocasia antiquorum*) and castor-oil plant (*Ricinus communis*) leaves and did not evoke responses of sensory cells in the lateral and medial sensilla styloconica of *Spodoptera litura*. This glucoside extract of the royal fern leaves stimulates receptors generally associated with deterrent. It is also possible that this compound may act as a behavioral deterrent, and from actual feeding tests, it is suggested that this compound may prevent feeding in some monophagous insects, such as *Bombyx mori*. The deterrent glucoside possesses a noncyclic aglycon.

Key Words—*Bombyx mori*, Lepidoptera, Bombicidae, Noctuidae, *Spodoptera litura*, sensilla styloconica, royal fern, *Osmunda japonica*, electrophysiological response.

INTRODUCTION

During a study of leaf carbohydrates, which may act as feeding stimulants to phytophagous insects in some plants, we have discovered glucosides in the leaves of the royal fern, *Osmunda japonica*. These compounds were not present in the leaves of taro, *Colocasia antiquorum* and of the castor-oil plant, *Ricinus*

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communis. It is known that Osmundae have ecdysial activity, which offers protection from attack by phytophagous insects (Matsuoka et al., 1969). The possibility exists that, in addition to phytoecdysones, other antifeedants are present. Therefore, we tested electrophysiologically whether these new compounds stimulate deterrent receptors in the maxillary sensilla styloconica of *Bombyx mori* and *Spodoptera litura* larvae.

METHODS AND MATERIALS

Insects. Larvae of *Bombyx mori* (137 × 146) (Lepidoptera: Bombicidae) and *Spodoptera litura* (Lepidoptera: Noctuidae) were reared on artificial diets, Silkmate and Insecta LF (Nihon Nosan Kogyo Co. Ltd.), respectively. Newly molted, unfed, last-instar larvae were used for electrophysiological investigations.

Preparation of Samples. One-gram dried powder samples of the castor-oil plant, *Ricinus communis*, of the royal fern, *Osmunda japonica* and of taro, *Colocasia antiquorum* were extracted with ethylether, and then extracted with 2 ml of 70% EtOH. After filtration, 10-μl extracts were injected on the column. Refractive index-detectable elution samples were dried and added to 10 ml distilled water containing trace NaCl solution (5×10^{-2} M) for testing of the electrophysiological response.

Electrophysiological Response. Electrophysiological responses were recorded from the lateral and medial sensilla styloconica (Ss) of larvae of *Bombyx mori* and *Spodoptera litura*. The electrophysiological methods used were the same as described previously (Ishikawa, 1963; Hirao and Arai, 1991). A head–thorax was amputated at the middle part of the thorax and secured with clay to a silver plate, which was used as the indifferent electrode. A glass capillary, which served as the stimulating-recording electrode, was applied externally over the tip of the hair. A silver wire inserted into the capillary was connected to a high-input impedance, cathode-follower preamplifier, followed by the main amplifier and oscilloscope. Responses were recorded from Ss-lateral and -medial. Impulse frequencies of five types, N_1, N_2, N_2', R, and S_1, were determined during the second sec of stimulation (see Hirao and Arai, 1991; Yazawa et al., 1991). Amplitude levels of the N type from inorganic salt receptor cells are $N_2 > N_2' \div N_1$, N_2 and N_2, and are obtained from Ss-medial of *Bombyx mori* and N_1 is obtained from Ss-lateral of *Bombyx mori* and *Spodoptera litura*.

High-Performance Liquid Chromatography (HPLC) with Refractive Index Detector (RID). The HPLC-RID methods used in the experiments were the same as described previously (Yazawa et al., 1992). Sinigrin or sinigrinlike substances are not determined in Shim pack column (Shimadzu Co., Ltd.) used in the present experiment.

Feeding Response. The P1 sample eluted with HPLC was dried in a rotary evaporator under vacuum and then added to an artificial diet that consists of agar (1 g) and water (30 ml) containing 10^{-1} M sucrose and 5×10^{-2} M inositol. The concentrations of test diet were prepared at 120, 12, and 1.2 ppm with water containing carbohydrates. The consumption of test diet during a 72-hr feeding period by newly ecdysed last-instar *Bombyx mori* was investigated.

Glucoside Hydrolysis with Beta-Glucosidase and Reaction of Liberated Glucose. Glucoside hydrolysis was performed using beta-glucosidase (Sigma), which liberates glucose from beta-bond glucosides. To differentiate between free glucose and estimated glucosides, specific detection of glucose by the God podlk and phenol methods was employed (Saloman and Johnson, 1959). God podlk (glucoseoxidase-peroxidase-chromogen reaction) was purchased from Nagase Biochemicals, Ltd. (Fukuchiyama, Japan).

UV Absorbance. The sample and standard salicin (Sigma) were dissolved in an HPLC mobile solution of acetonitoryl and water (7 : 3). The UV absorbance of 50-μl samples was measured using a UV-3100S spectrophotometer (Shimadzu). The absorbance spectrum of mobile solution was subtracted from that of the sample or salicin standard.

RESULTS

Figure 1 shows the HPLC pattern of sugar analysis from samples of standard (A), royal fern, *Osmunda japonica* (B), castor-oil plant, *Ricinus communis* (C), and taro, *Colocasia antiquorum* (D). Fructose, glucose, and inositol were found to be present in all three species. The level of sucrose of feeding stimulants is 60.12, 55.68, and 30.89 (mg/g dry weight) in the leaves of castor-oil plant, *Ricinus communis*; royal fern, *Osmunda japonica*; and taro, *Colocasia antiquorum*, respectively (Figure 1D). The HPLC pattern of the royal fern sample resembles that of the castor-oil plant but peaks 1 and *, which show 4.82 and 5.06 min retention times in the royal fern, are apparently absent in the castor-oil plant. The amount of the compound causing the small peak * is ca. 10% of that causing the large peak 1 (Figure 1B).

To clarify the taste responses, we tested the electrophysiological responses of Ss-lateral and Ss-medial in *Bombyx mori* and *Spodoptera litura* to the compound causing peak 1 (containing peak *) (indicated as P1), since the two phytophagous insects do not feed on the royal fern in nature. Because responses to deterrent substances such as sinigrin are only obtained from Ss-lateral in *Spodoptera litura* (Hirao et al., 1993), we first recorded from the Ss-lateral of *Spodoptera litura*. Figure 2A and 2B show responses to 0.05 M NaCl or a sample prepared from the carrier fluid of HPLC as controls. The salt receptor fires in response to both stimuli (N_1 in Figure 2). Figure 2C shows no response

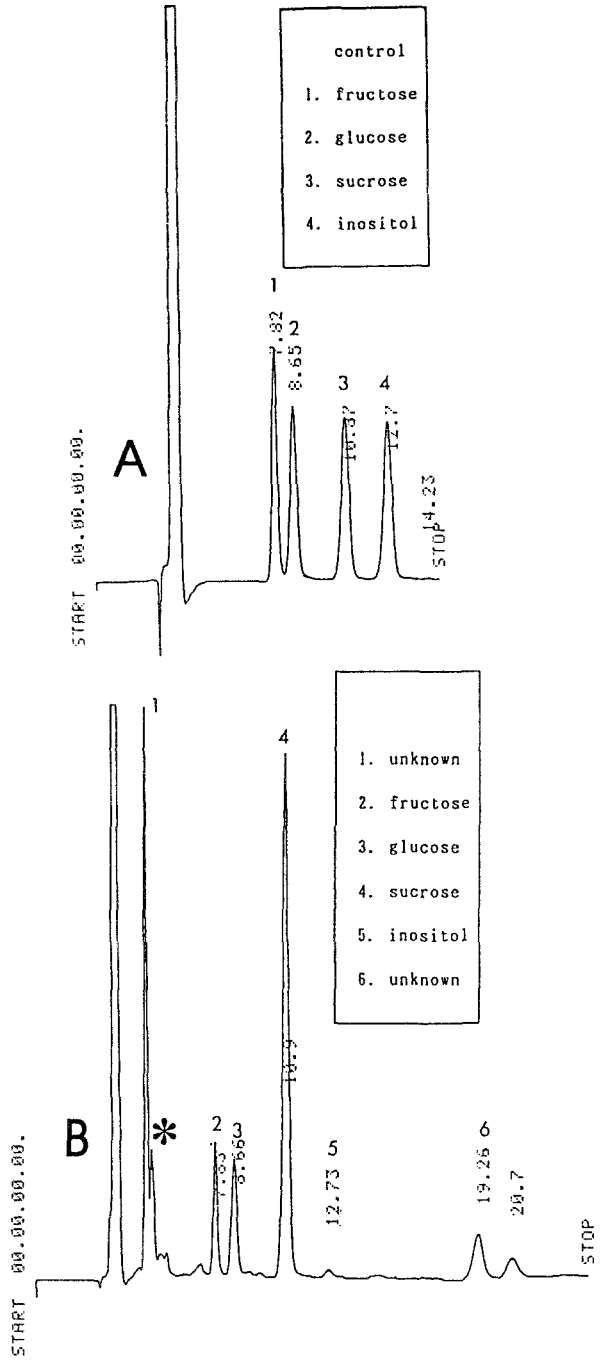

FIG. 1. The HPLC pattern of sugar analysis. (A) standard compounds; (B) royal fern, *Osmunda japonica*; (C) castor-oil plant, *Ricinus communis*; (D) taro, *Colocasia antiquorum*. *Small peak (10%) of eluted P1.

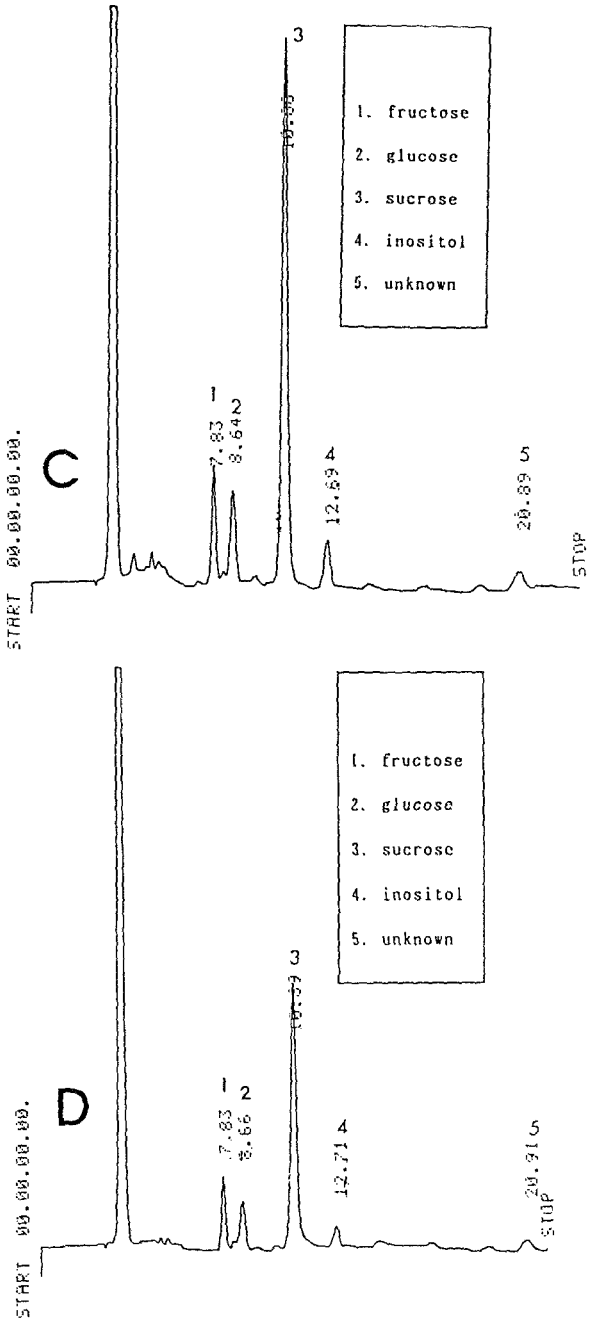

FIG. 1. Continued

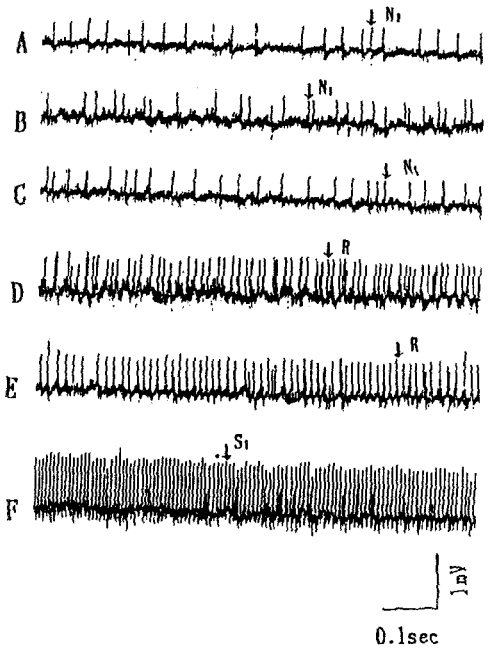

FIG. 2. The impulses discharged from Ss-lateral of *Spodoptera litura*. (A) NaCl (5×10^{-2} M), (B) sample prepared from carrier fluid of HPLC, (C) P1 sample prepared from 1 g dry powder of royal fern, *Osmunda japonica*, leaves in 10 ml distilled water containing trace NaCl; P1 is both peaks occurring at retention times of 4.82 and 5.06, (D) sinigrin (10^{-2} M) plus P1 sample (C), (E) sinigrin (10^{-2} M), (F) sucrose (10^{-3} M). N_1: impulses from inorganic salt receptor cells, R: impulses from "deterrent taste" receptor cells, S_1: impulses from sucrose receptor cells.

to P1 in Ss-lateral. Sinigrin (10^{-2} M) plus P1 or sinigrin (10^{-2} M) only elicits action potentials in another cell of this sensillum, the so-called R cells (deterrent receptor) (Figure 2D, E). Figure 2F depicts the response to sucrose solution (10^{-3} M) as the control and shows activity of predominantly one cell (S_1).

Figure 3 shows the impulses elicited from Ss-medial of *Bombyx mori* in response to deterrent substances; the Ss-lateral appear to be insensitive to deterrents (Hirao and Arai, 1990; Hirao et al., 1993). Figure 3A and 3B show the responses to NaCl or a sample prepared from the carrier fluid of HPLC as control. In this sensillum, the N_2 and N_2' impulses, based on the level of amplitude, originate in two different salt receptors. Figure 3C shows impulses recorded from the R receptor cell. Salicin (10^{-3} M) plus P1 show similar impulse patterns as with P1 only, but numerous impulses are observed when stimulated with salicin plus P1 (Figure 3D). The number of impulses elicited by salicin (10^{-3}

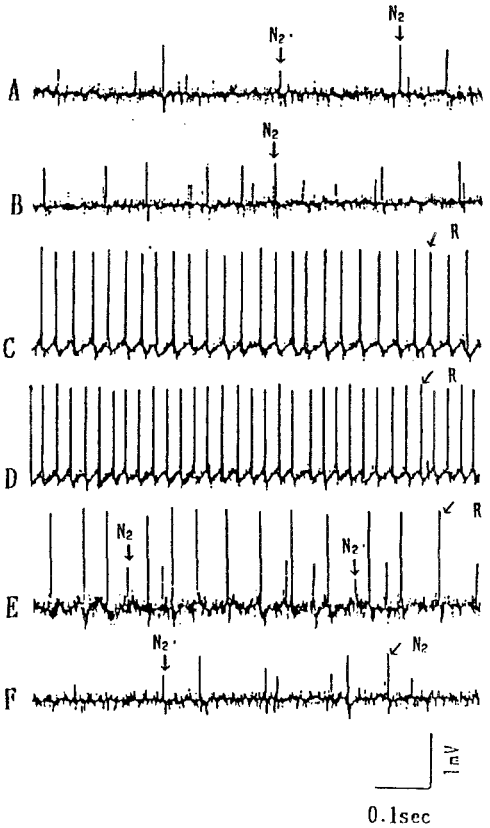

FIG. 3. The impulses discharged from Ss-medial of *Bombyx mori*. (A) NaCl (5×10^{-2} M), (B) sample prepared from carrier fluid of HPLC, (C) P1 sample prepared from 1 g dry powder of royal fern, *Osmunda japonica*, leaves in 10 ml distilled water containing trace NaCl; P1 is both peaks occurring at retention times of 4.82 and 5.06, (D) salicin (10^{-3} M) plus P1 sample (C), (E) salicin (10^{-3} M), (F) sinigrin (10^{-2} M). N_2, N_2': impulses from inorganic salt receptor cell, S_1: impulses from sucrose receptor cells, R: impulses from "deterrent taste" receptor cells.

M) only is lower (Figure 3E) than for P1 (Figure 3C) or salicin (10^{-3} M) plus P1 (Figure 3D). Sinigrin (10^{-2} M) elicits responses from the Ss-lateral of *Spodoptera litura* (Figure 2E), but not from the Ss-medial of *Bombyx mori* (Figure 3F) (see Hirao et al., 1993).

Figure 4 shows the impulses evoked in the Ss-medial of *Bombyx mori* by *Osmunda japonica* extracts at various concentrations. The number of impulses per second decreases at dilutions of 1/10 and 1/100 (Figure 4B,C). Figure 5 shows the dose–response curve of impulses discharged from R cells in the Ss-

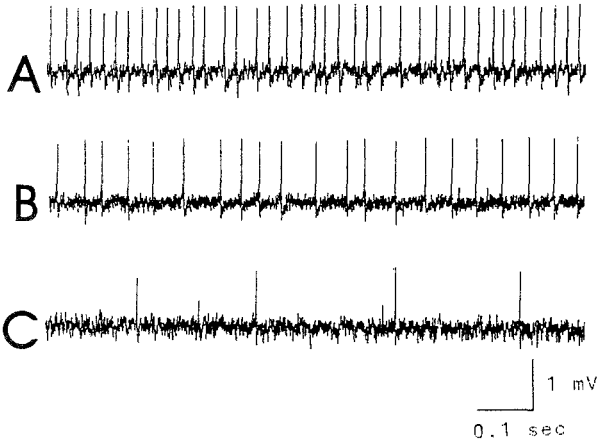

FIG. 4. The impulses discharged from R cells in Ss-medial of *Bombyx mori*. P1 is both peaks occurring at retention times of 4.82 and 5.06. (A) P1 sample prepared from 1 g dry powder of royal fern, *Osmunda japonica*, leaves in 10 ml distilled water containing trace NaCl. (B) 1/10 dilution sample of (A), (C) 1/100 dilution sample of (A).

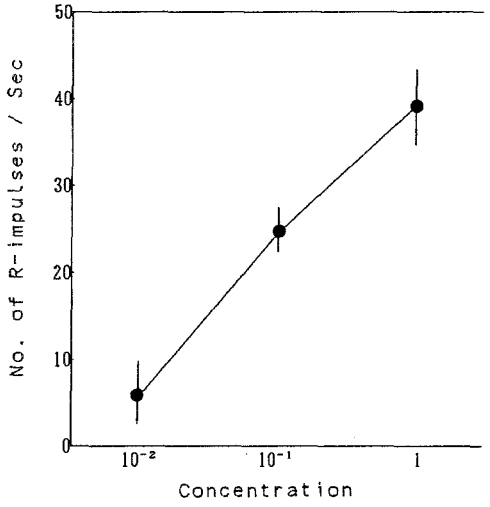

FIG. 5. The relationship between concentration of samples of P1 and the number of impulses discharged from R cells in the Ss-medial of *Bombyx mori*. P1 is both peaks occurring at retention times of 4.82 and 5.06. Vertical bar of each point indicates mean value of SEM ($N = 7$).

medial for *Osmunda japonica* extracts. Next, we carried out a consumption test of the artificial diet containing the P1 sample by *Bombyx mori* (Figure 6); it was found that P1 prevents feeding at a 120 ppm and that P1 clearly is a feeding deterrent.

From the results of the God podlk and phenol methods following beta-glucosidase hydrolysis of the sample, it was found that the sample contains glucosides with a beta-bond structure (sinigrin does not contain a beta-bond). Furthermore, from the chromatogram following beta-glucosidase hydrolysis to concentrated sample peak, re-collected through HPLC-RID, a peak indicating the presence of free glucose (R_t = 8.52 min) was detected (Figure 7A and B). Salicin is a substance typical of glucosides, possessing beta-bond. Therefore we compared the UV absorbance spectra of salicin and the sample. As shown in Figure 8, the UV absorbance pattern of salicin differs from that of the sample. The sample contains a noncyclic aglycon. A glucoside possessing a beta-bond and a noncyclic aglycon is methylglucoside. Therefore, we tested with HPLC the retention time of methylglucoside. It appeared, however, that the retention time of methylglucoside (6.1 min) differs from that of the sample.

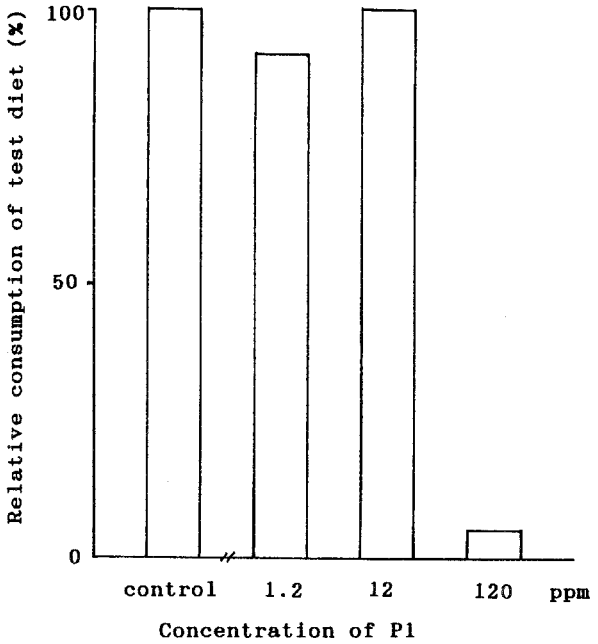

FIG. 6. The consumption of test diet during a 72-hr feeding period by newly ecdysed last-instar *Bombyx mori* larvae. Relative intake of test diet indicates the ratio to intake of P1-free diet (N = 10, at each concentration). P1 is both peaks occurring at retention times of 4.82 and 5.06.

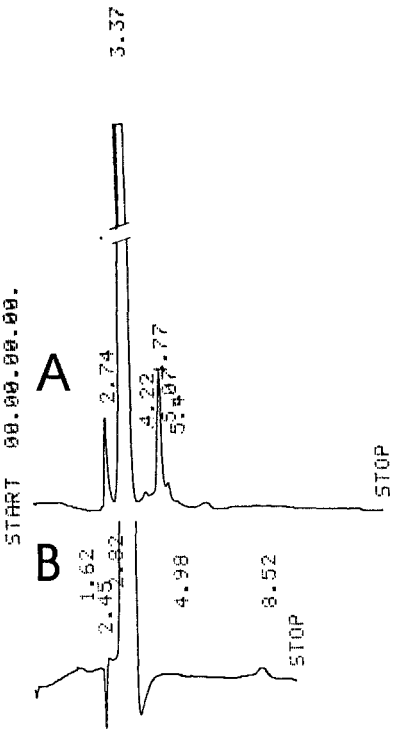

FIG. 7. Chromatograms of HPLC-RID. (A) Rechromatogram of sample peak collected through HPLC-RID and concentrated. (B) Chromatogram following beta-glucosidase (1 mg/1 ml PBS) addition to collected and concentrated sample peak. Beta-glucosidase hydrolysis was carried out for 10 min at 37°C.

DISCUSSION

The active compound present in the leaves of the royal fern, *Osmunda japonica*, is water- and alcohol-soluble and discharges the R receptor cells in *Bombyx mori*, but not in *Spodoptera litura*. The royal fern is an acceptable nonhost plant (De Boer et al., 1992) of *Spodoptera litura*. For sensitivity from other sensilla on the larvae tested, it is obvious that the P1 sample does not elicit electrical responses in Ss-lateral of *Bombyx mori* and in Ss-medial of *Spodoptera litura* (Shimizu et al., unpublished data). Therefore, it seems that these sensilla could not contain a receptor for this material.

A compound that stimulates the R receptor cells in the Ss-medial of *Bombyx mori*, but not the R receptor cells in the Ss-lateral of *Spodoptera litura*, is salicin (Hirao et al., 1993). Salicin is a glucoside, like the unknown compound in the royal fern extracts. However, the unknown compound is not identical to salicin,

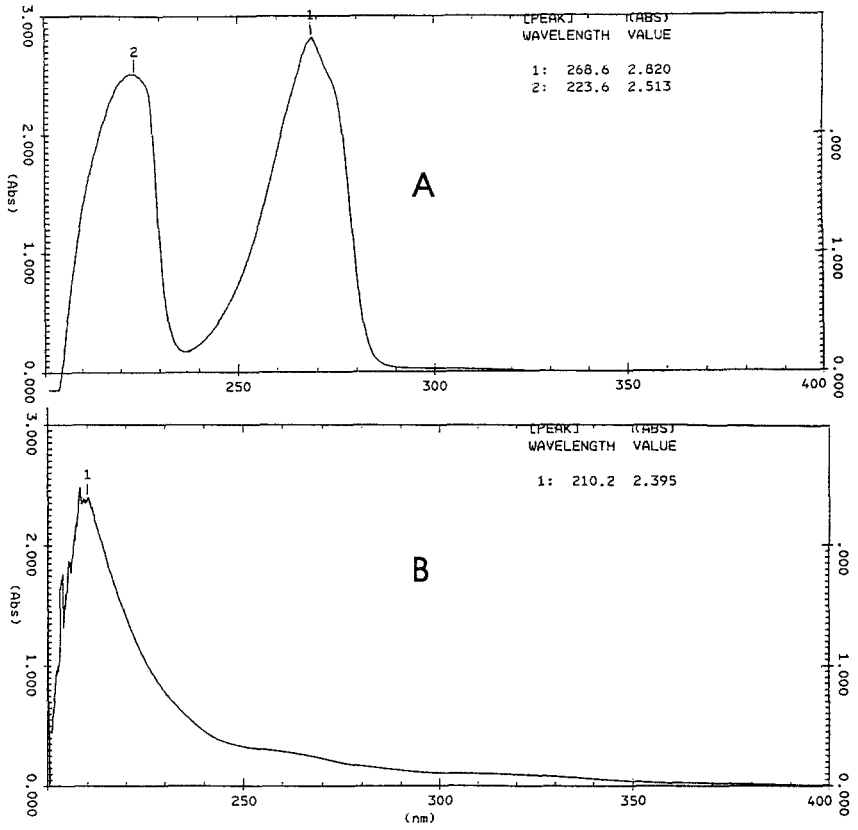

FIG. 8. The UV absorbance spectrum of salicin (A) and the isolated sample (B) with HPLC-RID.

which concluded from differences in their UV absorbance spectra. Compounds that may be detected by RID are sugars, alcohol, inorganic ions, amino sugars, and uronic acid. According to its beta-glucosidase degradation reaction, the unknown compound is a glucoside. Responses of the glucoside receptor in the lateral Ss have been reported in the larvae of *Mamestra brassicae* (Wieczorek, 1976) and *Pieris brassicae* (Schoonhoven, 1969), and these data may be helpful in the identification of our unknown compound.

From the Ss-medial response patterns to diluted samples (Figure 5) and from feeding responses (Figure 6), it may be suggested that this compound, which is heat stable (100°C for 5 min) in preparation of artificial diet, functions as a feeding deterrent at low concentrations to *Bombyx mori*, but not to *Spodoptera litura*. It thus has a species-specific activity. In *Morus alba*, no

deterrent substance that stimulates the Ss-medial of *Bombyx mori* has been found (Hirao and Arai, 1991; Yazawa et al., 1991). Other plant extracts that stimulate the R receptor of the medial Ss in *Bombyx mori* are those of *Ulmus pumila*, *Prunus donarium* (Hirao and Arai, 1991), and *Ulmus parvifolia* (Yazawa et al., 1991). This P1 is absent in the mulberry, *Morus alba* (Yazawa et al., 1992) and in sprouting broccoli, *Brassica oleracea* var. *italica* (Shimizu and Yazawa, 1993).

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DETERMINATION OF RELEASE RATES OF PHEROMONE DISPENSERS BY AIR SAMPLING WITH C-18 BONDED SILICA

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Abstract—Release rates of pheromones from commercial dispensers for mating disruption in *Lobesia botrana* and *Cydia pomonella* have been measured by sampling an air stream with C-18 bonded silica (Sep-Pak cartridges) followed by extraction and gas chromatographic analysis. The flow chamber is made from inexpensive materials that can be replaced when contaminated. The results correspond with those obtained by gravimetry; the method supplies additional information on the composition of the airborne material.

Key Words—Sex pheromone, release rate, pheromone dispenser, mating disruption, *Lobesia botrana*, *Cydia pomonella*, Lepidoptera, Tortricidae, (*E*,*E*)-8,10-dodecadien-1-ol, (*E*,*Z*)-7,9-dodecadienyl acetate.

INTRODUCTION

The effectiveness of mating disruption in pest control has been widely demonstrated (Ridgway et al., 1990). However, the manufacture of dispensers that release the necessary amount and quality of pheromone over the growing season has proven difficult. Therefore, developers and growers both need rapid methods of quality control.

The release of pheromones from dispensers has been measured by collecting and analyzing the entire effluvia (Hirooka and Suwanai, 1978; Weatherston et al., 1981; Golub et al., 1983; McDonough and Butler, 1983; Leonhardt et al., 1988; Van Der Kraan and Ebbers, 1990). To avoid errors arising from dead space and adsorption, we are using an open system that is described below.

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METHODS AND MATERIALS

A flow chamber was constructed by stacking clear polystyrene cups of 10.5–11.5 cm ID and 14 cm height whose bottoms had been removed and that were lined with polyvinylidene chloride foil (Saran wrap, Dow Chemical Company) to avoid absorption of pheromone into the polystyrene (Figure 1). A 12-V cooling fan attached to the bottom of the first cup pushed ambient air through the apparatus at 10 cm/sec, as measured by a ThermoAir 2 electronic anemometer (Schiltknecht Messtechnik AG, Gossau, Switzerland). The dispenser was hung from nylon gauze of 1.5 mm opening. The turbulence created by the stationary fan cut from 0.1 mm aluminum produced a fairly uniform distribution of the material evaporated, as was demonstrated with TiO_2 smoke.

A Sep-Pak C18 Cartridge (Waters Chromatography Division, Millipore Corporation, Milford, Massachusetts) connected to a water aspirator was placed in the airstream 15 cm above the dispenser for collection of volatiles. The flow was set at 1000 ml/min, as measured by a rotary flowmeter. The use of Sep-Pak cartridges for collecting airborne pheromones has previously been reported by McDonough et al. (1989).

Before the experiment, the cartridge was rinsed with 20 ml hexane from a syringe. Then 200 μl water was added to deactivate possible traces of free silica, dried with vacuum, and the internal standard containing 1000 ng decyl acetate

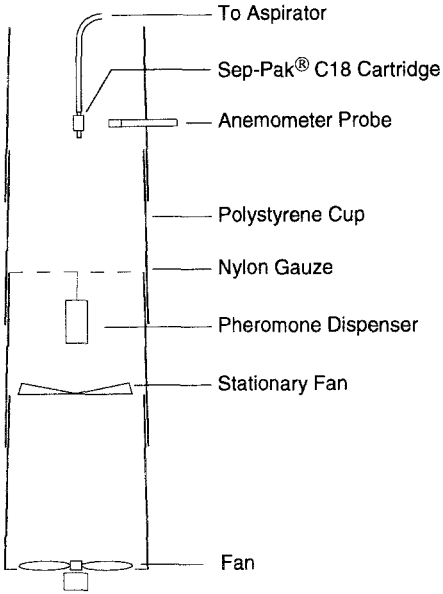

FIG. 1. Apparatus for release rate measurement.

(10:Ac) and 1000 ng tetradecyl acetate (14:Ac) in 50 μl hexane was applied to the cartridge.

Dispensers (unused or field-exposed) were kept in sealed polyethylene–polyvinylidene chloride-laminated paper bags at 4°C before release rate determination. A dispenser was placed in the apparatus 2 hr before the measurement. Then air was sampled for 1 hr. The cartridge was extracted with 4 ml hexane and the syringe standard containing 1000 ng pentadecane (15:Hy) in 50 μl hexane was added. One microliter of this was injected into the gas chromatograph for analysis. The amount of internal standard was the same in all experiments.

A Carlo Erba Mega Series 5350 gas chromatograph equipped with an on-column injector, a 30-m DB-5 fused silica column (0.33 mm, film thickness 0.35 μm, J&W Scientific, Folsom, California), and a flame ionization detector was used for analysis.

For quantitation, a standard solution containing a known amount of pheromone in 4 ml hexane was spiked with the syringe standard and 1 μl injected onto the GC column. The following values were entered into the calculation: p_x, peak area of pheromone in sample; p'_x, peak area of pheromone in standard solution; p_i, peak area of syringe standard (15:Hy) in sample; p'_i, peak area of syringe standard (15:Hy) in standard solution; a'_x, amount of pheromone in standard solution; q, ratio between the volume of air collected and the total volume flowing through apparatus (0.019 in our set-up); and t, time of air collection.

The release rate, r, is then calculated as follows:

$$r = \frac{p_x \cdot p'_i \cdot a'_x}{p_i \cdot p'_x \cdot t \cdot q}$$

where $(p'_x/p'_i \cdot a'_x)$ is the conversion factor of peak area to the amount of test compound in the standard solution.

RESULTS AND DISCUSSION

Recovery and Breakthrough. Heptadecane (17:Hy), dodecyl acetate (12:Ac), and (*E*,*E*)-8,10-dodecadien-1-ol (*E*8,*E*10–12:OH), 1000 ng each in 50 μl hexane, and the internal standard solution were added to a cartridge. The experiment was carried out with and without pretreatment of the cartridge with water. Half of the cartridges were extracted after 1 min, in the others, a 60-liter air blank was collected before extraction. The results are given in Table 1.

Recovery of acetates and hydrocarbons was at least 70% in all cases. The ratio between recovered 10:Ac and 14:Ac, which were used as internal standards, was constant, indicating that no significant breakthrough due to volatility

took place. Recovery of *E*8,*E*10–12:OH was between 50 and 60%; high variation was notably observed when no water was added to the cartridge before collection. Other experiments (not shown) indicated that without addition of water, the recovery is generally lower than in the presence of water.

In one case we extracted a cartridge with an additional 4 ml of dichloromethane. No significant amount of substances was found in the extract.

Release of Dodecyl Acetate. A dispenser was prepared by placing 40 mg 12:Ac in a piece of polyethylene tubing of 0.7 mm ID and 1.4 mm OD, which was sealed at both ends. This type of dispenser was used in previous field trials with the plum fruit moth, *Grapholita funebrana* (Arn et al., 1976). The results (Table 2) show some variation between determinations and a temperature

TABLE 1. PERCENT RECOVERY OF TEST COMPOUNDS (MEANS ± SE) FROM SEP-PAK C18 CARTRIDGES WITH PRETREATMENT AND AIR SAMPLING

Water added	yes	yes	no	no
Air sampled	none	601	none	601
Replicates (*N*)	5	4	1	5
Decyl acetate	83 ± 8	89 ± 5	98	83 ± 7
Dodecyl acetate	81 ± 6	81 ± 6	81	72 ± 2
Tetradecyl acetate	88 ± 2	86 ± 6	94	85 ± 7
(*E*,*E*)-8,10-Dodecadien-1-ol	71 ± 11	54 ± 7	64	53 ± 17
Heptadecane	80 ± 4	82 ± 8	80	77 ± 2

TABLE 2. RELEASE RATES OF DODECYL ACETATE FROM POLYETHYLENE TUBING (0.7 mm ID, 1.4 mm OD) AS MEASURED BY AIR COLLECTION AND WEIGHT LOSS

Date	Collection temperature (°C)	Air collection technique, release rate (μg/hr)	Weight loss technique	
			Release rate (μg/hr)	Sampling time (hr)
8/26/92	29	301		
		294		
9/30/92	26–27	114	145	5.5
		180		
		257		
		167		
9/30/92 to 10/1/92			163	15
10/1/92	26	177	117	12
		138		
		151		

dependence, but generally a good correlation between air collection and weight loss.

Release of (E,Z)- *and* (E,E)-*7,9-Dodecadienyl Acetate.* Dispensers used for mating disruption in *Lobesia botrana* (BASF Aktiengesellschaft, Limburgerhof, Germany) were exposed from 25 May to 26 August 1992. Two were facing south and exposed to daylight, and two were facing north and shaded. Unused dispensers were evaluated as a comparison.

The data (Table 3) show that dispensers exposed for three months still released between 50 and 100% the amount of unused dispensers. An effect of field exposure on isomer ratio could not be observed; one dispenser that had not been exposed showed a higher content of *E,E* isomer in the vapor phase than the rest; an unusually high proportion of *E,E* isomer was also found in the substance contained in its reservoir.

When a dispenser was shaken or placed on the side, the subsequent release rate was increased by a factor of up to 3, presumably because the surface of polymer in contact with the pheromone had been increased by this treatment.

Release of (E,E)-*8,10-Dodecadien-1-ol.* Dispensers of *E*8,*E*10–12:OH from Shin-Etsu Corporation (Biocontrol Ltd., Davis, California) for mating disruption in the codling moth, *Cydia pomonella*, were exposed as described for *Lobesia botrana*. Similar to the dispensers for *Lobesia botrana*, dispensers exposed for three months released about one third to one half that of unused ones, with only minor differences between those exposed to sunlight and those placed in the shade (Table 4). The values obtained for unused dispensers are of the same order as (although slightly higher than) those reported by Brown et al. (1992) and McDonough et al. (1992) for the same brand of dispenser (approximately 75 μg/hr at 29°C and 20 cm/sec airspeed).

TABLE 3. RELEASE RATES AND ISOMER COMPOSITION OF 7,9-DODECADIENYL ACETATE (*E,Z* AND *E,E*) OF BASF DISPENSERS OF DIFFERENT EXPOSURE HISTORY

Dispenser	Exposure	Collection temperature (°C)	Release rate of *EZ* + *EE* (μg/hr)	*EZ* in *EZ* + *EE* released (%)	*EZ* in *EZ* + *EE* in reservoir (%)
1	unexposed	25	132	68	59
2	unexposed	25	184	80	n.d.
3	3 months shade	26	89	77	73
4	3 months shade	26	163	79	n.d.
5	3 months sun	25	71	80	79
6	3 months sun	25	92	76	n.d.

TABLE 4. RELEASE RATES OF (*E*,*E*)-8,10-DODECADIEN-1-OL FROM TWO BATCHES OF SHIN-ETSU DISPENSERS

	Batch 21008		Batch 31116	
Dispenser exposure	Collection temperature (°C)	Release rate (μg/hr)	Collection temperature (°C)	Release rate (μg/hr)
unexposed	29	108	26	87
3 months sun	24	38	24	46
3 months shade	23	48	24	52

CONCLUSIONS

These results demonstrate that quality control of dispensers for mating disruption can be achieved by collection of volatiles in an open system with C-18 bonded silica. The advantage of this technique is the use of a simple and commercially available collection device and an inexpensive apparatus that can be replaced if contaminated.

Future versions of the apparatus could provide better control of temperature and airflow and allow simulation of various atmospheric conditions. The method allows monitoring the performance of individual dispensers throughout the season. Its advantage over gravimetric techniques lies in the possibility of determining the actual composition of the volatiles and to follow changes such as isomerization of pheromones during field exposure.

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AGGREGATION ARRESTANT PHEROMONE OF THE GERMAN COCKROACH, *Blattella germanica* (L.) (DICTYOPTERA: BLATTELLIDAE): ISOLATION AND STRUCTURE ELUCIDATION OF BLATTELLASTANOSIDE-A AND -B

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Abstract—The aggregation pheromone of the German cockroach, *Blattella germanica*, consists of attractant and arrestant, which can be detected by olfactometer and choice-chamber assay, respectively. Both were extracted from the frass-contaminated filter paper being used as a shelter. They were separated by solvent partition with *n*-butanol and water. The arrestant from the *n*-butanol phase was purified by open column chromatography and then successive HPLC isolated two major arrestant components. Spectral evidence from SI-MS, HR-EI-MS, and NMR experiments with pulse techniques provided possible structures as 1-(6α-chloro-4β,5β-epoxy-5β-stigmast-3β-yl)-β-D-glucopyranoside and 1-(6α-chloro-5β-hydroxy-5β-stigmast-3β-yl)-β-D-glucopyranoside, denoted as blattellastanoside-A and blattellastanoside-B, respectively. They represented arrestant activity as median effective doses (ED_{50}) at 0.044 (A) and 3.2 (B) nmol on 1.0 cm^2 of Whatman No. 1 filter paper.

Key Words—Dictyoptera, Blattellidae, *Blattella germanica*, German cockroach, cockroach, arrestant, orientation, pheromone, aggregation pheromone, steroid, blattellastanoside.

INTRODUCTION

The German cockroach, *Blattella germanica* (L.), is known to be gregarious, and its aggregation is believed to depend largely on an olfactory response to

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chemicals produced by the insect itself (Ledoux, 1945). The aggregation behavior was reinvestigated in depth, and the chemical cue responsible for the aggregation was found to be spotted onto the surface of their harboring place together with the frass (fecal materials). The insect of every instar and sex is involved in production as well as reception of the chemical; hence it was categorized as an aggregation pheromone (Ishii and Kuwahara, 1967, 1968).

Since then, the isolation and identification of the pheromone have been the objects of research. Several attempts were made and *p*-cresol (Nakajima, 1973), benzoic acid (Nakajima et al., 1974), fatty acids (Ritter and Persoons, 1975), and *l*-lactic acid (McFarlane and Alli, 1986) were isolated as candidates, although their activity was somewhat insufficient to account for the original activity.

These isolations were guided principally by the original bioassay method, which is based on the comparison between the numbers of insects on the sample and control filter papers standing vertically on the bottom of a cup (Ishii and Kuwahara, 1967, 1968; Ishii, 1970). This vertical paper assay has been adopted as the standard method for monitoring the cockroach aggregation and/or dispersion behavior (Bell et al., 1972; Nakayama et al., 1984; McFarlane, 1984; Ross and Tigner, 1986). Nevertheless, it hardly evaluates the behavioral mechanisms independently of each other, so consequently it often provides ambiguous outcomes (Sakuma and Fukami, 1985, 1991). Since the difficulty of this study could be ascribed to the lack of properly designed assay systems, the combined use of several behaviorally discriminating assay methods was believed to be appropriate. The behavioral analysis suggested the possibility that both attractant and arrestant components may function in the aggregation (Ishii and Kuwahara, 1967).

We began by investigating the remote olfactory behavior of the insects exerted by volatile attractants with a linear-track olfactometer (Sakuma and Fukami, 1985). Odor-conditioned anemotaxis and chemotaxis were evaluated quantitatively in the different airflow modes, and not only did the frass-contaminated paper exert strong responses on the insect but its methanol extract also did so (Sakuma and Fukami, 1990a). Isolation guided by the olfactometer assay afforded 1-dimethylamino-2-methyl-2-propanol, ammonia, and several alkylamines as hydrochlorides (Sakuma and Fukami, 1990b).

These amine hydrochlorides, however, exerted no arrest response on the test insects when the chemicals were applied to the vertical paper assay (Sakuma and Fukami, 1991). It is obvious from the original observation that the frass-contaminated paper promotes settlement of the insects (Ishii and Kuwahara, 1967). Therefore, it was suggested that the unknown arrestant component(s) of the aggregation pheromone still remained in the frass-contaminated paper (Sakuma and Fukami, 1991).

We improved the vertical paper assay so that it can distinguish the preference of each insect to the chemical from the interaction between individuals

and other indefinable factors. This choice-chamber assay uses many small cells, and the floor of each cell was contiguously divided into sample and control areas. Only one test insect is confined in the floor of each cell, and after a standard time, the preference observed in each cell was totaled to calculate the arrestant activity (Sakuma and Fukami, 1991).

Isolation of two major arrestant components (called blattellastanoside-A and blattellastanoside-B) from the extract of frass-contaminated paper as well as the structures have been briefly reported (Sakuma and Fukami, 1993a,b). In this article, the bioassay system, isolation, and structure elucidation of blattellastanosides-A and -B are described in detail.

METHODS AND MATERIALS

Insects. Blattella germanica (L.) were reared on insect diet pellets (Oriental Yeast Co., Tokyo) at 29 ± 1°C, 50 ± 10% relative humidity, and on a 14 : 10 hr light–dark regime (Sakuma and Fukami, 1985). Test insects were a mixture of first-and second-instar nymphs isolated from the colony at one to six days posteclosion. They were fed diet and water to satiation before the assay to exclude any behavior biased towards foraging.

Choice-Chamber Assay. In this assay (Sakuma and Fukami, 1991), strips of sample and control paper (Whatman No. 1, 2.2 × 24.2 cm, three strips each) were aligned alternately on a glass plate (15 × 20 × 0.2 cm), folded at both ends, and fixed with sticky tape onto the back side of the plate. A polystyrene grating (inner frame of an FC-2 freezing container, Nichiden-Rika Glass Co. Ltd., Kobe; 11 × 18 × 2 cm, divided into 5 × 8 = 40 cells) was placed on the plate so that the floor of each cell (2 × 2 cm) could be split into sample and control areas in the center. The grating had been coated with talcum powder to prevent the test insects from climbing walls and to confine them to the floor. One nymph was introduced into each cell and then the grating was covered with another glass plate and a paper card (white fine paper 128 g/m^2, 12.8 × 18.2 cm). The translucent card diffused 200 lux of fluorescent light illumination to 28 lux at the floor of the cell. The assembly was fastened by rubber bands and set on a level table. Nymphs on the sample area were counted by shifting the paper, while this exposure to light scrambled the insects and reset the behavioral process. Response was observed every 15 min 30 min after the start, and five observations of 40 subjects were totaled (N = 200). Assays were conducted during the day, at 25 ± 0.1°C, 50 ± 6% relative humidity.

Statistics. The response was expressed in terms of excess proportion index (I), i.e., the proportion of the difference between numbers of nymphs in sample and control areas to the total (Sakuma and Fukami, 1985). Doses were denoted as GFE, i.e., equivalent to 1 g of the original extract of the frass-contaminated

paper. Decimal dilutions in methanol were applied onto the paper strips (Whatman No. 1, 160 cm^2 in total). Probit analysis (parallel line assay) on sets of dose–response estimated median effective doses (ED_{50}) and relative potencies (R) to the origin (100%) (Finney, 1978; Sakuma and Fukami, 1985, 1990a,b) to compare the activity of each fraction.

Extraction and Fractionation of Shelter-Paper Material. After three months of contamination, the shelter paper (Toyo No. 2, 0.92 m^2/container) was soaked in methanol, and the extract was then concentrated with a flash-evaporator under reduced pressure. A container-full of shelter paper produced ca. 5 g of crude extract. The first lot (585 g) was the same as that used in the attractant isolation (Sakuma and Fukami, 1990b), and this together with the second lot (788 g), served for the isolation of arrestants.

The purification scheme, as well as relative weight and potency of the first lot, is illustrated in Figure 1. The original methanol extract was washed with *n*-hexane five times, concentrated to a viscous paste in vacuo, and then partitioned with *n*-butanol and water. Attractants were transferred to the aqueous layer (Sakuma and Fukami, 1990b), whereas all the arrestant activity remained in *n*-butanol. Basic and acidic components were extracted from the *n*-butanol layer with 1 N hydrochloric acid and 1 N ammonia water, respectively, but the activity remained in *n*-butanol as a neutral fraction. Throughout the process, the pH was controlled between 2 and 9 to preserve the activity. The neutral fraction was purified twice by stepwise elution from an ODS open column (Cosmosil 75 C_{18}-OPN; Nacalaitesque, Kyoto, 500 g). Elution with MeOH–H_2O–trifluoroacetic acid (90:1:10:0.2, v/v/v) gave an active fraction, and after readsorption of the fraction onto the column, the eluate with MeOH–H_2O–triethylamine (95:5:0.1, v/v/v) was found to be most active. The eluate was then applied onto a silica gel column (Silica gel 60, 0.063–0.200 mm, E. Merck, 500 g), and the elution with chloroform–MeOH (90:10, v/v) concentrated the active material into 0.5% of the original weight. This fraction was subjected to semi-preparative HPLC leading to the isolation of the chemicals afterwards.

HPLC Instruments and Conditions. The HPLC unit consisted of a rotary injector with a 1.0-ml sample loop, Altex 110A solvent metering pump, and Waters R401 differential refractometer (RID). A stainless steel column (25 × 2.0 cm ID) was loaded with ODS (Cosmosil 5C_{18}-AR, spherical 5 μm particle, Nacalaitesque, Kyoto) and was then dipped into a water bath, with the temperature controlled at 35 ± 0.1°C. The solvent system used for the reversed phase was MeOH–H_2O (92:8, v/v), flow rate at 9.9 ml/min. A Waters RCM 25 × 10 cartridge holder was loaded with a PrepPak cartridge (10 × 2.5 cm ID, packed with Prep Nova Silica HR 60 A 6μ, spherical 6-μm particle) and was pressurized before use. Chloroform–isopropanol (80:20, v/v), flow rate at 9.9 ml/min, was used as eluent for the silica gel column.

Spectral Instrumentation. The EI-MS spectra at 70 eV, including high-

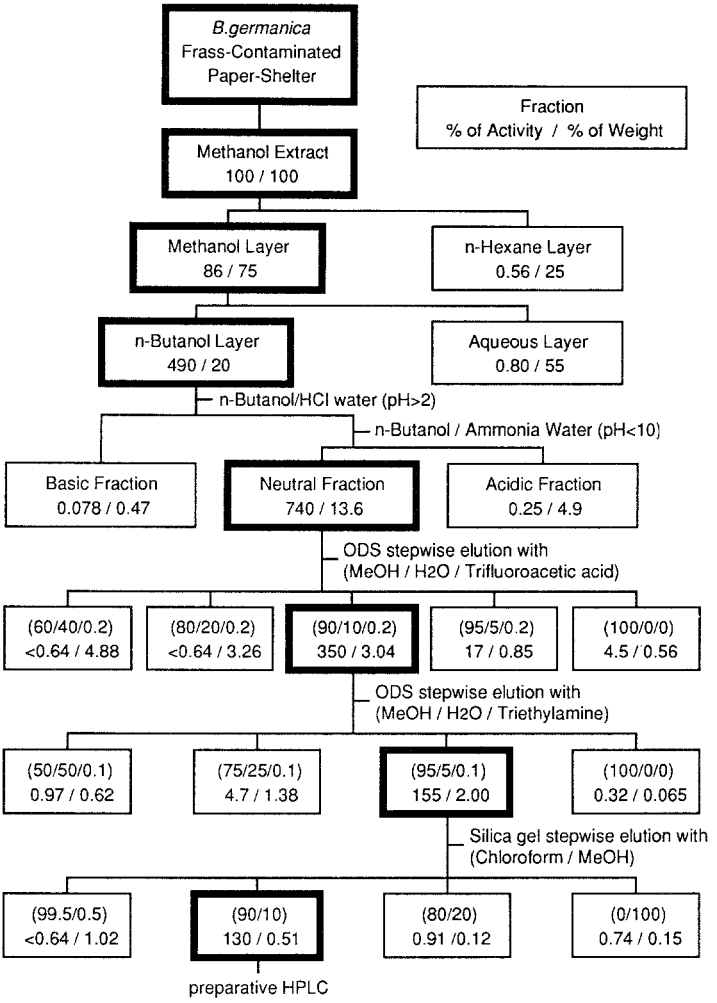

FIG. 1. Purification scheme of arrestant pheromones. Heavy bold frames indicate the active fractions. Figures in each frame represent the percentage of weight and activity in comparison to the original methanol extract of the frass-contaminated paper.

resolution mode (HR-EI-MS), were recorded on a Hitachi M-80 double-focusing mass spectrometer equipped with an M-003 data processing system at the Pesticide Research Institute, Kyoto University. An ion source temperature was kept at 180°C and sample materials were introduced with an in-beam direct insertion technique. The positive SI-MS spectra in glycerol matrix and negative SI-MS in *m*-nitrobenzyl alcohol were acquired from Hitachi Instrument Engineering

Co., Ltd. with a Hitachi M-2500 double-focusing mass spectrometer operating at a primary ion accelerating voltage of 8 kV onto Xe gas. Daughter ions of a candidate molecular ion were observed by B/E linked-scan operation. For NMR spectra, a JEOL GX-400 FT-NMR spectrometer at the Chemical Research Institute, Kyoto University, was used. Samples were dissolved in chloroform-*d* and the spectra were recorded at 25°C. In addition to ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra, those with various pulse techniques as ^1H–^1H homonuclear COSY with 45° pulse interval (H–H COSY), NOESY, ^1H–^{13}C heteronuclear COSY (H–C COSY), COLOC, DEPT, INEPT/N, and NOE difference spectra were recorded. The chemical shifts were referenced to TMS.

RESULTS

Isolation of Arrestants by HPLC

Aliquots of the prepurified material (30 GFE, 150 mg/500 μl) were subjected to reversed-phase chromatography with the ODS column. The eluate was collected into 15 fractions according to the RID signal, and four fractions with peak top at 20, 31, 38, and 47 min were found to be active. Rechromatography of each fraction with the silica gel column gave an active fraction as a single peak, and the corresponding arrestant components were denoted as compounds **4** (<4 mg), **1** (15.0 mg, $[\alpha]_D^{20}$ − 23.0° [c 1.0, ethanol]), **2** (147.6 mg, $[\alpha]_D^{20}$ + 3.67° [c 6.8, ethanol]), and **3** (<8 mg), respectively. The most active fraction was that for compound **1** (R = 42%), followed by **2** (6.5%), **3** (2.9%), and **4** (<1.9%). Compounds **1** and **2** were the major arrestants in terms of their activity and content, and were subjected to the spectral analysis, whereas compounds **3** and **4** were left for subsequent work.

Mass Spectra of Compounds 1 and 2

The molecular weight of each compound was estimated from the negative and positive SI-MS spectra (Table 1). The negative ion spectrum showed $(M\text{-}H)^-$ ion at *m/z* 625 (compound **1**), 627 **(2).** The positive ion spectrum afforded not only the $(M + H)^+$ ion at *m/z* 627 **(1),** 629 **(2),** but also its fragment ion at *m/z* 465 **(1),** 467 **(2),** verified by a B/E linked-scan operation as a dominant daughter ion. The leaving group of 162 m.u. was reduced to a (hexose − H_2O), so the fragment ion of each compound was assigned as $(\text{aglycon} + H)^+$: $(\text{Agl} + H)^+$.

The EI-MS spectrum did not present an observable $M^{+\cdot}$, but exhibited $Agl^{+\cdot}$ at *m/z* 464 **(1),** 466 **(2).** An HR-EI-MS system determined $Agl^{+\cdot}$ of compound **1** as $C_{29}H_{49}ClO_2$ (calc. 464.3418; obs. 464.3420), that of **2** as $C_{29}H_{51}ClO_2$ (calc. 466.3574; obs. 466.3491), and the common base peak ion

TABLE 1. SI-MS SPECTRAL DATA OF COMPOUNDS **1** AND **2**

Positive ions (glycerol matrix)			Negative ions (*m*-Nitrobenzylalcohol matrix)		
	m/z value			*m/z* value	
Fragment ion	**1**	**2**	Fragment ion	**1**	**2**
$[M + Na]^+$	649	651	$[M + matrix]^-$	779	781
$[M + H]^+$	627	629	$[M + Cl]^-$	661	663
$[M\text{-}H\text{-}H_2O]^+$	607	609	$[M - H]^-$	625	627
$[M\text{-}HCl + H]^+$	591		$[Agl\text{-}H_2O\text{-}H]^-$	445	
$[Agl^a + H]^+$	465	467			
$[Agl\text{-}H_2O + H]^+$	447	449			
$[Agl\text{-}HCl + H]^+$	429	431			
$[Agl\text{-}H_2O\text{-}HCl + H]^+$	411	413			
$[Agl\text{-}C_4H_7OH + H]^+$	393	395			

[a] Agl denotes aglycone.

at *m/z* 394 as $C_{25}H_{43}ClO$ (calc. 394.3000; **1** obs. 394.3042; **2** obs. 394.2986). The presence of a chlorine atom in each ion was supported by an accompanying isotopic ion at +2 m.u. The overall compositions with a hexose are $C_{35}H_{59}ClO_7$ **(1)** and $C_{35}H_{61}ClO_7$ **(2).**

EI-MS m/z (%). Compound **1:** 464 $[Agl]^+$ (7), 447 $[Agl\text{-}OH]^+$ (18), 429 $[Agl\text{-}OH\text{-}H_2O]^+$ (14), 428 $[Agl\text{-}HCl]^+$ (10), 412 $[Agl\text{-}OH\text{-}Cl]^+$ (17), 411 $[Agl\text{-}H_2O\text{-}Cl]^+$ (18), 395 $[Agl\text{-}C_4H_5O]^+$ (53), 394 $[Agl\text{-}C_4H_6O]^+$ (100), 359 $[Agl\text{-}C_4H_6O\text{-}Cl]^+$ (15), 281 $[Agl\text{-}C_{13}H_{27}]^+$ (17), Compound **2:** 466 $[Agl]^+$ (4), 448 $[Agl\text{-}H_2O]^+$ (7), 431 $[Agl\text{-}OH\text{-}H_2O]^+$ (37), 430 $[Agl\text{-}HCl]^+$ (41), 413 $[Agl\text{-}H_2O\text{-}Cl]^+$ (36), 412 $[Agl\text{-}H_2O\text{-}HCl]^+$ (36), 396 $[Agl\text{-}C_4H_6O]^+$ (47), 395 $[Agl\text{-}C_4H_7O]^+$ (55), 394 $[Agl\text{-}C_4H_8O]^+$ (100).

Structural Elucidation of Compound 2 with NMR Spectra

The NMR spectra were acquired on 102 mg of compound **2** in chloroform-*d*. The ^{13}C NMR spectrum showed 33 signals, but the interpretation of DEPT and H–C COSY spectra showed that two of them (δ_C 12.0, 56.2) overlapped. The total carbon signals thus amount to 35, which completely agreed with the estimate from the mass spectra.

β-D-Glucopyranoside. Five OH signals disappeared from the ^1H NMR spectrum by deuterium exchange, and eight signals for nine protons remained between δ_H 3.38 and 4.46. The H–H COSY spectrum showed two of them correlated with high-field protons in the aglycon, whereas six signals for seven protons correlated sequentially, suggesting a hexose sugar as a partial structure

(Figure 2). Large coupling constants between them (ca. $J = 8$), especially for 1′-CH and 2′-CH ($J = 7.6$), indicated a pyranose structure where all the methines locate in the vicinal diaxial geometry (Table 2). These characteristics implied a β-glucopyranose and the observed ^{13}C shieldings agreed with those of methyl-β-D-glucopyranoside (Stothers, 1972) (Table 3).

Skeleton of the Aglycon. Subtraction of the sugar from the total carbon signals gave 29 for the aglycon. The oxygenated or chlorinated carbons included two methines (-CH<X) at δ_C 67.4, 74.7, and one nonprotonated carbon (>C<X) at δ_C 76.1 (X denotes a hetero atom). This was consistent with the elemental composition as $C_{29}H_{51}ClO_2$ by HR-EI-MS. Since the compound consisted of only sp^3 carbons, all four unsaturations derived from four rings in the

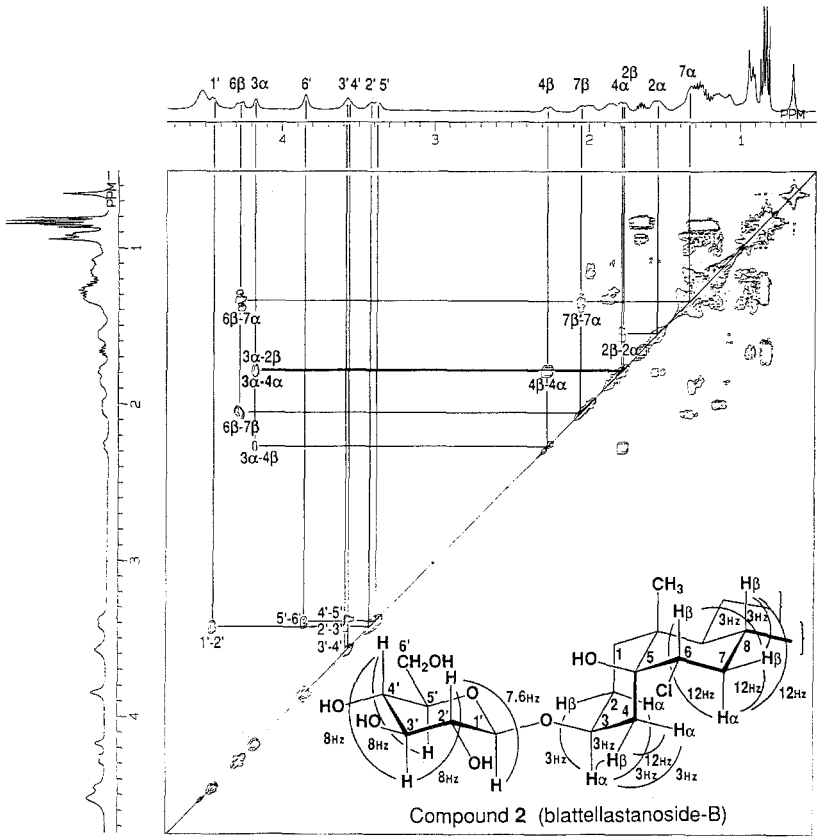

FIG. 2. H–H COSY spectrum of compound **2.** In relation to the substitution sites, the assignments of the cross-peaks are indicated as are the interpretations of the proton couplings with J values in Hz.

TABLE 2. ^{1}H NMR SPECTRAL ASSIGNMENT OF SUGAR OF COMPOUNDS **1** AND **2** (δ_H in ppm, Multiplicity, and J Values[a] in Hz)

Position	Compound **1**	Compound **2**
1′	4.60 d(7.6)	4.46 d(7.6)
2′	3.45 dd(7.6,8)	3.44 dd(7.6,8)
3′	3.62 dd(8,8)	3.57 dd(8,8)
4′	3.60 dd(8,8)	3.59 dd(8,8)
5′	3.40 m	3.38 m
6′	3.86 m	3.85 m

[a] J values were estimated from the coupling pattern.

TABLE 3. ^{13}C NMR SPECTRAL ASSIGNMENT OF SUGAR OF COMPOUNDS **1** AND **2** WITH REFERENCE TO METHYL β-D-GLUCOPYRANOSIDE (δ_c in ppm and Characterization of Carbons)

Position	Compound **1**	Compound **2**	Methyl β-D-glucopyranoside[a]
1′	101.8 −O-CH<O−	101.1 −O-CH<O−	104.2 −O-CH<O−
2′	73.8 −CH<OH	73.3 −CH<OH	74.1 −CH<OH
3′	76.4 −CH<OH	76.6 −CH<OH	76.9 −CH<OH
4′	70.1 −CH<OH	69.9 −CH<OH	70.7 −CH<OH
5′	75.8 −CH<O−	75.8 −CH<O−	76.8 −CH<O−
6′	62.2 $-CH_2-OH$	61.8 $-CH_2-OH$	61.9 $-CH_2$-OH

[a] Data from Stothers (1972).

skeleton resembling the basic structure of steroids. The DEPT experiment indicated that the aglycon comprised CH_3- (6), -CH_2- + -CH<X (13), -CH< + >C<X (8), and >C< (2), which completely agreed with the constitution of stigmastane as CH_3- (6), -CH_2- (13), -CH< (8), and >C< (2) (Table 4). The coupling pattern of six methyls as singlets (2), doublets (3), and a double doublet (1) also corresponded to the skeleton.

Sites of Substitution. The aglycone of compound **2** was expected to be a trisubstituted stigmastane with two methines (-CH<X) and one nonprotonated carbon (>C<X) attached to each of OH, Cl, and glycosidic function. The substitution sites were specified with respect to the spectral data.

Compound **2** had three nonprotonated carbons, two of which in the higher field (δ_C 43.0, 42.8) could be assigned as C10 and C13. The C10 shielding of compound **2** shifted downfield from that of 5α-cholestane (δ_C 35.5) and 5β-cholestane (δ_C 35.1) because of the substituent effects (Blunt and Stothers, 1977).

TABLE 4. ^{13}C NMR SPECTRAL ASSIGNMENT OF AGLYCONE OF COMPOUNDS **1** AND **2** WITH REFERENCE TO 5α-STIGMASTAN-3β-OL, 5β-CHOLESTAN-3β-OL (RING MEMBERS) AND β-SITOSTERYL ACETATE (SIDE CHAIN MEMBER)
(δ_c in ppm and Characterization of Carbons[a])

Position	Compound **1**	Compound **2**	5α-Stigmastan-3β-ol[b]	5β-Cholestan-3β-ol[c]
1	29.8 $-CH_2-$	26.4 $-CH_2-$	37.1 $-CH_2-$	30.0 $-CH_2-CH$
2	23.6 $-CH_2-$	25.8 $-CH_2-$	31.6 $-CH_2-$	27.9 $-CH_2-$
3	72.0 $-CH<$Ogl	74.7 $-CH<$Ogl	71.4 $-CH<$OH	67.1 $-CH<$OH
4	56.0 $-CH<$O$-$	28.8 $-CH_2-$	38.3 $-CH_2-$	33.6 $-CH_2-$
5	67.6 $>C<$ O$-$	76.1 $>C<$OH	44.9 $-CH<$	36.6 $-CH<$
6	58.5 $-CH<$Cl	67.4 $-CH<$Cl	28.8 $-CH_2-$	26.3 $-CH_2-$
7	41.8 $-CH_2-$	39.5 $-CH_2-$	32.1 $-CH_2-$	26.7 $-CH_2-$
8	36.0 $-CH<$	35.6 $-CH<$	35.6 $-CH<$	35.7 $-CH<$
9	48.2 $-CH<$	42.9 $-CH<$	54.4 $-CH<$	39.8 $-CH<$
10	38.4 $>C<$	43.0 $>C<$	35.5 $>C<$	35.1 $>C<$
11	21.5 $-CH_2-$	21.6 $-CH_2-$	21.3 $-CH_2-$	21.2 $-CH_2-$
12	39.6 $-CH_2-$	39.8 $-CH_2-$	40.1 $-CH_2-$	40.3 $-CH_2-$
13	42.8 $>C<$	42.8 $>C<$	42.6 $>C<$	42.7 $>C<$
14	55.6 $-CH<$	56.2 $-CH<$	56.6 $-CH<$	56.7 $-CH<$
15	24.2 $-CH_2-$	24.2 $-CH_2-$	24.3 $-CH_2-$	24.2 $-CH_2-$
16	28.1 $-CH_2-$	28.2 $-CH_2-$	28.3 $-CH_2-$	28.3 $-CH_3-$
17	56.2 $-CH<$	56.2 $-CH<$	56.3 $-CH<$	56.4 $-CH<$
18	12.0 $-CH_3$	12.0 $-CH_3$	12.1 $-CH_3$	12.1 $-CH_3$
19	18.7 $-CH_3$	16.9 $-CH_3$	12.3 $-CH_3$	23.9 $-CH_3$
				β-Sitosteryl acetate[d]
20	36.2 $-CH<$	36.2 $-CH<$	36.2 $-CH<$	36.2 $-CH<$
21	18.7 $-CH_3$	18.8 $-CH_3$	18.8 $-CH_3$	18.8 $-CH_3$
22	34.0 $-CH_2-$	34.0 $-CH_2-$	34.0 $-CH_2-$	34.0 $-CH_2-$
23	26.4 $-CH_2-$	26.4 $-CH_2-$	26.2 $-CH_2-$	26.2 $-CH_2-$
24	46.0 $-CH<$	45.9 $-CH<$	45.9 $-CH<$	45.9 $-CH<$
25	29.3 $-CH<$	29.2 $-CH<$	29.2 $-CH<$	29.3 $-CH<$
26	19.8 $-CH_3$	19.8 $-CH_3$	19.8 $-CH_3$	19.8 $-CH_3$
27	19.1 $-CH_3$	19.1 $-CH_3$	19.1 $-CH_3$	19.1 $-CH_3$
28	23.2 $-CH_2-$	23.1 $-CH_2-$	23.1 $-CH_2-$	23.1 $-CH_2-$
29	12.0 $-CH_3$	12.0 $-CH_3$	12.0 $-CH_3$	11.9 $-CH_3$

[a] Assigned with DEPT and/or H−C COSY spectra.
[b] Analyzed in the laboratory.
[c] Data from Blunt and Stothers (1977).
[d] Data from Seo et al. (1978).

Since the C19 shielding (δ_C 16.9) was also affected by the γ-substituent, it was suggested that a hetero atom had connected to C1, C5, or C9.

The DEPT and H–C COSY spectrum showed that one of the eight methines in stigmastane was substituted to become a nonprotonated carbon ($>C<X$) at

δ_C 76.1. This was ruled out from the sidechain and C-, D-ring members, and these shieldings were retained as those of the related steroid compounds (Table 4). For example, the C14 shielding of compound **2** (δ_C 56.2) remained unchanged from those of 5α-cholestane (δ_C 56.6) and 5β-cholestane (δ_C 56.7), which excluded the possibility of a γ effect by a hetero atom at C9. Thus C5 could be specified as the first site.

The stereochemistry at the connection between the A and B rings in a steroid compound is generally reflected on the ^{13}C shieldings of C19 angular methyl. Although the C19 signal of 5α-cholestane (*trans* isomer) is observed at δ_C 12.0, the more deshielded C19 of 5β-cholestane (*cis* isomer) appears downfield at δ_C 24.4 (Leibfritz and Roberts, 1973). The C18 signal of compound **2** remained at δ_C 12.0 overlapping with C29, but the C19 signal was observed at δ_C 16.9 because of the substituent effect. However, this did not provide the critical information for the stereochemistry, because it could be postulated on the one hand that the C19 signal of a *trans* isomer shifted downfield by an anti γ effect or, on the other hand, that the signal of the *cis* isomer shifted upfield by a γ (gauche) effect. Therefore the stereochemistry was investigated with the NOESY and NOE difference spectra. Since NOE was observed almost equally between 19-CH_3 and each of 1-CH_α (5%) and 1-CH_β (6%), this suggests a *cis* connection between A and B rings (Figure 3). This was also supported by the smaller NOE of 19-CH_3 with 4-CH_β (2%) in comparison with 6-CH_β (11%), 8-CH_β (10%), and 11-CH_β (12%). Thus compound **2** is more likely to be a 5β-isomer.

The fragmentation pattern of the EI-MS spectrum suggested the connecting site of the sugar. Elimination of the glucose moiety from the A ring would cause a retro Diels-Alder rearrangement, which left butadiene from the A ring to produce the base peak ion at *m/z* 394. According to this estimate, the β-D-

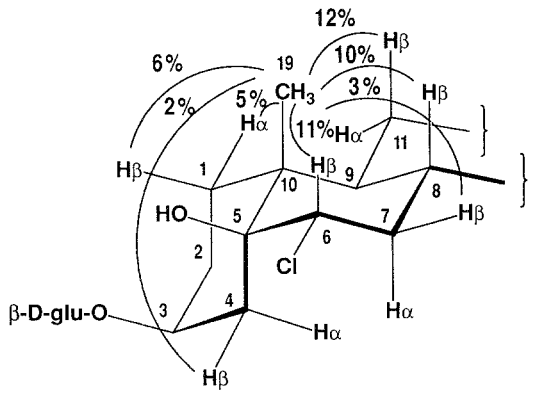

FIG. 3. NOE observed between 19-CH_3 and the other protons of compound **2**.

glucosyl function was the only substituent in the A ring and should have connected to C2 or C3. The COLOC spectrum showed a cross-peak between the corresponding oxygenated methine (δ_H 4.17) and C5 (δ_C 76.1), and this indicated the second site as C3.

The stereochemistry at C3 was investigated with the ^{1}H NMR (Table 5) and H–H COSY spectra (Figure 2). A pyramidal signal of 3-CH at δ_H 4.17 (3,3,3,3) indicated its equatorial position, which was supported by a square cross-peak between the geminal protons of 4-CH_2 at δ_H 1.78 (13,3) and 2.26 (13,3). This geometry in the 5β-isomer indicated that the glycosidic function is connected to 3β position.

The last site was limited to the B ring, because other possibilities were ruled out within the context of the above observations. The remaining methine with a hetero atom could only be C6 or C7. The corresponding methine at δ_H 4.27 shared cross-peaks with a pair of methylene protons at δ_H 1.33 (13,13,13) and 2.05 (13,3,3) (Figure 2). The higher-field proton appeared as a quartet, so the spin system included geminal and two vicinal diaxial geometries. Since the C5 proton had been substituted, the only possible proton was 7-CHα, and therefore the last substitution site could be specified at 6α equatorial.

Two substituents, OH and Cl, should be matched with C5 and C6. The α substituent effect of OH was relatively large for methine (+43.3 by 3β-OH) compared with quaternary carbon (+26.0 by 5α-OH), whereas Cl showed almost the same effect for both methine (+31.0 by 3β-Cl) and quaternary carbon (+33.6 by 9α-Cl) (Blunt and Stothers, 1977). Taking into account the enhancement by the β effect, the observed α effect for C5 quaternary carbon (+31.9) and C6 methine (+39.8) matched with those of OH and Cl, respectively. Consequently the structure of compound **2** was established as 1-(6α-chloro-5β-hydroxy-5β-stigmast-3β-yl)-β-D-glucopyranoside (Figure 4).

Assignment of Signals. As the discussion has so far already specified some signals related to the substitution, we may now outline the assignment of other signals. The ^{13}C shieldings of side-chain members were almost identical to those of β-sitosteryl acetate (Seo et al., 1978) and 5α-stigmast-3β-ol measured in the laboratory. The corresponding proton signals, specified with H–C COSY, showed reasonable cross peak patterns in the H–H COSY spectrum (Figure 4). The C14 (δ_C 56.2) and C17 (δ_C 56.2) methines and the C13 (δ_C 42.8) quaternary carbon were confirmed by the connectivity with 18-CH_3 (δ_H 0.65, s), as well as C10 (δ_C 43.0) with 19-CH_3 (δ_H 0.94, s), in the COLOC spectrum. The C- and D-ring members were not modified, so most of them retained the ^{13}C shieldings of the original skeleton. The A-ring members were allocated with regard to the H–H COSY connectivities. The assignment of the ^{13}C and ^{1}H NMR signals are listed in Tables 4 and 5, respectively.

TABLE 5. ^{1}H NMR SPECTRAL ASSIGNMENT OF AGLYCONE OF COMPOUNDS **1** AND **2** WITH REFERENCE TO 5α-STIGMASTAN-3β-OL (δ_H in ppm, Multiplicity,[a] and *J* Values[b] in Hz)

Position	Compound **1**	Compound **2**	5α-Stigmastan-3β-ol[c]
1α	1.57 ddd(**13**,4,2)	1.88 ddd(**13**,3,3)	0.95 ddd(**13,13,3**)
1β	1.24 ddd(13,13,2)	1.32 ddd(**13,13**,3)	1.70 ddd(**13,3,3**)
2α	1.67 dddd(**13,13**,4,2)	1.54 dddd(**13,13**,3,3)	1.79 dddd(**13,3,3,3**)
2β	1.57 dddd(13,4,2,2)	1.79 dddd(**13**,3,3,3)	1.39 dddd(**13,13,13,3**)
3α	4.23 ddd(4,3,2)	4.17 dddd(3,3,3,3)	3.58 dddd(**13,13,3,3**)
4α	3.83 d(**3**)	1.78 dd(**13**,3)	1.55 ddd(**13,3,3**)
4β		2.26 dd(**13**,3)	1.27 ddd(**13,13,13**)
5β(5α)			1.08 dddd(**13,13,3,3**)
6α			1.33 dddd(13,3,3,3)
6β	4.43 dd(**13,3**)	4.27 dd(**13,3**)	1.26 dddd(13,13,13,3)
7α	1.33 ddd(**13,13,13**)	1.33 ddd(**13,13,13**)	0.86 dddd(13,13,13,3)
7β	2.21 ddd(**13,3,3**)	2.05 ddd(**13,3,3**)	1.66 dddd(13,3,3,3)
8β	1.54 dddd(**13,13,12,3**)	1.53 m	1.33 dddd(13,13,12,3)
9α	1.08 m	1.23 m	0.60 ddd(**13,13,3**)
11α	1.40 dddd(**13**,3,3,3)	1.41 m	1.49 dddd9**13,3,3,3**)
11β	1.30 dddd(**13,13,13**,3)	1.26 m	1.26 dddd(**13,13,13,3**)
12α	1.12 ddd(**13,13**,3)	1.14 m	1.10 ddd(**13,13,3**)
12β	1.99 ddd(**13**,3,3)	1.98 ddd(**13**,3,3)	1.96 ddd(**13,3,3**)
14α	1.08 m	1.10 m	0.96 ddd(12,12,6)
15α	1.60 m	1.55 m	1.56 dddd(**13,12,6,2**)
15β	1.09 m	1.08 m	1.05 dddd(**13,12,12,6**)
16α	1.86 m	1.84 m	1.81 dddd(**13,12,12,8**)
16β	1.28 m	1.26 m	1.23 dddd(**13,10,6,2**)
17α	1.11 m	1.10 m	1.08 ddd(**10,8,8**)
18	0.67 s(3H)	0.65 s(3H)	0.65 s(3H)
19	1.03 s(3H)	0.94 s(3H)	0.80 s(3H)
20	1.34 m	1.32 m	1.33 dddq(8,8,8,7)
21	0.91 d(7, 3H)	0.91 d(7, 3H)	0.90 d(7, 3H)
22a[d]	1.00 m	0.99 m	1.00 dddd(13,8,8,8)
22b	1.32 m	1.32 m	1.31 dddd(13,8,8,8)
23a	1.13 m	1.13 m	1.13 dddd(13,8,8,8)
23b	1.17 m	1.17 m	1.17 dddd(13,8,8,8)
24	0.93 sextet(**8**)	0.93 sextet(**8**)	0.92 sextet(**8**)
25	1.66 dqq(**8,8,7**)	1.67 dqq(**8,8,7**)	1.62 dqq(**8,8,7**)
26	0.84 d(**8**, 3H)	0.84 d(**8**, 3H)	0.83 d(**8**, 3H)
27	0.82 d(**7**, 3H)	0.82 d(**7**, 3H)	0.81 d(**7**, 3H)
28a	1.22 m	1.22 m	1.22 ddq(13,8,8)
28b	1.26 m	1.26 m	1.25 ddq(13,8,8)
29	0.85 t(**8**, 3H)	0.85 t(**8**, 3H)	0.84 t(**8**, 3H)

[a] s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet.
[b] *J* values in bold type were confirmed with the coupling pattern, otherwise were estimated from the texture and outline of a cross-peak.
[c] Analyzed in the laboratory.
[d] a or b is a suffix to distinguish each methylene proton.

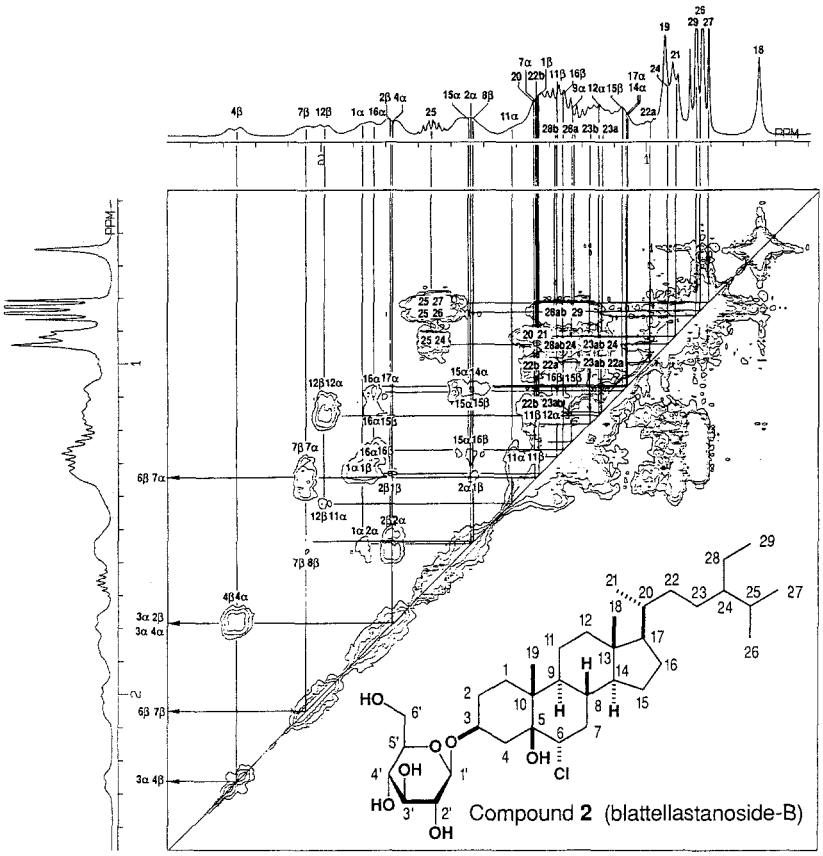

FIG. 4. Enlarged H–H COSY spectrum and the structure of compound **2.** The assignment of the cross-peaks in the upfield portion are indicated.

Structural Elucidation of Compound 1 with NMR Spectra

The NMR spectra were acquired on 15 mg of compound **1** in chloroform-*d*. The comparison of the H–C COSY spectrum of compounds **1** and **2** revealed that both compounds had an equal constitution, except that one methylene was replaced by oxygenated methine in **1.** The methine proton (δ_H 3.83) correlated with 3-CH_α (δ_H 4.23) in the H–H COSY spectrum (Figure 5). Since compound **1** had an elemental composition of 2H smaller than **2,** it was postulated that the 5β-hydroxy function in **2** was replaced by 4,5-epoxide in **1.** This estimate was supported by the following spectral evidence. The INEPT/N experiment recorded an exceptionally high J_{CH} (179.1 Hz) for 4-CH (δ_C 56.0) compared with that of the other oxygenated methines such as 3-CH (151.1 Hz),

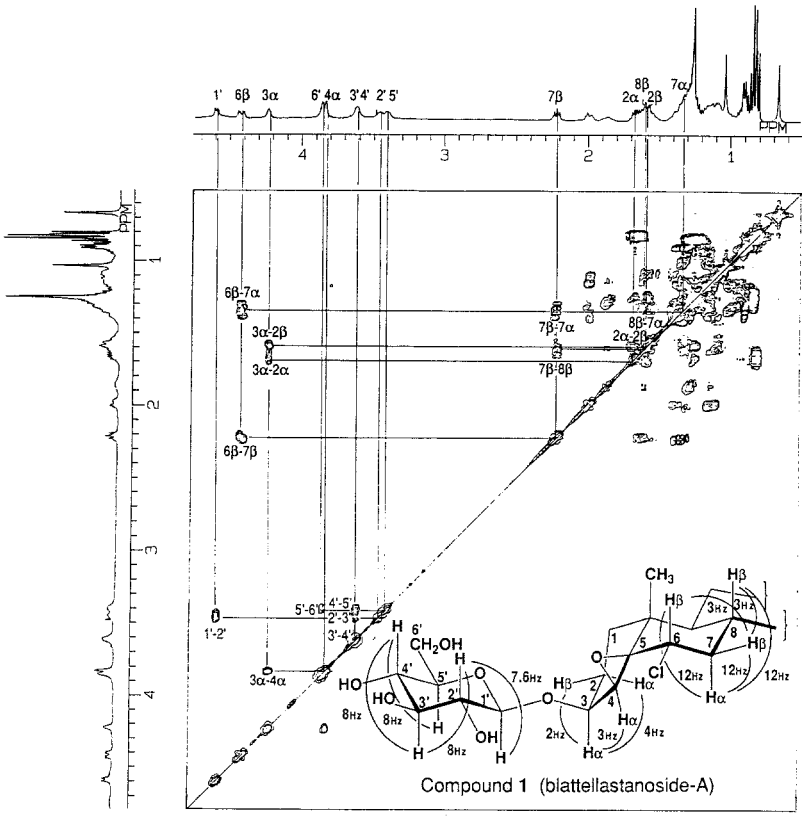

FIG. 5. H–H COSY spectrum of compound **1.**

suggesting the presence of an epoxide function on C4. The C5 shielding (δ_C 67.6) of compound **1** is shifted upfield from that of **2** (δ_C 76.1), which corresponded to the α substitution effect changing from that of OH to epoxide.

The stereochemistry of compound **1** at C5 was reflected in the shielding of C19 (δ_C 18.7) shifting downfield from that of **2** (δ_C 16.9), and this difference could be explained by the smaller γ effect of an epoxide compared with OH. This, together with the same evidence as in compound **1** in the NOE difference experiments, suggested that compound **1** was the same 5β isomer as **2.** The pyramidal shape of the 3-CH signal at δ_H 4.23 (4,3,2) indicated its equatorial position as well (Figure 5). All the other signals were almost identical to those of compound **2** and were assigned in the same manner as described above (Tables 2–5). This spectral evidence eventually permitted us to establish the structure of compound **1** as 1-(6α-chloro-4β,5β-epoxy-5β-stigmast-3β-yl)-β-D-glucopyranoside (Figure 6).

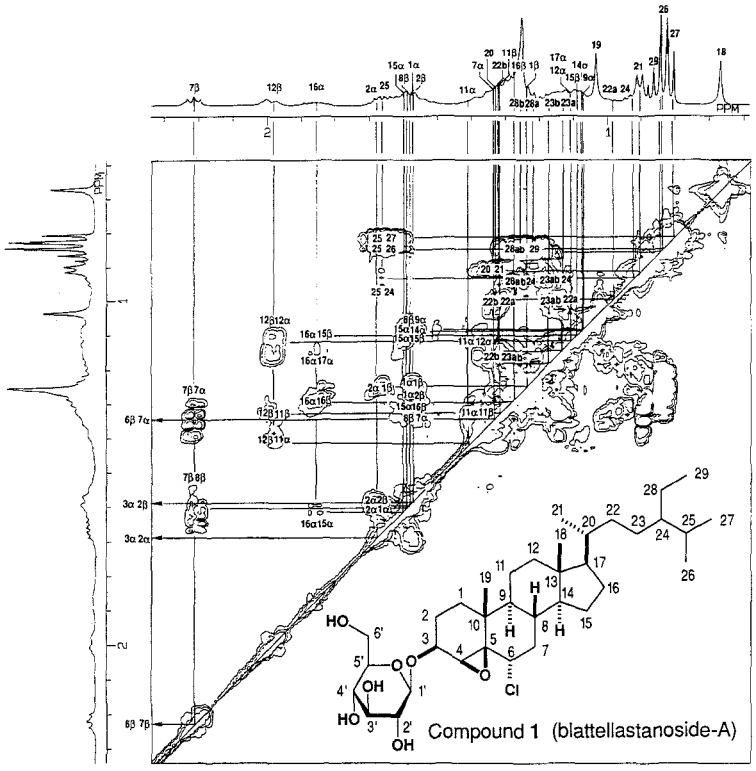

FIG. 6. Enlarged H–H COSY spectrum and the structure of compound **1**.

DISCUSSION

In the final stage of the purification, the arrestant activity of the most potent fraction containing compound **1** was retained apparently at the same level as the original extract. However, the activity had increased up to 740% after partitioning with *n*-butanol and water, and then decreased in the subsequent processes of chromatography. The enhancement of the activity was probably due to the removal of the coexisting masking substances transferred into the aqueous phase, and the technical as well as decompositional loss of active materials might have resulted in the reduction of the activity in the subsequent processes. Nevertheless, the active principle could be attributed to both compounds **1** and **2**, because they were unique in the activity and no synergistic effect between the other fractions was observed throughout the purification. Since both compounds **1** and **2** were as yet unidentified, they were designated as blattellastanoside-A and blattellastanoside-B, respectively (Sakuma and Fukami, 1992, 1993b). Choice-

chamber assay of both blattellastanosides represented the arrestant activity in ED_{50} at 0.044 (A) and 3.2 (B) nmol impregnated on 1.0 cm^2 of Whatman No. 1 filter paper (Sakuma and Fukami, 1993b).

Both blattellastanoside-A and -B have characteristic steroid skeletons and chlorine atoms, which are uncommon in insect pheromones. While in vertebrates, steroid pheromones such as androstenone (5α-androst-16-en-3-one), a sex pheromone of the boar, *Sus domestica*, and probably of other mammals too (Claus, 1979), and the spawning pheromone of the goldfish, *Carassius auratus*, determined as 17α,20β-dihydroxy-4-pregnen-3-one (Dulka et al., 1987), have been identified, no such steroid pheromones appear to have been discovered in insects, save for a trail pheromone of the tent caterpillar, *Malacosoma americanum* (Fitzgerald and Edgerly, 1979, 1982) identified as 5β-cholestan-3,24-dione (Crump et al., 1987).

A stigmastane skeleton of both blattellastanosides can be categorized in a phytosterol, which reflects the possible origin of the compounds. The laboratory diet used in the experiment contained wheat flour and soybean powder (although each percentage was not mentioned), both of which are rich in phytosterols like β-sitosterol and stigmasterol. It is generally considered that insects lack the complete biosynthetic pathway of steroid skeleton from mevalonic acid (Ritter and Wientjens, 1967), and so *B. germanica* probably modifies these phytosterols or their glycosides to blattellastanosides.

Few chlorinated compounds seem to have been identified as pheromones of arthropods. The only example so far might be 2,6-dichlorophenol produced by female ticks as the sex attractant pheromone (Berger et al., 1972). Both blattellastanoside-A and -B have chlorine atoms on C6. The chlorine in blattellastanoside-B is accompanied by a hydroxyl group on C5 in the *trans* position, which could be a product of the cleavage of an epoxide group by hydrochloric acid in the purification process. However, 4β,5β-epoxide of blattellastanoside-A remained unchanged under such conditions, so the possibility of the artifact is unlikely.

Nevertheless, this idea of an artifact also provides some hints for the biosynthesis of both compounds. The chlorine atom on C6 common to both compounds implies the priority of the chlorination reaction, so that the following process may be considered: The hydrochlorination of Δ5 double bond of a common precursor may come first to produce blattellastanoside-B, and then the oxidation of 5β-hydroxyl group to 4β,5β-epoxide may provide blattellastanoside-A. The idea that blattellastanoside-B is a precursor of blattellastanoside-A would be reflected by the biological significance of blattellastanoside-A, showing ca. 70 times higher specific activity than blattellastanoside-B. This hypothetical process should, however, be confirmed by tracing the biosynthetic pathway.

The site of pheromone production was experimentally specified as the eighth

to tenth (terminal) segments of the abdominal tip, and then the histological study suggested rectal pads as the glandular organ (Ishii and Kuwahara, 1967). Hitherto it was thought that the pheromone was emitted from the rectal pads into the lumen when fecal materials pass through the anterior part of the rectum. Recently, however, the site of pheromone production was reinvestigated in the same experimental system except that the choice-chamber assay was used instead of the vertical paper assay (Sakuma and Fukami, 1991). Although the pheromonal activity of the abdominal tip extract was confirmed, further examination suggested that the pheromone is secreted from the center part of the supraanal plate surrounding the anus. The trace characterization of both blattellastanosides in the possible pheromone glands is expected to specify the production site as well as the origin of the compounds.

Both blattellastanosides are the less volatile compounds, and they function as contact chemicals. The assay on antenn-and/or palpectomized males indicated that the pheromone was perceived with both antennae and maxillary palpi through contact chemoreception (Sakuma and Fukami, 1991). The cockroach aggregation pheromones so far identified include volatile materials like undecane, tetradecane, and ethyl caproate from *Blaberus craniifer* (Burmeister) (Brossut et al., 1974); undecane, 4-heptanone, 2,6-dimethyl-4-heptanone, and octanol from *Eublaberus distanti* (Brossut, 1979); and 1-dimethylamino-2-methyl-2-propanol and alkylamines from *Blattella germanica* (Sakuma and Fukami, 1990).

These findings were obtained from olfactometer assays, which were designed to detect the volatile attractants, whereas the isolation of blattellastanosides was guided by choice-chamber assay, which allows direct contact of the test insect to the sample material. The final recognition of the aggregation site as well as the conspecific individuals may be conducted by direct contact with the arrestants, which would be a key to opening the social behavioral program of the group formation, and therefore the use of a choice-chamber type assay that detects arrestants is required together with an olfactometer.

Airborne attractants evaporate from the substrate to guide insects toward the odor source by inducing the orientation behavior of insects such as odor-conditioned anemotaxis and chemotaxis (Kennedy, 1977), whereas arrestants of low volatility are a durable signal on a stationary object, although this will be difficult for receivers to detect (Harper, 1991). In the case of *B. germanica*, the approaching insects will search intensively for the odor source in the vicinity, and when they come across a patch of arrestants, which is practically the attractant odor source itself, an inverse orthokinesis response terminates the insects' locomotion as the final recognition of the shelter. Consequently the combined use of attractants and arrestants will enhance the capability of the local search of the insects.

Interspecific responses with some species specificity were observed between various cockroaches in the vertical paper assay. Although *B. germanica* nymphs

aggregated on the paper conditioned with *Periplaneta fuliginosa* (Serville) when it was compared with the clean paper, the insects preferred the contaminated paper of conspecifics to that of the other species (Ishii, 1970). The less-strict species specificity was, however, also observed between various cockroaches other than *B. germanica* (Bell et al., 1972), and this ambiguity may be due to the vertical paper assay, which does not discriminate behavioral components (Sakuma and Fukami, 1991). If the observed responses were executed by arrestants alone, these interspecific responses might suggest that the aggregation arrestant pheromones of cockroaches are the similar compounds as blattellastanosides, i.e., steroid derivatives, but this is still unclear from the present findings.

Recently both blattellastanosides-A and -B were synthesized from β-sitosterol (Fukamatsu and Mori, 1993; Mori and Fukamatsu, 1993; Mori et al., 1993), and the synthetic materials were completely identical to the natural products with respect to the HPLC retention time, EI-MS spectra, ^{13}C NMR spectra as well as the cross-peak pattern of the H–H COSY spectra. Moreover, the structures that included the stereochemistry of the synthesized materials were confirmed by the X-ray analysis of 6α-chloro-4β,5β-epoxy-5β-stigmastan-3-one derived from the aglycone of blattellastanoside-A in the synthetic pathway. Since both synthetic blattellastanosides exhibited arrestant activity equivalent to the natural ones, i.e., ED_{50} at 0.040 (A) and 1.4 (B) nmol/1.0 cm^2 of filter paper, the structures of both compounds were believed to be confirmed.

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Pellioditis pellio: SUBSTITUTED PHENOLIC COMPOUNDS AS CHEMOATTRACTANTS

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Abstract—Vanillin, vanillic acid, and other related compounds are chemoattractants either for nematodes or arthropods. We tested vanillin, vanillic acid, and 2,4-dihydroxybenzaldehyde in an in vitro bioassay system for their ability to attract *Pellioditis pellio* males and females. Females were not attracted to any of the three compounds tested. Males responded to vanillin and vanillic acid, but not to 2,4-dihydroxybenzaldehyde. Males responded to vanillin and vanillic acid when these compounds were present at 10^{-5} M concentration. Vanillin at 10^{-3} and 10^{-7} M concentrations was not attractive.

Key Words—*Pellioditis pellio*, Nematoda, chemoattraction, behavior, vanillin, 4-hydroxy-3-methoxybenzaldehyde, vanillic acid, 4-hydroxy-3-methoxybenzoic acid, 2,4-dihydroxybenzaldehyde, β-resorcylaldehyde.

INTRODUCTION

Using an in vitro bioassay without barriers, Eveland and Fried (1990) showed heterosexual (male to female) and homosexual chemoattraction among females of *Pellioditis pellio*. Only male to female heterosexual attraction occurred using barriers of 12,000–25,000 M_r pore size. These findings suggest the occurrence of at least two chemoattractants of an unknown chemical nature.

Jaffe et al. (1989) showed that the substituted phenolic acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid), elicited both attractancy and coiling behav-

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ior in the soybean cyst nematode *Heterodera glycines*. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a component of the sex pheromone of the insect, *Eurygaster integriceps* (Ubik et al., 1975; Vrkoc et al., 1977). Furthermore, vanillin and vanillyl alcohol occur in gland secretions of male leaf-footed bugs (Aldrich et al., 1979).

In this in vitro study we tested whether substituted phenolic compounds also attract adult *Pellioditis pellio*.

METHODS AND MATERIALS

Pellioditis pellio were cultured following their isolation from the lumbricoid oligocaete *Apporectodea caliginosa* recovered from soil in Long Beach, California, U.S.A., as described by Eveland et al. (1990). Chemoattraction studies were done with 3- to 7-day-old worms picked from cultures containing both male and female worms.

Vanillic acid (4-hydroxy-3-methoxybenzoic acid), vanillin (4-hydroxy-3-methoxybenzaldehyde), and 2,4-dihydroxybenzaldehyde (β-resorcylaldehyde) (Aldrich Chemical Co., Milwaukee, Wisconsin) were dissolved in double glass-distilled water (DDW). To overcome relative insolubility, the DDW was initially adjusted to pH 8.0 with 0.1 N NaOH, and the solutions were then adjusted to pH 7.0 with 0.1 N HCl before use.

The bioassays done were in polycarbonate chambers, as described by Eveland and Fried (1990). A paper punch was used to make disks from Whatman No. 1 filter paper. Two stacks of six disks each were placed 10 mm apart into channels. One stack was saturated with 10 μl of 10^{-3}–10^{-7} M concentrations of test chemicals, and the control stack was saturated with 10 μl of pH-adjusted DDW.

Worm migration was studied using a closed-circuit video system described previously (Eveland and Fried, 1990). The distance between the responder worm and the test disk was measured at 5-min intervals for 30 min, and the percent of the starting distance was calculated. Student's t test was used to test significance of migration of each group of worms.

RESULTS

Worms distributed themselves evenly around the zero point when placed into the bioassay without a chemical stimulus. Males responded significantly ($P = 0.05$) to disks containing 10^{-5} M vanillin after 10 min or 10^{-5} M vanillic acid after 25 min (Figure 1, Table 1). Differences in migration patterns of the two groups did not differ significantly. However, males did not respond to 10^{-3} or 10^{-7} vanillin or vanillic acid. Males were significantly repelled by 2,4-

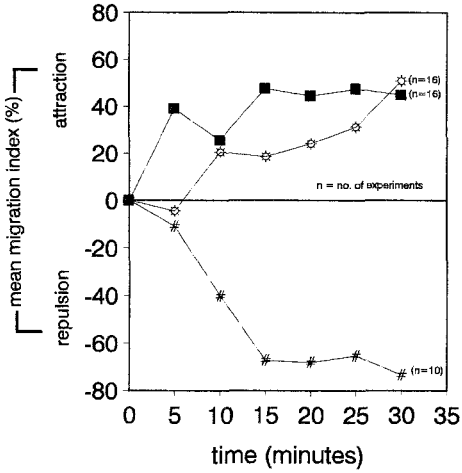

FIG. 1. *Pellioditis pellio* attraction to substituted phenolic compounds. Observations were recorded at 5-min intervals using a closed-circuit video recording system and the mean migration index was calculated. ■ = vanillin, ○ = vanillic acid, # = 2, 4-dihydroxybenzaldehyde.

TABLE 1. PROBABILITIES (STUDENT'S *t* TEST)

Minutes	Vanillin	Vanillic acid	2,4-D[a]
5	0.035	0.78	0.58
10	0.24	0.25	0.15
15	0.026	0.32	0.042
20	0.044	0.21	0.041
25	0.035	0.063	0.056
30	0.042	0.004	0.022

[a]2,4-Dihydroxybenzaldehyde.

dihydroxybenzaldehyde at 15, 20, and 30 min. Their migration pattern differed significantly from attraction to both vanillin and vanillic acid after 10 min (Figure 1, Table 1). Females did not respond to any concentration of the three compounds tested (data not shown).

DISCUSSION

This study suggests that substituted phenolic compounds such as vanillin or vanillic acid may function as chemoattractants for male *Pellioditis pellio*. From experiments using barriers of varied pore sizes, Eveland and Fried (1990)

postulated a heterosexual chemoattractant in *P. pellio* between 12,000–25,000 M_r. Because vanillin and vanillic acid have formula weights of 152.14 and 168.14, respectively, it appears more likely that the conclusions drawn from those experiments were due to differences in diffusion through the membranes.

There are structural similarities between vanillin and vanillic acid; both have a methoxy group at position 3 and a hydroxyl group at position 4. In contrast, 2,4-dihydroxybenzaldehyde lacks a functional group at position 3; instead, it has a hydroxyl group at position 2. Vanillin and vanillic acid have an aldehyde group and a carboxyl group at position 1, respectively. It is possible that these two compounds occur together in nature because of conversion through oxidation and reduction. Compounds with a methoxy group at position 4 and a hydroxyl group at position 3, or hydroxyl groups at positions 3 and 4 may also be attractive for *P. pellio* males.

Jaffe et al. (1989) reported a "paralytic-like" effect on male *Heterodera glycines* when vanillic acid concentrations exceeded 10^{-5} M. In our studies, neither 10^{-3} nor 10^{-7} M vanillin attracted males, presumably because these concentrations were not optimal.

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CHIRALITY OF SYNERGISTIC SEX PHEROMONE COMPONENTS OF THE WESTERN HEMLOCK LOOPER *Lambdina fiscellaria lugubrosa* (HULST) (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—Bakers' yeast reduction of (2*E*)-3-(2′-furanyl)-2-methyl-2-propenal yielded the synthetic intermediate, (2*S*)-3-(2′-furanyl)-2-methylpropanol, of high chiral purity (>97% ee) for the synthesis of the enantiomers of 2,5-dimethylheptadecane and 7-methylheptadecane, two synergistic sex pheromone components of the western hemlock looper (WHL), *Lambdina fiscellaria lugubrosa* Hulst. In electrophysiological bioassays, (7*S*)- but not (7*R*)-7-methylheptadecane elicited strong antennal responses by male WHL antennae. In field trapping experiments, addition of (7*S*)- but not (7*R*)-7-methylheptadecane to (5*R*,11*S*)-5,11-dimethylheptadecane, the major sex pheromone component of WHL, increased attraction. Attraction to (5*R*,11*S*)-5,11-dimethylheptadecane in combination with (7*S*)-7-methylheptadecane was further enhanced by the addition of (5*S*)- but not (5*R*)-2,5-dimethylheptadecane. Similarly, attraction to (5*R*,11*S*)-5,11-dimethylheptadecane combined with (5*S*)-2,5-dimethylheptadecane increased when (7*S*)- but not (7*R*)-7-methylheptadecane was added as a third component. We conclude that (7*S*)-7-methylheptadecane and (5*S*)-2,5-dimethylheptadecane are the synergistic sex pheromone components of WHL. The synthetic methodology described is applicable to the synthesis of chiral methyl-branched pheromones in other orders of the Insecta, particularly Coleoptera, Diptera and Orthoptera.

Key Words—Sex pheromone, enantiomer, Lepidoptera, Geometridae, western hemlock looper, (5*R*)-2,5-dimethylheptadecane, (5*S*)-2,5-dimethylheptadecane, (7*R*)-7-methylheptadecane, (7*S*)-7-methylheptadecane, chirality.

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INTRODUCTION

5,11-Dimethylheptadecane (5,11-dime-17Hy) is the major sex pheromone component of the eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria* (Guen.) (Gries et al., 1991a,b) and the western hemlock looper (WHL) *L. f. lugubrosa* (Hulst) (Gries et al., 1993a). As shown in recent electrophysiological studies and field trapping experiments, of the four stereoisomers of 5,11-dime-17Hy, only (5*R*,11*S*)-5,11-dime-17-Hy elicited antennal responses by male EHLs and WHLs and attracted male loopers in the field (Li et al., 1993).

2,5-Dimethylheptadecane (2,5-dime-17Hy) is a synergistic sex pheromone component in both EHL and WHL. While 7-methylheptadecane (7-me-17Hy) is present in the EHL, it is a third synergistic pheromone component only in the WHL (Gries et al., 1993a). Biotransformation is widely used in the synthesis of chiral synthons of high chiral purity, and it provides many ways to chiral natural products (Fuganti, 1990). We report the bakers' yeast synthesis of and antennal responses and field attraction to the enantiomers of 7-me-17Hy and 2,5-dime-17Hy in the WHL.

METHODS AND MATERIALS

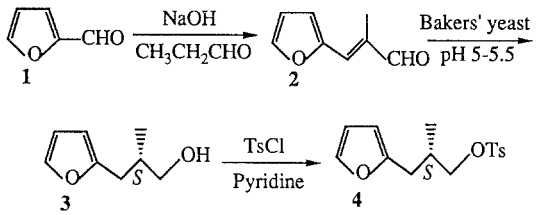
General Procedures for Synthesis and Characterization

Evaporations were carried out under reduced pressure below 45°C. Infrared spectra (Perkin-Elmer 599B spectrophotometer) were obtained from a neat film or suspension of the pure compound in Nujol on NaCl plates. Gas–liquid chromatography (GLC) was conducted employing a Hewlett Packard 5880 gas chromatograph equipped with a fused silica column (30 m × 0.25 mm ID) coated with DB-1 (J&W Scientific, Folsom, California). Thin-layer chromatography (TLC) plates were prepared from silica gel 60G. For detection of compounds, plates were sprayed with 10% sulfuric acid and heated. Chromatographic separations were carried out as previously described (Still et al., 1978). Optical rotations were measured on an Autopol II automatic polarimeter. Concentrations for optical rotations were reported in grams per 100 ml of solvent. Nuclear magnetic resonance (NMR) spectra (Bruker WU-400 spectrometer) were taken in $CDCl_3$ at 400 MHz (*J* values in hertz). Mass spectra were obtained on a Hewlett Packard 5985B mass spectrometer equipped with a fused silica column (30 m × 0.25 mm ID) coated with DB-1. High-resolution mass spectra were generated on Kretos M580 mass spectrometer.

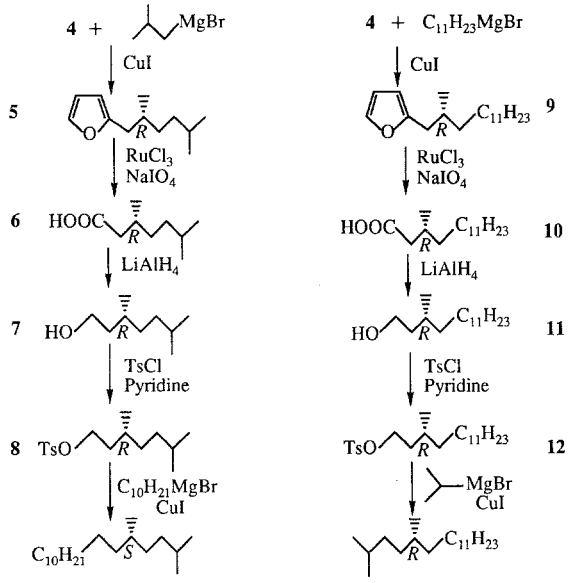
Syntheses of Individual Compounds

(2S)-3-(2'-Furanyl)-2-methylpropanyl p-*toluenesulfonate* ***4*** *(Scheme 1).* This compound was synthesized as previously reported (Fuganti et al., 1988): $[\alpha]_D^{24}$ + 6.9(*c*6.4, $CHCl_3$).

(2'R)-2-(2', 5'-Dimethylhexyl)-furan **5** *(Scheme 2).* A solution of 2-methylpropyl magnesium bromide made from magnesium (2.6 g) and 1-bromo-2-methylpropane (4.19 g) in ether (30 ml) was cooled to −78°C under argon. Following addition of CuI (0.581 g) to the solution, **4** (3.00 g) in THF (40 ml) was added dropwise. The mixture was stirred overnight while the temperature was slowly increased to room temperature. After quenching with water and extracting (5×) with hexane–ether (1 : 1), the combined extracts were washed (2×) with saturated NaCl, dried over Na_2SO_4, concentrated and purified by column chromatography with hexane as eluent to yield **5** (1.12 g, 61% yield): $[\alpha]_D^{24}$ + 2.8(*c*5.05, $CHCl_3$); IR (film): 2955, 2926, 2851, 1596, 1507, 1467,



SCHEME. 1. Chiral synthesis of (2*S*)-3-(2'-furanyl)-2-methylpropanyl tosylate.



SCHEME. 2. Chiral synthesis of enantiomers of 2,5-dimethylheptadecane.

1384, 1147, 1080, 1008, 928, 793, 723, 599 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.30 (1H, dd, J = 1.8, 0.7, H-5), 6.27 (1H, dd J = 1.8, 3.0, H-4), 5.97 (1H, dd, J = 3.0, 0.7, H-3), 2.61 (1H, dd, J = 14.8, 6.0, H-1′), 2.42 (1H, dd, J = 14.8, 7.9, H-1′|), 1.77 (1H, m), 1.49 (1H, m), 1.33 (1H, m), 1.17 (3H, m), 0.88 (3H, d, J = 6.7, CH_3), 0.86 (3H, d, J = 6.6, CH_3), 0.85 (3H, d, J = 6.6, CH_3); ^{13}C NMR ($CDCl_3$) δ: 155.7(C-2), 140.7(C-5), 110.0(C-4), 105.7 (C-3), 36.3, 35.5, 34.4, 32.9, 28.2, 22.8, 22.5, 19.7; EI-MS: m/z 180(2, M^+), 123(2), 109(9.5), 82(48), 81(59), 57(100); Anal. calcd. for $C_{12}H_{20}O$: C, 80.00%; H, 11.11%. Found: C, 80.11%; H, 11.25%.

(3R)-3,6-Dimethylheptanoic Acid ***6*** *(Scheme 2).* Starting material **5** (650 mg) was dissolved in acetonitrile (14.4 ml), carbon tetrachloride (14.4 ml) and water (36.1 ml). $NaIO_4$ (16.6 g) and $RuCl_3$ (162 mg) were added separately to the solution. After stirring overnight, the reaction mixture was filtered through a Celite pad and the filtrate extracted (5×) with chloroform. The combined organic extracts were extracted (3×) with 5% NaOH. The combined basic extracts were washed (3×) with chloroform, acidified with concentrated HCl, and extracted (5×) with ether. The ether extracts were dried over Na_2SO_4 and evaporated to yield crude **6,** which was directly used as synthetic intermediate without further purification.

(3R)-3,6-Dimethylheptanol ***7*** *(Scheme 2).* $LiAlH_4$ (300 mg) was slowly added to crude **6** in ether (15 ml). After stirring overnight at room temperature, the reaction mixture was slowly quenched with saturated NaCl and 10% HCl. Following extraction (5 ×) with ether, the combined extracts were washed (3×) with saturated NaCl, dried over Na_2SO_4, concentrated, and purified by column chromatography to yield **7** (400 mg, 77% yield from **5**): $[\alpha]_D^{24}$ + 2.9(c3.07, $CHCl_3$); IR (film): 3331, 2955, 2870, 1467, 1383, 1366, 1061, 1005, 961 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 3.67 (2H, td, J = 6.6, 7.2, H-1), 1.46 (2H, m), 1.49 (2H, m), 1.37 (1H, m), 1.29 (1H, m), 1.14 (3H, m), 0.89 (3H, d, J = 6.6, CH_3), 0.87 (3H, d, J = 6.6, CH_3), 0.86 (3H, d, J = 6.6, CH_3).; CI-MS: m/z 201(27, M + 57), 143(44, M^+ − 1), 127(100); Anal. calcd. for $C_9H_{20}O$: C, 75.00%; H, 13.89%. Found: C, 75.01%; H, 13.79%.

(3R)-3,6-Dimethylheptyl p-*Toluenesulfonate* ***8*** *(Scheme 2).* *p*-Toluenesulfonyl chloride (1.1 g) was added to **7** (330 mg) in pyridine (8 ml) at 0°C. After stirring overnight at room temperature, the reaction mixture was quenched with water and saturated NaCl and extracted (5×) with hexane–ether (1:1). The combined extracts were washed (2×) with 10% HCl, 5% NaOH, and saturated NaCl, dried over Na_2SO_4, concentrated, and purified by column chromatography to yield **8** (500 mg, 73% yield): $[\alpha]_D^{24}$ + 1.2(c3.26, $CHCl_3$); IR (film): 2956, 2927, 2870, 1598, 1468, 1361, 1189, 1098, 1020, 944, 888, 815, 764, 665, 555 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.81 (2H, d, J = 7.6, H-2′), 7.35 (2H, d, J = 7.6, H-3′), 4.06 (2H, m, H-1), 2.45 (3H, s, CH_3), 1.67 (1H, m), 1.42 (3H, m), 1.18 (1H, m), 1.05 (3H, m), 0.89 (3H, d, J = 6.6, CH_3), 0.87 (3H, d, J

= 6.6, CH_3), 0.86 (3H, d, J = 6.6, CH_3).; ^{13}C NMR ($CDCl_3$) δ: 144.6, 133.5, 129.8, 127.9, 69.1, 36.0, 35.7, 34.3, 29.5, 28.2, 22.7, 22.4, 21.6, 19.2.

(5S)-2,5-Dimethylheptadecane (Scheme 2). Bromodecane (0.75 g) in ether (10 ml) was added to iodine-activated magnesium (246 mg) to yield decyl magnesium bromide. Cu(I)I (86 mg) was added at −78°C followed by the addition of **8** (200 mg) in THF (5 ml). The reaction continued overnight while the temperature increased from −78°C to room temperature. The reaction mixture was then quenched with 10% HCl, extracted (3×) with hexane, dried over Na_2SO_4, concentrated, passed through a silica gel column with hexane as eluent, and concentrated to yield crude (5*S*)-2,5-dime-17Hy. Activated molecular sieve 5Å was added to the solution of crude (5*S*)-2,5-dime-17Hy in isooctane, and kept in the oven at 110°C for 2 hr to remove straight long-chain hydrocarbon impurities. IR, GC, and EI-MS characteristics of (5*S*)-2,5-dime-17Hy were identical to those of standard racemic 2,5-dimethylheptadecane (Gries et al., 1991a, 1993a). The HR-MS result for molecular ion: 268.3120; calculated result for $C_{19}H_{40}$: 268.3130.

(2'R)-2-(2'-Methyltetradecyl)-furan **9** *(Scheme 2).* Synthesis was identical to that of **5** except undecyl magnesium bromide was used. Product **9** was obtained in 51% yield: IR (film): 2926, 2853, 1596, 1506, 1465, 1378, 1147, 1008, 929, 885, 793, 722, 599 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.30 (1H, dd, J = 1.8, 0.7, H-5), 6.27 (1H, dd, J = 1.8, 3.0, H-4), 5.97 (1H, dd, J = 3.0, 0.7, H-3), 2.60 (1H, dd, J = 14.8, 6.0, H-1'), 2.42 (1H, dd, J = 14.8, 7.9, H-1'), 1.77 (1H, m), 1.25 (21H, m), 1.13 (1H, m), 0.88 (3H, t, J = 6.6, CH_3), 0.87 (3H, d, J = 6.6, CH_3); ^{13}C NMR ($CDCl_3$) δ: 155.7(C-2), 140.7 (C-5), 110.0(C-4), 105.7(C-3), 36.7, 35.5, 32.7, 31.9, 29.9, 29.7, 29.4, 27.0, 22.7, 19.6, 14.1; EI-MS: m/z 279(9, M^+ + 1), 278(40, M^+), 151(9), 138(10), 137(26), 110(12), 109(100), 85(18), 82(72), 81(76), 71(24), 57(33), 55(14), 53(11); Anal. calcd. for $C_{19}H_{34}O$: C, 82.01%; H, 12.23%. Found: C, 82.05%; H, 12.11%.

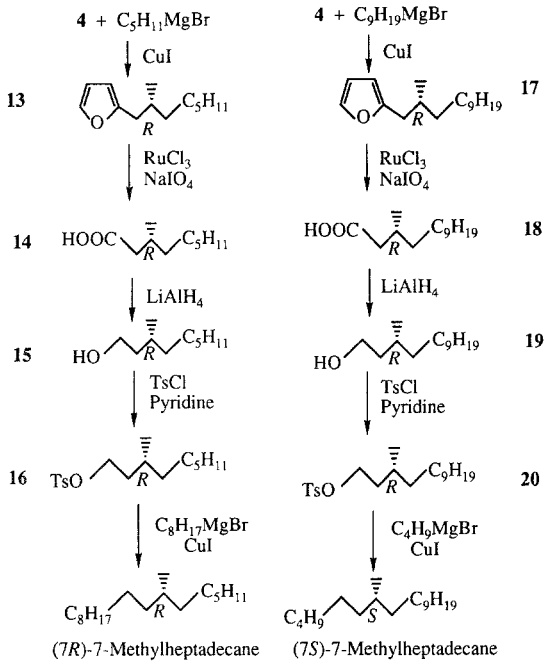
(3R)-3-Methylpentadecanol **11** *(Scheme 2).* Synthesis was identical to that of **7** except **9** was used instead of **5.** Product **11** was obtained in 52% yield: $[\alpha]_D^{24}$ + 1.8 (c3.25, $CHCl_3$); IR (film): 3332, 2922, 1458, 1377, 1054 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 3.69 (2H, m, H-1), 1.60 (1H, m), 1.58 (1H, s), 1.36 (1H, m), 1.26 (21H, m), 1.15 (2H, m), 0.89 (3H, d, J = 6.6, CH_3), 0.88 (3H, t, J = 7.2, CH_3); CI-MS: m/z 299(10, M^+ + 57), 241 (21, M^+ − 1), 225(100); Anal. calcd. for $C_{16}H_{34}O$: C, 79.34%; H, 14.05%. Found: C, 79.10%; H, 13.99%.

(3R)-3-Methylpentadecyl p-*Toluenesulfonate* **12** *(Scheme 2):* Synthesis was identical to that for **8** except **11** was used instead of **7.** Product **12** was obtained in 66% yield: $[\alpha]_D^{24}$ + 3.9(c1.55, $CHCl_3$); IR (film): 2924, 2853, 1598, 1466, 1366, 1189, 1178, 1098, 945, 889, 814, 763, 664 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.81 (2H, d, J = 7.6, H-2'), 7.35 (2H, d, J = 7.6, H-3'), 4.06 (2H, m,

H-1), 2.45 (3H, s, CH_3), 1.62 (1H, m), 1.38 (1H, m), 1.26 (22H, m), 1.05 (1H, m), 0.87 (3H, t, J = 7.2, CH_3), 0.79 (3H, d, J = 6.6, CH_3); Anal. calcd. for $C_{23}H_{40}SO_3$: C, 69.70%; H, 10.10%. Found: C, 69.77%; H, 9.97%.

(5R)-2,5-Dimethylheptadecane (Scheme 2). Synthesis was identical to that of (5*S*)-2,5-dime-17Hy except **12** and 2-propyl magnesium bromide were used instead of **8** and decyl magnesium bromide. IR, GC, and EI-MS characteristics of (5*R*)-2,5-dime-17Hy were identical to those of racemic 2,5-dime-17Hy (Gries et al., 1991a, 1993a). The HR-MS result for molecular ion: 268.3143; calculated result for $C_{19}H_{40}$: 268.3130.

*(2'R)-2-(2'-Methyloctyl)-furan **13** (Scheme 3).* Synthesis was identical to that of **5** except pentyl magnesium bromide was used. Product **13** was obtained in 53% yield: $[\alpha]_D^{24}$ + 3.4(*c*4.09, $CHCl_3$); IR (film): 2957, 2925, 2855, 1596, 1507, 1459, 1378, 1147, 1008, 929, 885, 794, 723, 599 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.30 (1H, dd, J = 1.8, 0.7, H-5), 6.27 (1H, dd, J = 1.8, 3.0, H-4), 5.97 (1H, dd, J = 3.0, 0.7, H-3), 2.60 (1H, dd, J = 14.8, 6.0, H-1'), 2.42 (1H, dd, J = 14.8, 7.9, H-1'), 1.77 (1H, m), 1.26 (9H, m), 1.14 (1H, m), 0.88 (3H, t, J = 6.7, CH_3), 0.88 (3H, d, J = 6.6, CH_3); ^{13}C NMR ($CDCl_3$) δ: 155.7(C-2), 140.7(C-5), 110.0(C-4), 105.7(C-3), 36.7, 35.5, 32.7,



SCHEME. 3. Chiral synthesis of enantiomers of 7-methylheptadecane.

31.9, 29.5, 27.0, 22.7, 19.6, 14.0; EI-MS: *m/z* 194(17, M^+), 137(16), 109(32), 81(100), 71(21), 57(32), 53(31), 41(52).

(3R)-3-Methylnonanol ***15*** *(Scheme 3).* Synthesis was identical to that of **7** except **13** was used instead of **5.** Product **15** was obtained from **13** in 76% yield: $[\alpha]_D^{24}$ + 3.1(*c*4.20, $CHCl_3$); IR (film): 3333, 2957, 2925, 2856, 1459, 1378, 1059 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 3.69 (2H, m, H-1), 1.60 (1H, m), 1.55 (1H, s), 1.36 (1H, m), 1.28 (9H, m), 1.15 (2H, m), 0.89 (3H, d, J = 6.6, CH_3), 0.88 (3H, t, J = 7.2, CH_3); ^{13}C NMR ($CDCl_3$) δ: 61.3, 40.1, 37.2, 31.9, 29.6, 26.9, 22.7, 19.7, 14.0; CI-MS: *m/z* 215(24,M^+ + 57), 157(27, M^+ − 1), 141(100); Anal. calcd. for $C_{10}H_{22}O$: C, 75.95%; H, 13.92%. Found: C, 75.80%; H, 13.85%.

(3R)-3-Methylnonyl p-*Toluenesulfonate* ***16*** *(Scheme 3).* Synthesis was identical to that of **8** except **15** was used instead of **7.** Product **16** was obtained in 63% yield: $[\alpha]_D^{24}$ + 1.4(*c*5.70, $CHCl_3$); IR (film): 2926, 2856, 1598, 1458, 1362, 1189, 1177, 1098, 944, 892, 814, 762, 664 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.81 (2H, d, J = 7.6, H-2′), 7.35 (2H, d, J = 7.6, H-3′), 4.06 (2H, m, H-1), 2.45 (3H, s), 1.63 (1H, m), 1.43 (1H, m), 1.25 (10H, m), 1.05 (1H, m), 0.87 (3H, t, J = 7.2, CH_3), 0.79 (3H, d, J = 6.6, CH_3); ^{13}C NMR ($CDCl_3$) δ: 144.6, 133.5, 129.8, 127.9, 69.1, 36.6, 35.8, 31.8, 29.5, 29.3, 26.7, 22.6, 21.6, 19.2, 14.0; CI-MS: *m/z* 313(5, M^+ + 1), 141(100).

(7R)-7-Methylheptadecane (Scheme 3). Synthesis was identical to that for (5*S*)-2,5-dime-17Hy except **16** and octyl magnesium bromide were used instead of **8** and decyl magnesium bromide. IR, GC, and EI-MS characteristics of (7*R*)-7-me-17Hy were identical to those of standard racemic 7-me-17Hy (Gries et al., 1991a, 1993a). The HR-MS result for molecular ion: 254.2956; calculated result for $C_{18}H_{38}$: 254.2973.

(2′R)-2-(2′-Methyldodecyl)-furan ***17*** *(Scheme 3).* Synthesis was identical to that of **5** except nonyl magnesium bromide was used. Product **17** was obtained in 70% yield: $[\alpha]_D^{24}$ + 2.9(*c*5.52, $CHCl_3$); IR (film) 2926, 2853, 1596, 1506, 1465, 1378, 1147, 1008, 929, 885, 793, 722, 599 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.30 (1H, dd, J = 1.8, 0.8, H-5), 6.27 (1H, dd, J = 1.8, 3.1, H-4), 5.97 (1H, dd, J = 3.1, 0.8, H-3), 2.60 (1H, dd, J = 14.8, 5.9, H-1′), 2.42 (1H, dd, J = 14.8, 7.8, H-1′), 1.79 (1H, m), 1.25 (17H, m), 1.14 (1H, m), 0.88 (3H, t, J = 6.7, CH_3), 0.88 (3H, d, J = 6.6, CH_3); ^{13}C NMR ($CDCl_3$) δ: 155.7(C-2), 140.7(C-5), 110.0(C-4), 105.7(C-3), 36.7, 35.5, 32.7, 31.9, 29.9, 29.7, 29.3, 27.0, 22.7, 19.6, 14.0; EI-MS: *m/z* 250(10, M^+), 137(10), 109(80), 82(95), 81(100), 71(70), 57(85).

(3R)-3-Methyltridecanol ***19*** *(Scheme 3).* Synthesis was identical to that of **7** except **17** was used instead of **5.** Product **19** was obtained from **17** in 79% yield: $[\alpha]_D^{24}$ + 3.5(*c*4.85, $CHCl_3$); IR (film): 3334, 2923, 2852, 1466, 1378, 1057 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 3.68 (2H, m, H-1), 1.60 (1H, m), 1.55 (1H, s), 1.38 (1H, m), 1.26 (18H, m), 1.14 (1H, m), 0.89 (3H, d, J = 6.6, CH_3),

0.88 (3H, t, J = 7.2, CH_3); Anal. calcd. for $C_{14}H_{30}O$: C, 78.50%; H, 14.02%. Found: C, 78.34%; H, 13.92%.

(3R)-3-Methyltridecyl p-*Toluenesulfonate* ***20*** *(Scheme 3).* Synthesis was identical to that of **8** except **19** was used instead of **7.** Product **20** was obtained in 50% yield: $[\alpha]_D^{24}$ + 1.0(c3.85, $CHCl_3$); IR (film): 2925, 2853, 1598, 1466, 1365, 1189, 1177, 1098, 945, 888, 814, 764, 664 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.81 (2H, d, J = 7.6, H-2′), 7.35 (2H, d, J = 7.6, H-3′), 4.06 (2H, m, H-1), 2.45 (3H, s), 1.62 (1H, m), 1.42 (1H, m), 1.26 (18H, m), 1.05 (1H, m), 0.87 (3H, t, J = 7.2, CH_3), 0.79 (3H, d, J = 6.6, CH_3); Anal. calcd. for $C_{21}H_{36}SO_3$: C, 68.48%; H, 9.78%. Found: C, 68.49%; H, 9.94%.

(7S)-7-Methylheptadecane (Scheme 3). Synthesis was identical to that for (5*S*)-2,5-dime-17Hy except **20** and butyl magnesium bromide were used instead of **8** and decyl magnesium bromide. IR, GC, and EI-MS characteristics of (7*S*)-7-me-17Hy were identical to those of racemic 7-me-17Hy (Gries et al., 1991a, 1993a). The HR-MS result for molecular ion: 254.2962; calculated result for $C_{18}H_{38}$: 254.2973.

Electrophysiological Studies

WHL larvae were collected near Blue River, British Columbia and reared to the adult stage in the laboratory at 20°C, 70% relative humidity, and a photoperiod of 15 : 9 hr light–dark. Male and female pupae were kept separately in Petri dishes to avoid mating of emergent moths. Two- to three-day-old males were used for gas chromatographic–electroantennographic analysis (GC-EAD) (Arn et al., 1975) employing a Hewlett Packard 5890A gas chromatograph equipped with a DB-210 coated, fused silica column (30 m × 0.25 mm ID). In four replicates, responses of male WHL antennae to 10 pg of each enantiomer of 7-me-17Hy were recorded.

Field Bioassay

Field experiments were set up near Blue River, British Columbia, in randomized complete blocks with traps and blocks at 15- to 20-m intervals. Along a forest margin, green Unitraps (Phero Tech Inc., Delta, British Columbia) were suspended 1.5–2 m above ground and 0.5–1 m inside the foliage of various coniferous and deciduous trees. Traps were baited with rubber septa (Sigma Chemical Co., St. Louis, Missouri) impregnated with test chemicals in HPLC-grade hexane. A Dichlorvos cube (~1 cm^3) (Green Cross Co., Mississauga, Ontario) in each trap ensured rapid killing and retainment of captured moths.

The first five-treatment, 10-replicate experiments tested (5*R*,11*S*)-5,11-dime-17Hy alone (10 μg) and in binary combination with either (7*R*)-7-me-17Hy (10μg), (7*S*)-7-me-17Hy (10 μg), (7*R*)-7-me-17Hy and (7*S*)-7-me-17Hy combined at 10 μg each, or racemic 7-me-17Hy (20 μg). The second five-

treatment, 20-replicate experiments tested (5*R*,11*S*)-5,11-dime-17Hy (10 μg) in binary combination with (7*S*)-7-me-17Hy (10 μg) and in ternary combination with either (5*S*)-2,5-dime-17Hy (10 μg), (5*R*)-2,5-dime-17Hy (10 μg), (5*S*)-2,5-dime-17Hy, and (5*R*)-2,5-dime-17Hy combined at 10 μg each, or racemic 2,5-dime-17Hy (20 μg). A final five-treatment, 20-replicate experiment tested (5*R*,11*S*)-5,11-dime-17Hy and (5*S*)-2,5-dime-17Hy in binary combination at 10 μg each and in ternary combination with either (7*R*)-7-me-17Hy (10 μg), (7*S*)-7-me-17Hy (10 μg), (7*R*)-7-me-17Hy and (7*S*)-7-me-17Hy combined at 10 μg each, or racemic 7-me-17Hy (20 μg).

Statistical Analysis

Statistical analysis was conducted with a SAS statistical package. To meet the requirements of normality and homoscedasticity, data were transformed by $\log_{10}$ or $\log_{10}(x + 10)$ and subjected to analysis of variance followed by Scheffé's test.

RESULTS

Biotransformation of (2*E*)-3-(2′-furanyl)-2-methyl-2-propenal **2** to (2*S*)-3-(2′-furanyl)-2-methylpropanol **3** (>97% ee) by bakers yeast (Scheme 1) yielded a synthetic intermediate of high chiral purity for the synthesis of enantiomers of 2,5-dime-17Hy (Scheme 2) and 7-me-17Hy (Scheme 3). All four enantiomers were obtained by Cu(I)I catalyzed coupling of Grignard reagents and tosylates derived from (2*S*)-3-(2′-furanyl)-2-methylpropanol.

(7*S*)-7-Me-17Hy but not (7*R*)-7-me-17Hy elicited strong electrophysiological responses (~6 mV) by male WHL antennae (Figure 1). In field experiments, (7*S*)-7-me-17Hy but not (7*R*)-7-me-17Hy significantly increased attraction to the major sex pheromone component (5*R*,11*S*)-5,11-dime-17Hy (Figure 2). (7*S*)-7-Me-17Hy, (7*S*)- and (7*R*)-7-me-17Hy combined and racemic 7-me-17Hy equally enhanced attraction (Figure 2). (5*S*)- but not (5*R*)-2,5-Dime-17Hy significantly increased attraction to (5*R*,11*S*)-5,11-dime-17Hy combined with (7*S*)-7-me-17Hy (Figure 3). Racemic 2,5-dime-17Hy or (5*R*)- and (5*S*)-2,5-dime-17Hy combined were less synergistic (Figure 3). Addition of (7*S*)-but not (7*R*)-7-me-17Hy significantly increased attraction to (5*R*,11*S*)-5,11-dime-17Hy combined with (5*S*)-2,5-dime-17Hy (Figure 4). (7*S*)-7-Me-17Hy, (7*S*)- and (7*R*)-7-me-17Hy combined and racemic 7-me-17Hy equally enhanced attraction (Figure 4).

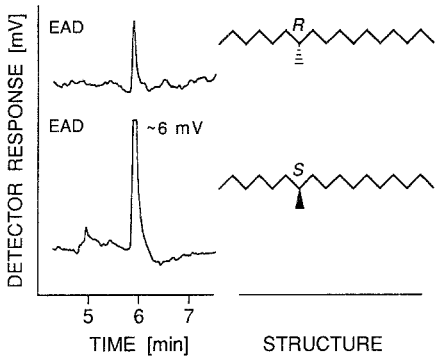


FIG. 1. Representative GC-EAD recording of a male WHL antenna responding to 10 pg of separately injected enantiomers of 7-me-17Hy (Hewlett Packard 5890A, DB-210 column, 1 min at 70°C, 20°C/min to 130°C, 2°C to 220°C).

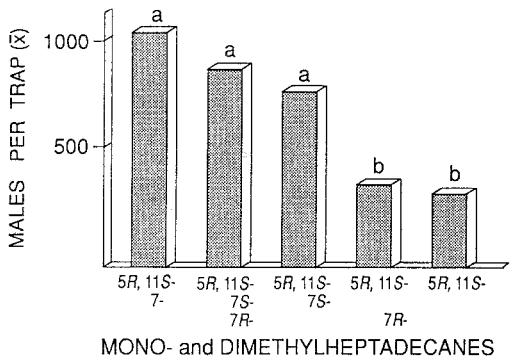


FIG. 2. Mean catches of male WHL in Unitraps baited with candidate chiral pheromone components. Each component was tested at 10 μg except racemic 7-me-17Hy was tested at 20 μg, 27 August–7 September 1992, Blue River, British Columbia, $N = 10$. Bars superscripted by the same letter are not significantly different (ANOVA, followed by Scheffé's test, $P < 0.05$).

DISCUSSION

Chiral methyl-branched pheromones have been identified in the Lepidoptera (Francke et al., 1987, 1988; Gries et al., 1991a,b, 1993a,b; Grula and Taylor, 1979; Sugie et al., 1984; Tamaki et al., 1979), Coleoptera (Chuman et al., 1987; Guss et al., 1982, 1983; Silverstein et al., 1980; Suzuki and Sugawara, 1979; Tanaka et al., 1986; Yarger et al., 1975), Diptera (Carlson et al., 1978; Linley and Carlson, 1978; Sonnet et al., 1979), and Orthoptera (Nishida et al., 1975). Chirality of pheromone components was determined among others in the

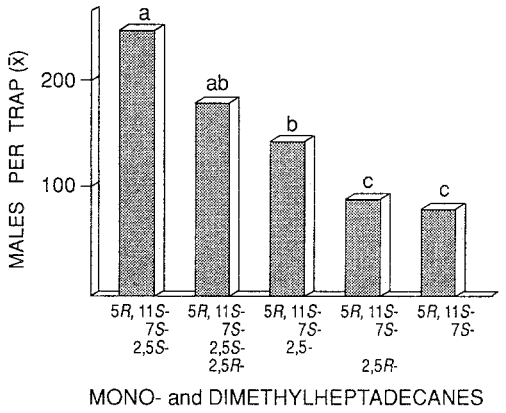


FIG. 3. Mean catches of male WHL in Unitraps baited with candidate chiral pheromone components. Each component was tested at 10 μg except racemic 2,5-dime-17Hy was tested at 20 μg, 9–19 September 1992, Blue River, British Columbia, $N = 20$. Bars superscripted by the same letter are not significantly different (ANOVA, followed by Scheffé's test, $P < 0.05$).

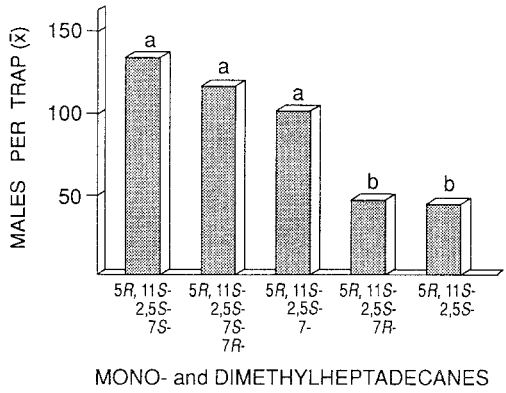


FIG. 4. Mean catches of male WHL in Unitraps baited with candidate chiral pheromone components. Each component was tested at 10 μg except racemic 7-me-17Hy was tested at 20 μg, 19 September–16 October 1992, Blue River, British Columbia, $N = 10$. Bars superscripted by the same letter are not significantly different (ANOVA, followed by Scheffé's test, $P < 0.05$).

moths *Leucoptera scitella* (Zeller) (Tóth et al., 1989) and *Lambdina fiscellaria* (Li et al., 1993); the redheaded pine sawfly, *Neodiprion lecontei* (Fitch) (Matsumura et al., 1979), and in the tsetse fly, *Glossina pallidipes* (Austen) (McDowell et al., 1985).

Chiral methyl-branched pheromones have been synthesized from monomethyl (3*R*)-3-methylglutarate (Carpita et al., 1989), chiral citronellic acid (Mori, 1989), 2-methylbutan-1-ol (Bestmann et al., 1988, 1990), through conjugate addition of alkyl copper complexes to chiral crotonates (Helmchen and Wegner, 1985), and from methoxylbornyl sulfoxides (Li, 1993), as well as by other routes (Mori, 1989). In applying Fuganti's method (Fuganti et al., 1988) to the chiral synthesis of 7-me-17Hy and 2,5-dime-17Hy, ease of preparation and chiral purity of enantiomers were enhanced over classical chemical methods. This method employs bakers' yeast to transform (2*E*)-3-(2′-furanyl)-2-methyl-2-propenal **2** to (2*S*)-3-(2′-furanyl)-2-methylpropanol **3** (Scheme 1). As **3** is of extremely high chiral purity (>97% ee), is bifunctional, and readily reacts with various potential coupling components, it is a valuable chiral starting material for many chiral syntheses (Fuganti et al., 1988; Fuganti, 1990).

Synthesis of (5*S*)-2,5-dime-17Hy (Scheme 2) exemplifies the use of **3.** The *p*-toluenesulfonate **4** was coupled in the presence of 10% copper(I) iodide with 2-methylpropyl magnesium bromide to yield **5.** Use of Li_2CuCl_4 as alternative catalyst always gave poor yields of **5.** Oxidation with $NaIO_4$ in the presence of catalytic $RuCl_3$ converted **5** to the corresponding acid **6** (Ferraboschi et al., 1990). Chiral purity of **6** was >97% ee, as determined by GC analysis of the corresponding (1*R*)-1-phenylethylamide (Li, 1993, Carpita et al., 1989). Reduction of **6** with $LiAlH_4$ in THF yielded **7,** and esterification with *p*-toluenesulfonyl chloride and pyridine gave **8.** Coupling chiral **8** with decyl magnesium bromide yielded the final product, (5*S*)-2,5-dime-17Hy, with very high chiral purity. This coupling also gave cross-coupling hydrocarbon impurities, which made purification and characterization difficult, but fortunately, all these impurities were biologically inactive in electrophysiological and field trapping studies of WHL. (5*R*)-2,5-Dime-17Hy (Scheme 2) and (7*S*)-7-me-17Hy and (7*R*)-7-me-17Hy (Scheme 3) were synthesized following the same route.

Strong antennal responses by male WHL antennae to (7*S*)- but not (7*R*)-7-me-17Hy (Figure 1) suggested that the *S* enantiomer is the synergistic pheromone component. In field experiments (Figures 2 and 4), (7*S*)-7-me-17Hy significantly enhanced attraction to (5*R*,11*S*)-5,11-dime-17Hy alone or in combination with (5*S*)-2,5-dime-17Hy, while (7*R*)-7-me-17Hy was neither attractive nor inhibitory. GC-EAD recordings with enantiomers of 2,5-dime-17Hy were not obtained. All males emerged from field-collected pupae had died before chiral synthesis of 2,5-dime-17Hy was completed, and field collected adult males were of insufficient quality to conduct EAD recordings. However, field experiments clearly demonstrated that (5*S*)-2,5-dime-17Hy is a synergistic pheromone component in WHL (Figure 3).

In contrast with findings in the gypsy moth, *Lymantria dispar* (L.) (Cardé et al., 1977, 1978; Plimmer et al., 1977) and the geometrid moths *Itame occiduaria* (Packard) and *I. brunneata* (Thunberg) (Millar et al., 1990), the presence

of non-species-specific pheromone enantiomers in field trapping experiments did not inhibit attraction of male WHL. Only (5*R*)-2,5-dime-17Hy in combination with (5*S*)-2,5-dime-17Hy or in racemic 2,5-dime-17Hy slightly reduced attraction (Figure 3).

In conclusion, (5*R*,11*S*)-5,11-dime-17Hy (Li et al., 1993), (7*S*)-7-me-17Hy, and (5*S*)-2,5-dime-17Hy comprise the sex pheromone of the WHL. The WHL sex pheromone constitutes the first example of a (lepidopteran) pheromonal blend with three chiral components. Bakers' yeast produced **3** is demonstrated to be a versatile chiral synthon of high chiral purity for the synthesis of chiral methyl-branched pheromones in the Insecta.

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EVIDENCE FOR A MULTICOMPONENT SEX PHEROMONE IN *Eriborus terebrans* (GRAVENHORST) (HYM.: ICHNEUMONIDAE), A LARVAL PARASITOID OF THE EUROPEAN CORN BORER

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Abstract—Sex pheromone activity of *Eriborus terebrans* (Gravenhorst) (Hymenoptera: Ichneumonidae) was recovered from acetone rinses of flasks that previously contained females. The acetone flask rinses elicited the following male responses: upwind anemotaxis, casting, hovering, landing, wing-fanning, and mating attempts with other nearby males. Activity of the acetone flask rinse lasted up to four days on a glass substrate. Polar component and nonpolar components were demonstrated in the acetone flask rinse. The polar component elicited male behavioral responses similar to those by the acetone flask rinse, although retention of males at the pheromone source and the period of wing-fanning were of shorter duration. Chromatography data and chemical derivatization indicated that the polar component had the properties of a carboxylic acid with an additional oxygen-containing functional group. The nonpolar component acted as a synergist since it was inactive alone but increased male behavioral responses when added to the polar component. Florisil open column chromatography suggested that the nonpolar component was a hydrocarbon(s).

Key Words—Sex pheromone, multicomponent pheromone, parasitoid, bioassay, Hymenoptera, Ichneumonidae, *Eriborus terebrans*, Lepidoptera, Pyralidae, *Ostrinia nubilalis*.

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INTRODUCTION

The use of natural enemies for biological control of insects is an integral part of a successful integrated pest management (IPM) program. An understanding of the chemical ecology of insect parasitoids can help to make their use more efficient. This includes the study and identification of sex pheromones of parasitoids, since such pheromones can be used to assess the activity of parasitoids, to monitor their density, and to predict rates of host parasitism. Morse and Kulman (1985) trapped the yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) and its parasitoid, *Syndipnus rubiginosus* Walley, using their respective synthetic sex pheromones. This information, along with tree height, was used to predict subsequent sawfly-caused defoliation of white spruce trees. Furthermore, sex pheromones can be useful as a chemotaxonomic character because the chemical composition of sex pheromones and/or the ratio of their components are often species-specific (Roelofs and Brown, 1982; Doré et al., 1986).

Sex pheromones of three species of Hymenopterous parasitoids have been identified (Robacker and Hendry, 1977; Eller et al., 1984; Swedenborg and Jones, 1992; Swedenborg et al., 1993), but only two have been verified with synthesis and field studies. The occurrence of sex pheromones has been demonstrated in a few dozen species among at least nine families (Aphelinidae, Braconidae, Chalcididae, Cynipidae, Eulophidae, Ichneumonidae, Pteromalidae, Scelionidae, and Trichogrammatidae). Evidence for sex pheromones in Hymenopterous parasitoids and their courtship behavior have been reviewed in detail (Shu, 1992).

Eriborus terebrans (Gravenhorst) (Hymenoptera: Ichneumonidae) is a larval parasitoid of the European corn borer, *Ostrinia nubilalis* (Hübner). It was introduced to the United States from Europe and Asia in 1920s and 1930s and is now established in the corn-growing areas (Rolston et al., 1958; Hill et al., 1978; Andreadis, 1982; Lewis, 1982; Winnie and Chiang, 1982).

Preliminary data showed that the presence of *E. terebrans* females elicited attraction and wing-fanning of conspecific males, indicating the existence of a female sex pheromone. This paper presents the evidence for a multicomponent sex pheromone in *E. terebrans* and describes the isolation and preliminary characterization of the pheromone.

METHODS AND MATERIALS

Insects

The colony of *E. terebrans* used in this study was field collected in Minnesota. *E. terebrans* and its host, *O. nubilalis*, were reared according to Ma et al. (1992) and Guthrie et al. (1971). Cocoons of *E. terebrans* were kept indi-

vidually to isolate sexes. Voucher specimens of the parasitoid have been deposited in the University of Minnesota Insect Collection (Shu, 1992).

Pheromone Collection (Flask Rinse)

Active pheromone extracts were obtained by confining four virgin females (1–3 days old) in a 125-ml clear glass flask at ca. 25°C, 85% relative humidity, and 16:8 hr light–dark for 24 hr. The females were removed and the flask was rinsed with 2 ml hexane once (hexane flask rinse, HFR) and 2 ml acetone twice (acetone flask rinse, AFR) by swirling the flask with the solvent for 30 sec each time. Both HFR and AFR were concentrated under a nitrogen stream and kept separately in a freezer at −30°C until used. Some females were used repeatedly for pheromone collection. Females used more than once were fed with honey and water in a cotton sponge between collections. One female equivalent (FE) was equivalent to the total acetone rinse of a flask exposed to one female for 24 hr. Only AFR was used in this study. HFR was used for detailed studies of hydrocarbons (Shu and Jones, 1993; Shu et al., 1993).

Pheromone Isolation

Isolation of AFR. Initially, AFR was separated into various fractions with Florisil open column chromatography consecutively eluted with aliquots of hexane, then 2.5, 5.0, 7.5, 10.0, 25.0, and 50.0% ether in hexane, ether, acetone, and methanol. Later, separation of AFR into two fractions was accomplished in the following way. AFR was dried under a stream of nitrogen and taken up in diethyl ether, which was then extracted three times in a separatory funnel with water saturated with $NaHCO_3$. The ether layer (neutral phase) was saved, dried over $MgSO_4$, and stored at −30°C until used. The water extracts were combined, then acidified carefully with dilute HCl and extracted with ether three times. The water layer was discarded. The ether layers (polar phase) were combined, dried over $MgSO_4$ and either stored in a freezer at −30°C or used immediately.

Isolating Polar Component. High-performance liquid chromatography (HPLC) of the polar phase was conducted with a 25-cm × 0.46-cm Lichrosorb RP-8 column (5 μ, Alltech) employing a 1% aqueous phosphoric acid–methanol solvent running isocratically at a flow rate of 1 ml/min. The aqueous phosphoric acid–methanol ratio was 20:80. Collection of fractions began at 3 min after injection. A total of five 1-ml fractions was collected. Methanol in fractions was reduced under a stream of nitrogen, and the residue in water was extracted with ether. The ether extracts were then dried over $MgSO_4$.

Preparative thin-layer chromatography (TLC) of AFR was conducted with 5-cm × 20-cm Redi/Plate plates precoated with Silica Gel G (Analtech Inc., 250 μm thickness). The plates were activated overnight at 110 °C, then cooled

to room temperature before use. Plates were developed at room temperature with hexane–diethyl ether–acetic acid (70:30:2, v/v/v). Spots were visualized under UV light after spraying the plates with a 0.05% ethanol solution of rhodamine B. Four fractions were collected. Fraction 1 was recovered by scraping off the band from an unsprayed plate at a location corresponding to a detected spot of 10-hydroxydecanoic acid (R_F = 0.05) on a sprayed plate. Fraction 3 corresponded to a detected spot of octanoic acid (R_F = 0.41). Fraction 2 was from a band between fraction 1 and fraction 3, and fraction 4 was from a band above fraction 3. The recovery procedure involved placement of the scraped gel into a glasswool-stopped pipet, which was then eluted with 2 ml acetone. Fractions 1 and 2 were combined, rechromatographed, and recovered in the same manner as described above.

Isolation of Neutral Phase. The neutral phase was fractionated by open column chromatography on Florisil (2.5% water by weight). The column was eluted consecutively with aliquots of hexane, 5%, 7.5%, 10%, 25%, and 50% ether in hexane, ether, acetone, and methanol. Each fraction was bioassayed alone and in combination with the polar phase. Later, separations of the neutral phase were accomplished by collecting only two fractions from a Florisil column, one with hexane and one with ether. The volume of each eluant was twice the void volume of the column. Fractions were stored at −30°C until used.

Derivatization

Diazomethane in ether (CH_2N_2-ether) was obtained from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to Black (1983). To esterify the extracts, freshly prepared diazomethane–ether with 10% methanol (volume ratio to CH_2N_2-ether) was added to the crude extracts dried under a stream of nitrogen (Schlenk and Gellerman, 1960). The solution was kept at room temperature for at least 1 hr. It was then stored in −30°C if not used. For a control, the crude extracts were dried under a stream of nitrogen and taken up in 10% methanol ether.

Female Body Rinse

Female body rinse (FBR) was obtained by soaking virgin females in either hexane or acetone. After soaking for a day, the extracts were drawn off and the bodies were rinsed with acetone three times. The extracts and subsequent acetone rinses were combined and concentrated under nitrogen.

Gas Chromatography (GC)

Hydrocarbons were analyzed by GC with a nonpolar column (DB-1) as described in Shu and Jones (1993).

Bioassay

Bioassays were conducted in a rectangular wind tunnel (140 cm long × 90 cm wide × 94 cm high), which consisted of screens on each end, wood-framed glass walls (two side walls and top), and a plastic bottom. The wind-tunnel was set up in a ventilated laboratory room in which a 16:8 hr light–dark regime was maintained. A temperature of 22–26°C and 55–70% relative humidity were maintained in the wind tunnel. A 6-cm-square wood-board platform was erected at a height of 38 cm at 40 cm from the upwind end to hold a watchglass or a female-holding cage. A 22.5-in.-diam. fan was used at one end to draw air out of the wind tunnel at ca. 0.25 m/sec at the center of the tunnel. Air from the downwind end was exhausted from the room via a chemical hood. Two banks of four 40-W fluorescent lights above the wind tunnel ran parallel with the long axis of the tunnel. These lights were turned on when conducting bioassays. All the tests were performed between the first and third hour of the photo phase. A group of 50 unmated males, 3 days old or older, were used in every bioassay. Each group was used for no more than five consecutive days. No more than six bioassays were conducted each day. Diluted honey in cotton sponges and water in cotton wicks were provided in the wind tunnel. A virgin female (1–3 days old), when tested, was housed in a copper cylinder (4 × 6 cm) with one end screened and other end stopped with a watchglass and placed on the platform. Rinses or isolated fractions were applied on 6.5-cm watchglasses, and except for tests of effects of AFR age, all the chemical preparations were left to dry at the room temperature prior to bioassays for 24 hr.

The number of male landings and the number of wing-fannings in a period of 5 min were recorded as the response criteria for all the bioassays except where otherwise noted. Since males were not captured upon landing, the same male could be counted more than once. Only one wing-fanning per landing, if any, was recorded.

All replications were conducted with new chemical preparations except for the test of effects of AFR age. A completely randomized block design was used for all the tests. All treatments in a test were bioassayed in random order on the same day, which was regarded as a block. Data were transformed with log $(y + 1)$ to stabilize the variance. Multiple comparisons were made using Duncan's multiple-range tests at $\alpha \leq 0.05$ (Duncan, 1955) after F test indicated significance. SAS computer program was used.

RESULTS

When virgin females or AFR were present in the wind tunnel, male behavior included upwind flight (anemotaxis), casting, hovering, landing, wing-fanning, and attempts to copulate with other males.

One FE AFR was significantly more active than one virgin female and than control in terms of both male landings and male wing-fannings ($\alpha \leq 0.05$). One virgin female was significantly more active than control ($\alpha \leq 0.05$). The mean numbers (four replicates) of male landings and male wing-fannings on the AFR-treated watchglass were 126.25 (SE = 7.21) and 49.75 (SE = 3.87) respectively, while those on the female-holding cage were 89.25 (SE = 5.45) and 32.5 (SE = 1.39), respectively. The mean numbers of male landings and male wing-fannings on the control solvent-treated watchglass were 23.5 (SE = 2.02) and 0, respectively.

The watchglass treated with AFR remained active up to four days (Figures 1 and 2). AFR on watchglasses between 0.5 FE and 2 FE was most active when it was 24 hr old. The 4 FE AFR maintained about the same activity from day 1 to day 4. The optimal dose for AFR when 1 day old was 2.5 FE.

The pheromonal activity of AFR would not pass through a Florisil column. Neither a single Florisil fraction nor combination of all the Florisil fractions could restore the activity of AFR. Consequently the activity in AFR was partitioned between ether and water saturated with $NaHCO_3$. The neutral phase (ether) was active, but additional activity was obtained by acidifying the water (saturated with $NaHCO_3$) and reextracting with ether (polar phase). Both the

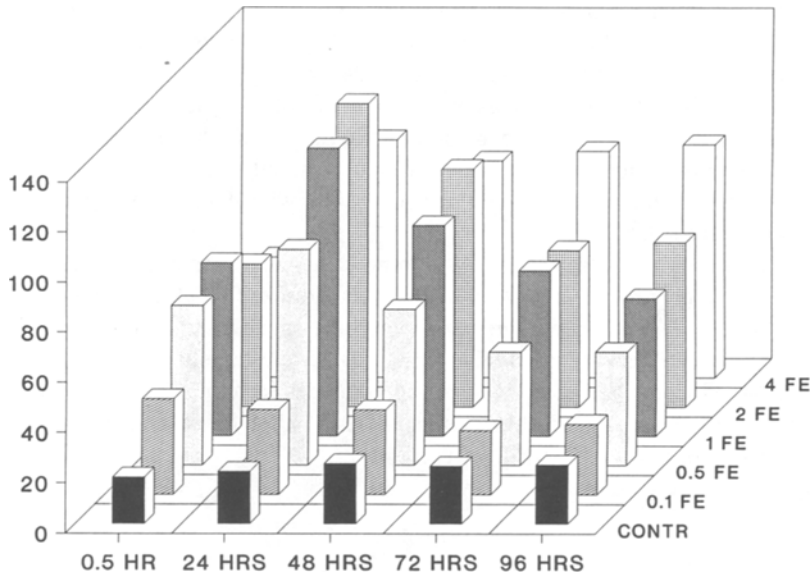

FIG. 1. Effect of AFR ages measured in male landings. The mean number of male landings is represented by y axis (mean based on six replications). FE = female equivalent; CONTR = control.

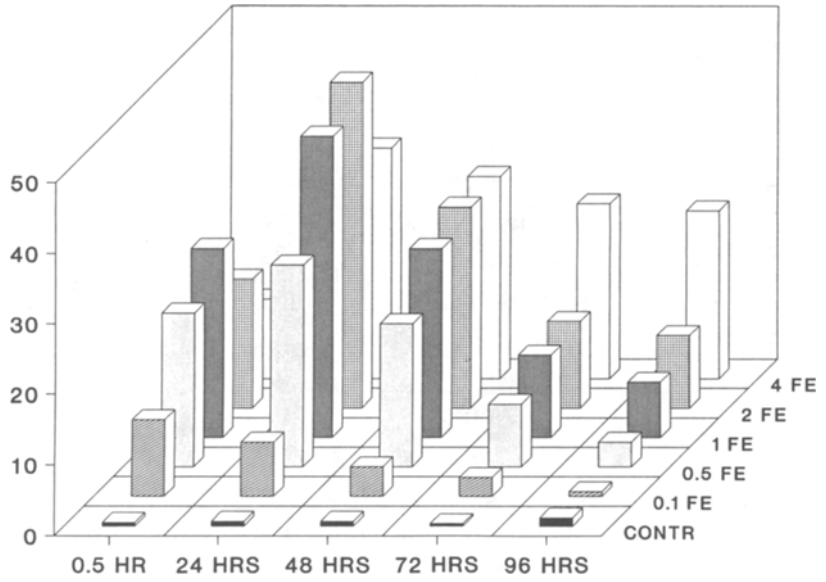

FIG. 2. Effect of AFR ages measured in male wing-fannings. The mean number of male wing-fannings is represented by *y* axis (mean based on six replications). FE = female equivalent; CONTR = control.

polar phase (1 FE) and the neutral phase (1 FE) were active, but not as active as AFR (1 FE) (Figure 3). Male behavior, in response to the polar phase, was similar to that obtained with AFR, but observations indicated that the retention of males on the watchglasses and the duration of their wing-fanning were briefer when the polar phase was present compared to AFR. Doubling the dose of the polar phase (2 FE) did not increase activity. Combination of 1 FE polar phase and 1 FE neutral phase increased the activity significantly, implying that more than one component was present.

When the polar phase was further analyzed by HPLC, fraction 2 elicited the greatest behavioral activity and there was some activity in fraction 1 (Table 1). The retention times of 10-hydroxydecanoic acid and octanoic acid were 4.3 min and 6 min, respectively. The retention time of 10-hydroxydecanoic acid corresponded to fraction 2, while the solvent (acetone) came off in fraction 1. Therefore, the active polar component was similar in polarity to 10-hydroxydecanoic acid under the chromatography conditions.

When AFR was chromatographed by TLC, both TLC fractions 1 and 2 were active (Table 2), although TLC fraction 2 was more active than TLC fraction 1 in terms of male wing-fannings. TLC fraction 1 had an R_F equivalent to that of 10-hydroxydecanoic acid. TLC fraction 3 had an R_F equivalent to that

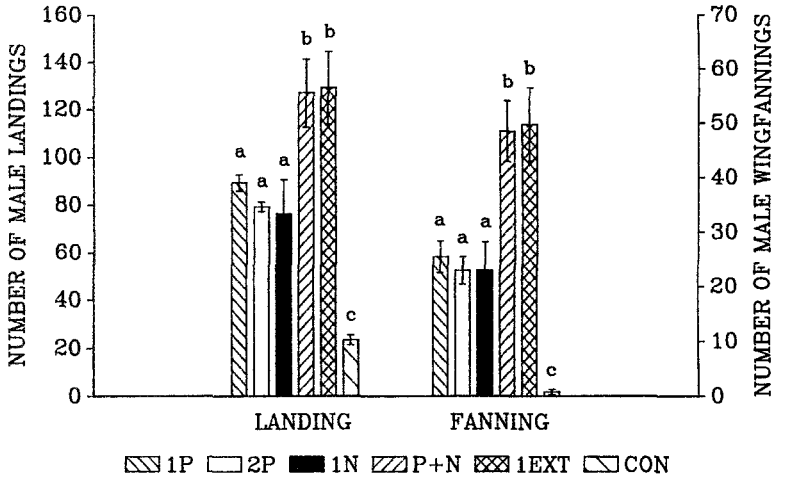

FIG. 3. Male responses (mean ± SE) to isolated fractions of AFR. The figure is composed of two graphs. The x axis represents landings and wing-fannings. The number of male landings is represented by y axis on the left and the number of male wing-fannings is represented by y axis on the right (data based on four replications). The different letters above bars in the same category indicate significant difference at $\alpha \leq 0.05$. 1P = 1 FE (female equivalent) polar phase; 2P = 2 FE polar phase; 1N = 1 FE neutral phase; P + N = 1 FE polar phase plus 1 FE neutral phase; 1EXT = 1 FE AFR; CON = control.

TABLE 1. MEAN NUMBER OF MALE RESPONSES TO HPLC FRACTIONS (2 FE) OF POLAR PHASE[a]

Fraction	Collection start to end (min)	Responses	
		Male landings	Male wing-fannings
1	3-4	45.8a	2.8a
2	4-5	64.0b	11.8b
3	5-6	19.3c	0
4	6-7	18.3c	0
5	7-8	17.3c	0
Control		20.5c	0

[a]The mean was obtained from four replications; HPLC was equipped with a Lichrosorb RP-8 column using a solvent system of 1% aqueous phosphoric acid–methanol (20:80, v/v) running isocratically. The means followed by different letters in the same column are significantly different at $\alpha \leq 0.05$.

TABLE 2. MEAN NUMBER OF MALE RESPONSES TO TLC FRACTION (5 FE) OF POLAR FRACTIONS[a]

Fraction	R_F value	Responses	
		Male landings	Male wing-fannings
1	0.05	55.0a	19.0a
2	0.18	61.8a	25.8a
3	0.41	22.5b	0.5b
4		24.3b	0
Control		19.0b	0.3b

[a]The means were obtained from four replications; TLC plate was precoated with Silica Gel G and developed in a solvent system of: hexane–ether–acetic acid (70:30:2, v/v/v). Polar fractions = TLC fractions 1 and 2 of AFR. The means followed by different letters in the same column are significantly different at $\alpha \leq 0.05$.

of octanoic acid. Therefore, the polar component of the sex pheromone had an R_F smaller than that of a fatty acid and equal to or greater than that of a hydroxy acid.

When AFR was esterified with diazomethane, it lost most activity. The mean numbers (six replications) of male landings and wing-fannings on watch-glasses treated with the esterified AFR (1 FE) were 33.3 (SE = 5.401) and 7.7 (SE = 1.5), respectively, while those with AFR (1 FE) (taken up in the 10% methanol ether) were 98.5 (SE = 10.01) and 48.2 (SE = 5.48), respectively. The number of male landings and the number of male wing-fannings elicited by the esterified AFR were significantly different from those by unmodified AFR at $\alpha \leq 0.05$.

To further characterize the neutral phase of AFR, it was fractionated by open column chromatography on Florisil. Preliminary data suggested that only the hexane Florisil fraction was synergistically active. The neutral phase was consequently separated into two fractions—hexane Florisil fraction and ether Florisil fraction. Bioassay with a combination of either hexane Florisil fraction (2 FE) or ether Florisil fraction (2 FE) with the polar phase (1 FE) indicated that the hexane fraction from the neutral phase contained a component of the sex pheromone (Figure 4). The component was essentially inactive alone, but could increase activity to the polar phase, thus acting as a synergist. The hexane Florisil fraction elicited some male wing-fannings probably from older males. Observations indicated that when males became older and/or were more experienced with sex pheromone, they responded slightly to hydrocarbons (hexane Florisil fraction) alone and to other males with upwind anemotaxis, landing, and even wing-fanning.

Extraction of virgin female bodies of *E. terebrans* with hexane or acetone

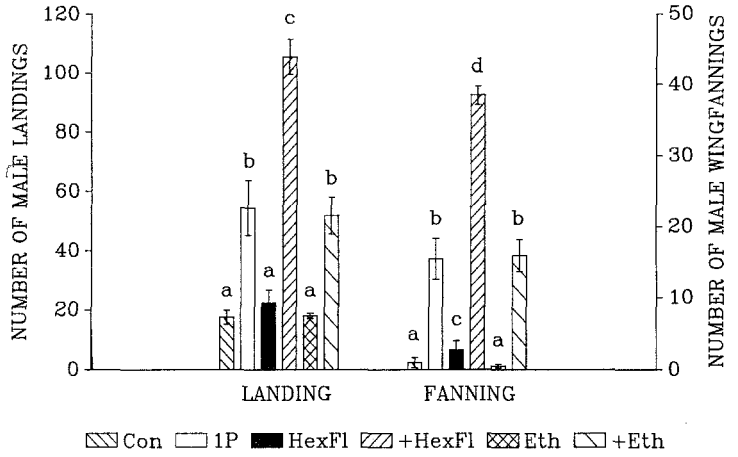

FIG. 4. Male responses (mean ± SE) to isolated fractions of the neutral phase by Florisil column. The figure is composed of two graphs. The *x* axis represents landings and wing-fannings. The number of male landings is represented by the *y* axis on the left and the number of male wing-fannings is represented by *y* axis on the right (data based on four replications). The different letters above bars in the same category indicate significant difference at $\alpha \leq 0.05$. Con = control; 1P = 1 FE polar phase; HexFl = 2 FE hexane Florisil fraction; +HexFl = 1 FE polar phase + 2 FE hexane Florisil fraction; Eth = 2 FE ether Florisil fraction (eluted just after hexane); +Eth = 1 FE polar phase + 2 FE ether Florisil fraction.

yielded extracts with very little activity (Table 3). However, FBR (by soaking females in hexane and rinsing the bodies with acetone after hexane extracts were drawn off) contained both the polar component (the polar phase) and the hydrocarbon component (hexane Florisil fraction of the neutral phase). These two components, isolated from FBR, were very active together, and the polar component from FBR was active alone.

Based on GC analysis, soaking the females in hexane yielded about 15 times as much hydrocarbon as in AFR. It appeared that *E. terebrans* males responded to a range of ratios of hydrocarbons to polar component (Table 3, Figure 4).

DISCUSSION

Active extracts were obtained by rinsing the female-holding container (glass flask) with acetone twice after rinsing with hexane once. HFR was slightly active, but much less active than AFR, indicating that most pheromone activity was still on the glass after rinsing with hexane. The sex pheromone was appar-

TABLE 3. MEAN NUMBER OF MALE RESPONSES TO FEMALE BODY RINSES (FBR) AND ACETONE FLASK RINSES (AFR) IN 2.5 MINUTES[a]

Treatments	*N*	Mean number of male responses	
		Landings	Fannings
0.2 BE FBR	9	8.9	1.8
1 BE FBR	4	7.8	3
0.2 BE PC(FBR)	5	26.6	8
0.2 BE PC(FBR) + 0.2 BE HexFl(FBR)	2	51.5	21.5
1 FE PC(AFR) + 15 FE HexFl(AFR)	2	100	52.5
1 FE AFR	2	80	39.5
Control	4	8.3	0

[a] Treatments were not blocked. Data were not statistically analyzed. BE (body equivalent) = total extracts obtained by soaking one virgin female in hexane; PC = polar component (polar phase), isolated as described in Shu and Jones (1993); HexFl = hexane Florisil fraction.

ently adsorbed strongly by glass, which indicated that the pheromone component(s) was very polar. This was supported by other evidence discussed below. Furthermore, GC analysis indicated that HFR contained the same profiles of hydrocarbons as those in AFR, but in greater quantities (4×).

The activity of AFR of *E. terebrans* on glass lingered for several days. Sex pheromones of several other hymenopteran parasitoids have been reported to remain active for a long time. For example, a cube of plaster of Paris, soaked in an aqueous suspension of ground-up pupal shells of *Tortrix viridana* (L.) from which females of *Phaeogenes invisor* Thunberg had emerged, remained attractive to the males for at least a week (Cole, 1970). Dead female bodies of *Lariophagus distinguendus* (Först) stayed attractive over periods of months (Assem et al., 1980). The reason for this phenomenon is unknown. In the case of *E. terebrans*, the fact that the pheromone was polar and thus strongly adsorbed to glass and/or the possible high molecular weight of the pheromone might account for the longevity.

Near the optimal dose, AFR became most active when they were 24 hr old. The significance of this is unclear. Consequently, all the male responses to the pheromone in this study were assayed after the pheromone substance had been applied to watchglasses for 24 hr, except for the test of effects of AFR age.

The activity of *E. terebrans* AFR could not be recovered from the Florisil column probably because the major pheromone component(s) was too polar and adsorbed to the Florisil too strongly to be eluted with an organic solvent. However, the pheromonal activity in AFR could be obtained with a liquid–liquid

extraction method. The separation seemed incomplete since the neutral phase was active as well as the polar phase.

The active compound(s) in the polar phase had the properties of a carboxylic acid with an additional oxygen-containing function group. A carboxylic acid, being a "strong" acid relative to most organic compounds, would form a salt with a "weak" base ($NaHCO_3$) that would be extracted with water and would be converted, after acidifying the solution, back to the carboxylic acid that would be extracted with ether. Comparison of retention times on HPLC indicated that the polar component was more polar than octanoic acid and about as polar as 10-hydroxydecanoic acid. The compound(s) in the polar component had the properties of either a carboxylic acid with less than eight carbons or a carboxylic acid with an additional polar function group. Fractionation by TLC was strictly by class and based on polarity (Holloway and Challen, 1966). Therefore, the R_F of the polar component suggested that the compound(s) had the properties of a carboxylic acid with an additional oxygen-containing function group, assuming that the sex pheromone chemicals are composed of the three elements, carbon, hydrogen and oxygen.

Diazomethane reacts with carboxylic acids to form methyl esters. AFR lost most of its activity, after esterification with diazomethane, suggesting that the polar component had the properties of a carboxylic acid. Base-hydrolysis of the esterified AFR failed to recover activity, which made purification of the polar compound(s) and subsequent identification by this method impossible.

The second component of *E. terebrans* pheromone was isolated from the neutral phase of AFR. The neutral phase was eluted from a Florisil column with hexane followed only by ether. The ether Florisil fraction should contain all compounds that would come off the Florisil column when eluted with a gradient from 2.5% ether in hexane to 100% ether. Bioassays indicated that this ether fraction did not contain any pheromonal component. The synergistic component in the neutral phase was obtained from a Florisil column with hexane, indicating that it was a hydrocarbon(s).

Extraction of virgin female bodies of *E. terebrans* with hexane or acetone (FBR) failed to yield an active extract. This could be due to several reasons. For example, an inhibitory agent or compounds, which reacted with or masked the sex pheromone, might have been extracted. Vinson (1972) reported that ethyl acetate extracts of females of *Campoletis sonorensis* (Cameron) elicited all the sexual behavioral elements in males. However, extracting females with ethyl acetate over 5 or 6 hr reduced the activity of the extracts. An unbalanced ratio of pheromone components also could reduce activity. Cuticular hydrocarbons have been reported to be sex pheromone components of two hymenopteran insects (Bartelt et al., 1982; Swedenborg and Jones, 1992). Because the cuticular hydrocarbons are easy to extract, soaking insects in an organic solvent might

obtain the hydrocarbon component in excess of the natural ratio. Such extracts may not be active because of the wrong ratio of components.

In the case of *E. terebrans*, the ratio of hydrocarbon component to polar component in sex pheromone of *E. terebrans* seems to be not critical, since the males actively responded to a range of ratios of hydrocarbons to polar component (Table 3, Figure 4). Furthermore, the polar component and hydrocarbons, isolated from FBR, were very active in concert, while FBR itself was not active, suggesting FBR contained an extra component(s) rendering FBR inactive.

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SEASONAL VARIATION OF EXUDATE OF *Cistus ladanifer*

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Abstract—The production of labdanum exudate by *Cistus ladanifer* L. is highly seasonal, reaching a maximum concentration during summer and a minimum concentration in winter. Because this exudate strongly absorbs in the wavelength range of 260–400 nm (the near-UV–visible range), it may be important biologically as an UV–visible filter. Separation of exudate components has been achieved by reverse-phase high-performance liquid chromatography (HPLC). The retention times of HPLC chromatograms and the spectral characteristics (absorption and fluorescence) of the exudate identify flavonoids as the most relevant chromophores regarding the potency of the exudate as a UV–visible filter. HPLC studies show that kaempferol-3-(*O*)methyl, kaempferol-3,7-di(*O*)methyl, and apigenin-4′-(*O*)methyl are the most enriched flavonoids in the exudate. Other flavonoids [apigenin, apigenin-7-(*O*)methyl, apigenin-7,4′-di(*O*)methyl, kaempferol-3,4′-di(*O*)methyl and kaempferol-3,7,4′-tri(*O*)methyl] are present in the exudate as minor components, e.g., each contributes by less than 10% to total flavonoids. The ratio of kaempferols to apigenins of the exudate also shows seasonal variation (maximum value in summer and minimum in spring). However, due to the similar absorption spectra of both groups of flavonoids, this has a minor influence on the exudate's ability to filter near-UV–visible radiation.

Key Words—Flavonoids, seasonal variation, *Cistus ladanifer* L., UV filter, labdanum exudate.

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INTRODUCTION

Plants have adapted to hostile environments by a variety of morphological and physiological mechanisms. Biochemical adaptations have resulted in a complex pool of secondary metabolites. In particular, the flavonoids have been suggested to function as natural antioxidants, specific enzyme inhibitors, regulators of plant growth and development, microbial and animal toxins, and near-UV light screens (Fimin et al., 1986; Harborne, 1986; Hedin et al., 1983; McClure, 1975; Peters et al., 1986; Tomás-Llorente et al., 1990).

Flavonoids strongly absorb UV–visible radiation between 260 and 380 nm, a wavelength range associated with enhanced mutagenesis, DNA thymine dimerization, and photolysis of NAD and NADP, all capable of causing cellular death (Swain, 1970). Prior studies have shown that plant tissues exposed to high UV irradiance accumulate large amounts of flavonoids, mostly in the epidermal cells and in their vacuoles (Caldwell, 1971; Les and Sheridan, 1990; Hahlbrook et al., 1982; Knogge and Wessenböck, 1986; Matern et al., 1983; McClure, 1986; Schnabl et al., 1986; Swain, 1975). These studies lend support to the hypothesis that flavonoids serve as plant UV filters. Although flavonoids occur in epidermal cells of most plants, there can be variation in tissues within the same plant, or from plant to plant. These patterns have been attributed both to environmental and genetic bases (Bohm, 1987; Les and Sheridan, 1990; Roberts and Haynes, 1986; Swain, 1970). Swain (1970, 1975) suggested that flavonoids may have played a major role in the colonization of land by plants.

We used the Mediterranean species *Cistus ladanifer* L. to test the hypothesis that flavonoids might function ecophysiologically as UV filters, because *C. ladanifer* is a pioneer species (Herrera, 1984; Nuñez, 1989) and is able to colonize land exposed to irradiance higher than 360 cal/m^2/day (Matias, 1990). Leaves of this plant are also known to exude a sticky mixture containing flavonoids (J. De Pascual et al., 1974; Proksch and Gülz, 1984), whose physiological relevance is not well understood.

If the labdanum exudate of *C. ladanifer* is biologically important to protect this plant against the damaging effects of near-UV–visible solar radiation, it can be predicted that its secretion should be seasonal, attaining a maximum during summer. The aim of this study is to test this hypothesis.

METHODS AND MATERIALS

Specimens of *C. ladanifer* were sampled at the Sierra de los Conejeros, located near Alburquerque in the county of Badajoz (Spain). This site is rich in *C. ladanifer*, and its proximity (40 km from Badajoz) facilitates collection and processing within 1 hr. This mountainous site is 340–360 m above sea level, with an average annual rainfall of 500 mm (Cabezas et al., 1983). The rainy

months are October, November, March, and April. Annual average minimum and maximum temperatures are 10.7 and 19.8°C, respectively (Cabezas and Escudero, 1989).

Sample Collection. We examined exudate production in four tissue types: (1) mature leaves (nascent in the spring and exude high levels of labdanum during the summer), (2) sticky senescent leaves, characteristically darker with a brown–red color, (3) nascent summer leaves, active in labdanum secretion, and (4) upper part of photosynthetic stems, in which exudate is also evident. Samples were standardized to 17 g of each tissue type (Chaves, 1991), and were collected during a nearly two-year interval (May 1990 until December 1991).

Sample Handlings. The extracts of labdanum exudate were prepared as follows: Using forceps, 0.7-g pieces of ethanol-soaked Whatman 118 filter paper were wiped over the surface of freshly cut leaves and stems until all visible traces of exudate were removed. Usually this operation was repeated several times, with special care taken to avoid putting pressure on the biological samples during handling.

Filter paper samples were then transferred to glass flasks, and 10 ml of absolute ethanol was added to extract the exudate from the paper. Under mild stirring the ethanol solution soon turned yellow–green. The flasks were then carefully sealed to avoid losses by evaporation, and stored at 4°C until use.

Sample Analysis. Absorption spectra were recorded with a diode array Hewlett Packard 8451A, from 250 to 700 nm. The samples were diluted in absolute ethanol to ensure that the maximum absorbance was less than 1.0 optical density at the peak wavelength in the spectrophotometer cuvette.

HPLC chromatograms were carried out in a LCD Analytical HPLC instrument equipped with an integrator, and with a Nucleosil 5 C_{18} column of 150 × 4 mm. In each case, 20 μl of diluted ethanol extract was injected. Initially all samples were analyzed at wavelengths of 260, 280, 300, and 345 nm. After noting only minor differences between detection at 260 and 280 nm and that detection at 300 nm yields a mixed result between 280 and 345 nm, we decided to complete the study with the detection set at 260 and 345 nm.

Chemicals. The following chemicals were used as standards: cinnamic acid, hydrocinnamic acid, benzyl alcohol, dimethyl- and diethyl-phthalate, apigenin (all from Aldrich Chemical Co.); apigenin-7-(*O*)methyl, kaempferol-3,7-di(*O*)methyl, kaempferol-3,7,4′-tri(*O*)methyl (all kindly supplied by Dr. J. Gonzalez-Urones, University of Salamanca, Spain); other apigenins and kaempferols [kaempferol-3(*O*)methyl, kaempferol-3,7-di(*O*)methyl, kaempferol-3,4′-di(*O*)methyl, apigenin-4′(*O*)methyl, apigenin-4′,7-di(*O*)methyl and apigenin-7-(*O*)methyl] were kindly supplied by Drs. Thomas Vogt (Botanisches Institut, Universität Köln, Germany) and E. Wollenweber (Institut für Botanik, Technische Hochschule Darmstadt, Germany). All other chemicals were purchased from Merck, Darmstadt, Germany, or Carlo Erba, Milano, Italy.

RESULTS

Spectral Properties of Labdanum Exudate. The labdanum exudate is a sticky mixture that is clearly noticeable on leaves of *C. ladanifer* from the end of May until the end of October. Figure 1 shows the absorption spectra of samples taken in May, August, and October, with strong absorption in the near-UV to visible wavelength range up to 400 nm and peaks at 260, 310, and 350 nm. On the basis of previous studies (J. De Pascual et al., 1974; Proksch and Gülz, 1984), several flavonoids are likely components of the extracts of labdanum exudate, namely, apigenin-7-(*O*)methyl, kaempferol-3,7-di(*O*)methyl and kaempferol-3,7,4′-tri(*O*)methyl. This is further supported by the comparison of the absorption and fluorescence spectra of the exudate (Figure 1) with that of purified flavonoids (Figure 2).

The mild treatment used to prepare the samples of exudate does not extensively damage the epidermal plant cells. Leaves treated to extract the labdanum exudate in situ in plants, under low sunlight irradiance, do not become senescent and slowly (in several days) recover their sticky appearance, and their ability to produce exudate remains largely unimpaired. Moreover, epicuticular flavonoids have been shown to be predominantly aglycones, as opposed to intracellular (vacuolar) flavonoids, which are highly glycosylated (Vogt et al., 1987a, b; Proksch and Gülz, 1984; Wollenweber and Dietz, 1981). Flavonoid glycosides have been shown to be well resolved and separated from aglycone flavonoids by paper chromatography using H_2O as eluant, due to the much lower R_f values of the glycones (Markham, 1982). Chromatograms run under these

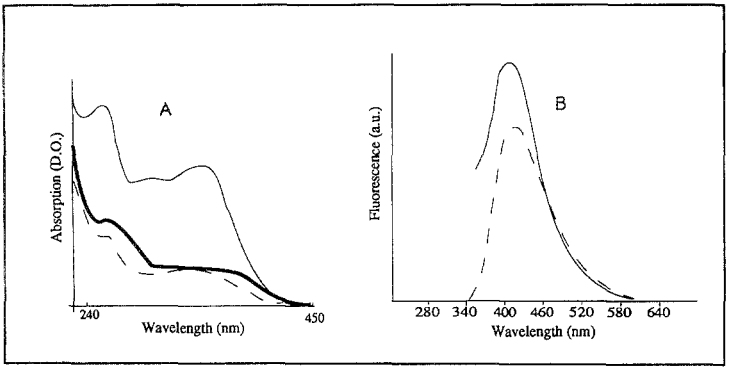

FIG. 1. Absorption and fluorescence emission spectra of the exudate of *Cistus ladanifer*. The spectra shown correspond to samples taken on May 29 (broken line), on August 30 (solid line), and on October (bold solid line). (A) Absorption spectra in methanol. (B) Fluorescence emission spectra recorded with an excitation wavelength of 320 nm in ethanol (a.u. stands for arbitrary unit).

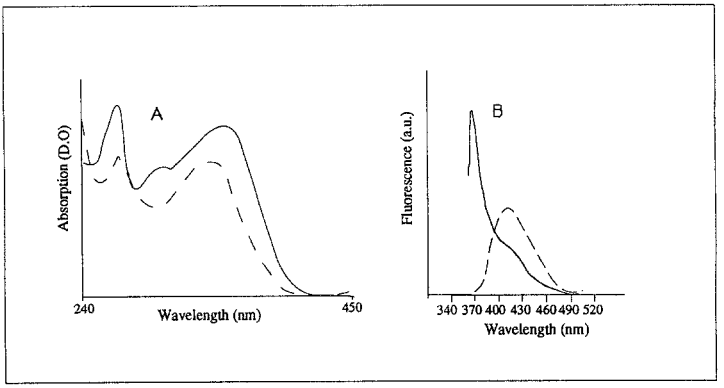

FIG. 2. (A) Absorption spectra of the flavonoids kaempferol-3-(*O*)methyl (solid line) and apigenin (broken line) in methanol. (B) Fluorescence emission spectra (a.u. stands for arbitrary unit) of the flavonoids kaempferol-3,7-di(*O*)methyl (broken line) and apigenin-7-(*O*)methyl (solid line), recorded with an excitation wavelength of 320 nm in ethanol.

experimental conditions show that the components of our samples of labdanum exudate do not move significantly from the application point. On the contrary, bidimensional paper chromatography (Whatman 3 MM paper) of samples using $CHCl_3$–CH_3COOH–H_2O, (30 : 15 : 2 v/v ratios) as the eluant for the first dimension and CH_3COOH–H_2O–HCl (30 : 10 : 3 v/v ratios) for the second dimension, conditions suitable for the resolution of aglycone flavonoids (Markham, 1982), shows that most of the sample moves from the application point, yielding four separate spots when observed under UV light. Therefore, we conclude that the content of flavonoid glycosides is negligible in our samples of labdanum exudate.

As shown in Figure 3, the absorption properties of the exudate reach their maximum value during August, lagging a bit behind the maximum irradiance flux of sunlight in this country, which is usually near the end of July in a normal year. Absorption spectra of the extracted exudate vary little between mature or nascent summer leaves. Absorption at both 260 and 345 nm show a parallel temporal pattern during summer, rising several-fold from the beginning of June and rapidly diminishing by the end of October. The period of maximum absorption of the extracts of labdanum exudate also correlates with the higher amount of sticky exudate of the leaves. The decay of the absorption of the exudate extracts in the autumn correlates well with rainy periods at the beginning of autumn, after approximately two to three months of dryness. In contrast to what it is observed by the end of the spring, this exudate is not noticeable again until the warm season starting in May of the next year. In contrast, after an occasional

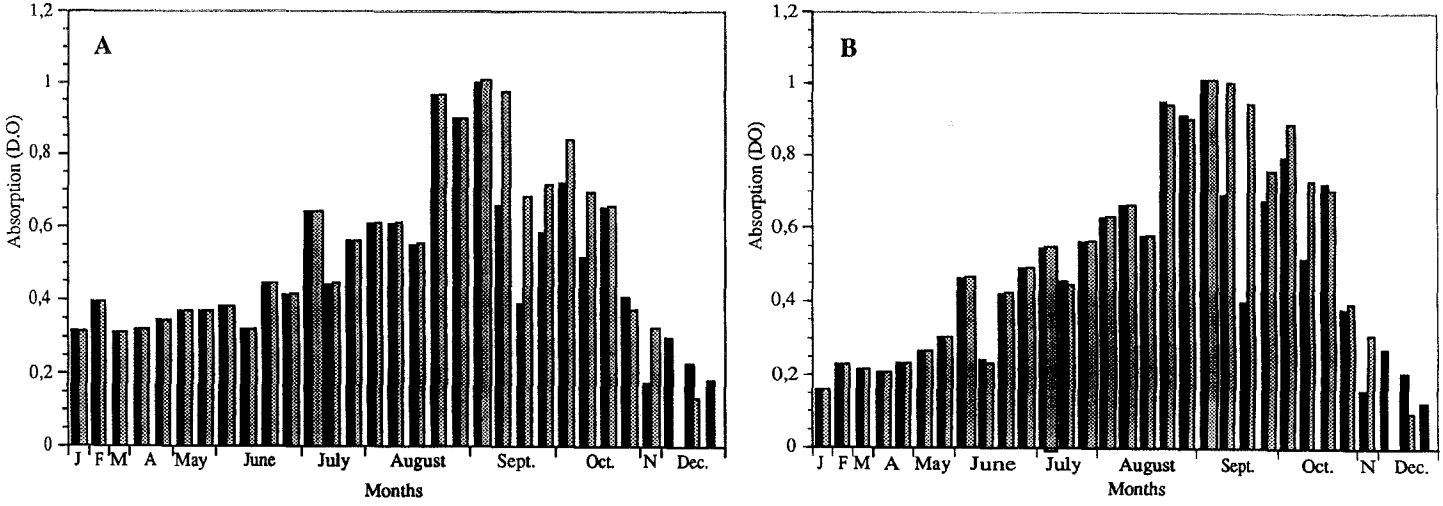

FIG. 3. Seasonal variation of absorption at 260 nm (A) and 345 nm (B) of ethanolic extracts of labdanum exudate from mature leaves (black bars) and from nascent summer leaves (stippled bars). J, F, up to Dec are abbreviations of the consecutive months of the year; when several samples were taken during different weeks of a given month they have been arranged chronologically.

summer storm, mature and nascent summer leaves continue secreting the exudate, giving them a sticky appearance until the beginning of autumn, indicating that during the summer the labdanum exudate lost due to occasional rainfall is rapidly restored by stimulated secretion during this season.

HPLC Studies of Labdanum Exudate Components: Seasonal Variation. HPLC can be used to separate mixtures of a wide variety of organic compounds, (see, e.g., Lim, 1986). HPLC studies have been carried out in an attempt to resolve this mixture into its major components in order to assess the possibility that seasonal variation of the labdanum secretion could be due to changes of major components relative to each other during summer and early autumn.

To detect seasonal changes in relative efficiency of different components of the labdanum exudate serving as a UV–visible filter, a fractional separation by reverse-phase HPLC of components was used. With Nucleosil C_{18} columns, methanol–water (75:25 v/v) as eluant, and a flow rate of 0.5 ml/min, we succeeded in separating the chromophores into four major peaks with approximate retention times (R_t) of 3, 7, 13, and 32 min (Figure 4). Also included in the Figure 4 are the HPLC chromatograms of blanks corresponding to these experimental conditions (e.g., ethanol extract of Whatman 118 paper), which only show small peaks centered at 3.7 and 4.3 min. Most nonflavonoid components of the exudate absorbing at 260 nm, such as cinnamic and hydrocinnamic acids, benzyl alcohol, and phthalates (Ohno et al., 1981), show R_t values lower than

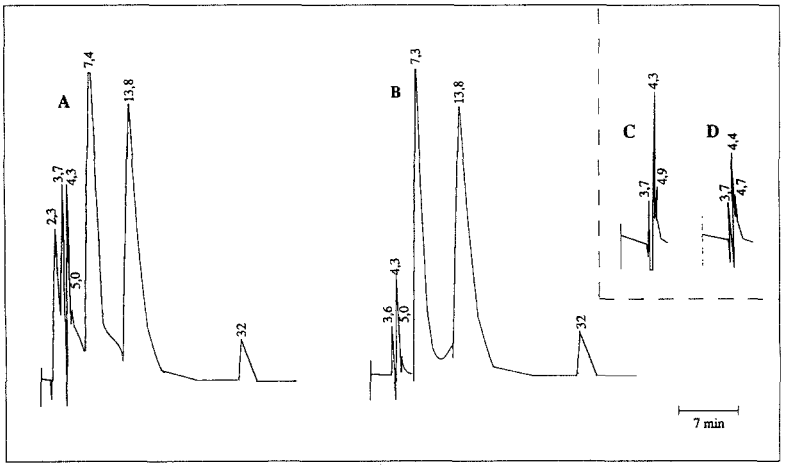

FIG. 4. HPLC chromatograms of ethanol exudate extracts from mature labdanum leaves (see text for further experimental details). (A and B) Chromatograms obtained with detection wavelengths of 260 and 345 nm, respectively. Insert (C and D): the blanks run with ethanol extracts of Whatman 118 paper using detection wavelengths of 260 and 345 nm, respectively.

5 min under these HPLC conditions (not shown) and are likely to be the predominant components of the peak of R_t of 3 min. On the contrary, purified flavonoids grouped into R_t values covered by the peaks of 7 min [apigenin, apigenin-4′-(*O*)methyl and kaempferol-3-(*O*)methyl], 13 min [apigenin-7-(*O*)methyl, kaempferol-3,4′-di(*O*)methyl, and kaempferol-3,7-di(*O*)methyl], and 32 min [apigenin-4′,7-di(*O*)methyl and kaempferol-3,7,4′-tri(*O*)methyl] consistently show strong absorption at 345 nm. Thus, the ratio between the area under the peak of 3 min and the total area under the peaks with absorbance reading at 260 nm, which ranges from 8 to 12% in the different samples analyzed, give an approximate measurement of the relative contribution of the nonflavonoid components of the exudate to its ability to filter near-UV light. This result shows that flavonoids account for most of the near-UV–visible filter potency of the exudate. Therefore, we analyzed further the seasonal pattern of the areas under peaks of R_t values 7 and 13 min of the exudate.

Figure 5 shows that in all cases the absorbance (either at 260 or 345 nm) of the HPLC peaks at 7 and 13 min reaches maximum values by the end of August, then decays slowly during September and more rapidly during October. A statistically significant delay of the onset of the rising phase is observed in absorbance at 345 nm of the peak centered at 13 min, which suggests a delayed induction of some flavonoid components of the exudate. This is revealed as well by the spectral ratio (A_{345}/A_{260}) of the whole extract versus time (Figure 6). The labdanum exudate extracted from both mature and nascent summer leaves shows an identical temporal pattern from May to October, while no significant seasonal variation was observed in extracts from senescent leaves and from photosynthetic stems.

To further identify the flavonoid components of the exudate, samples were resolved into eight major peaks with absorption at 350 nm by reverse-phase HPLC, using Nucleosil C_{18} columns with the following solvent mixture as eluant: H_2O plus 0.5% phosphoric acid–methanol–acetonitrile–tetrahydrofurane (56:16:6:22 v/v) and a flux rate of 0.7 ml/min (Figure 7). The peak at a retention time of 2–3 min largely comes from poorly resolved contaminants of the solvent since it is present in blank HPLC chromatograms of the eluant, plus nonflavonoid components of the exudate (see above). The remaining peaks have been identified using purified flavonoids as standards. The relative contribution of different flavonoids to the absorption at 350 nm of the exudate has been estimated from the area of the peaks of the chromatogram. The results obtained for samples prepared at the beginning of the spring (March) and mid-summer (August) are listed in the Table 1. These results clearly show that kaempferol-3-(*O*)methyl is the flavonoid more enriched in this exudate, and that all of them are kaempferols and apigenins with different levels of *O*-methylation. In addition, our results show a seasonal change of the composition of flavonoids in

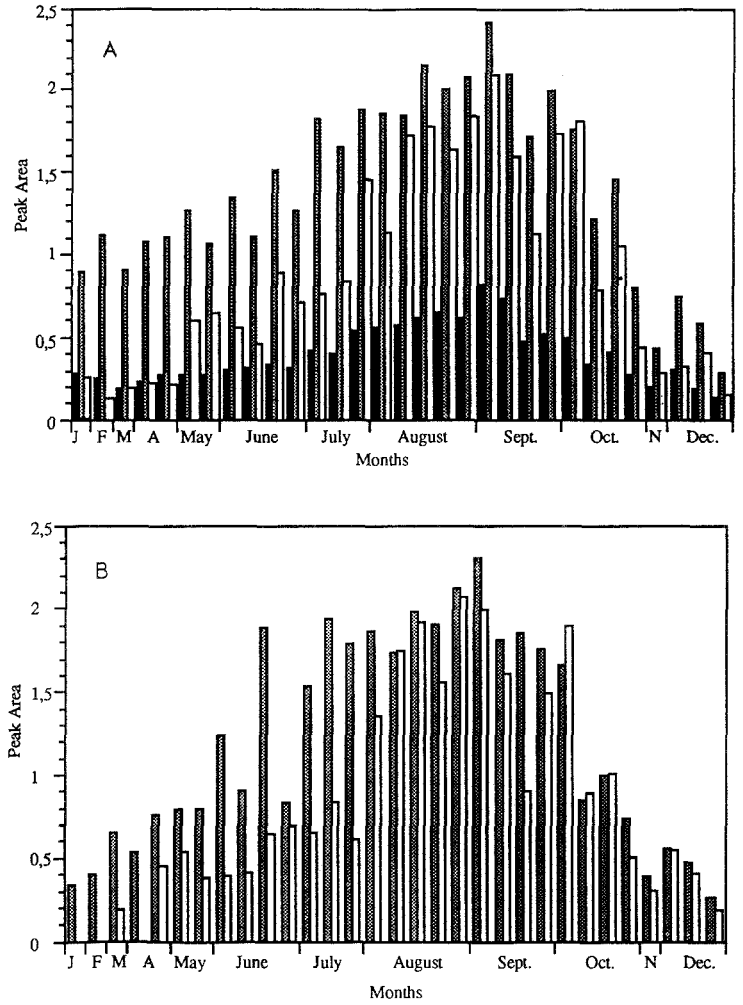

FIG. 5. Seasonal variation of the peak area of HPLC chromatograms of ethanol extracts of labdanum exudate from mature leaves with a detection wavelength of 260 nm (A) and 345 nm (B). Different bars correspond to peak areas of approximate retention times (min) of 3 (black), 7 (shaded), and 13 (white). The abscissae have been plotted as in Figure 3.

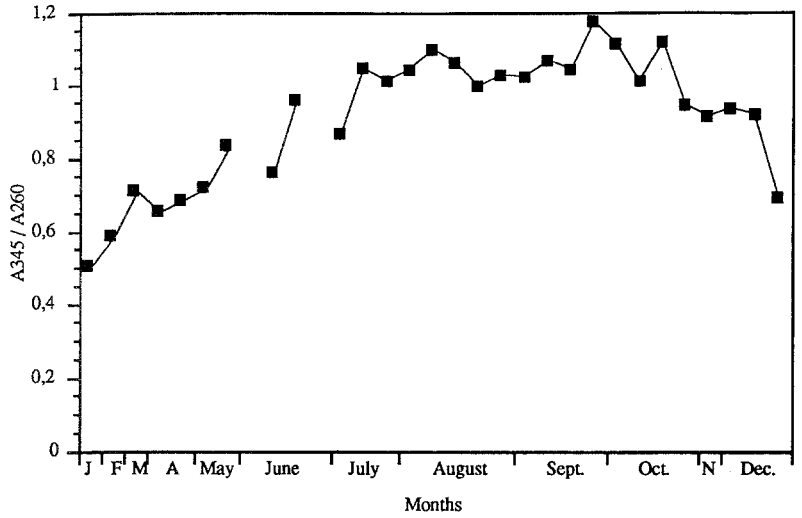

FIG. 6. Seasonal variation of the ratio between the absorption at 345 nm (A_{345}) and 260 nm (A_{260}) of ethanol extracts of labdanum exudate from mature leaves. The abscissa has been plotted as in Figure 3.

labdanum exudate. The relative ratio of kaempferols to apigenins is increased in August compared to March, mostly due to the decrease of apigenin and apigenin-4′-(*O*)methyl peaks and the increase of kaempferol-3,7-di(*O*)methyl.

DISCUSSION

This study shows the occurrence of important seasonal variations in the absorbance between 260 and 400 nm in the labdanum exudate extracted from leaves of *C. ladanifer*. The maximum absorbance is attained during the warmest months in this part of Spain, July and August, and lagging somewhat toward the period of highest sunlight irradiance by approximately mid-July (Font-Tullot, 1984). When the exudate is obtained from the stems, however, a different pattern is obtained, for there is no significant and continuous rise of the absorbance from May to October but only random fluctuations (results not shown). Taken together, these results support the hypothesis that the exudate is produced in the leaves and then slowly flows down the stem, adhering to it due to its viscosity and physical chemical properties.

C. ladanifer colonizes largely eroded lands, with a thin soil and low tree population (Bolaños and Guinea, 1949; Nuñez, 1989), and therefore it is highly exposed to sunlight irradiance. This exudate has excellent advantages as a nat-

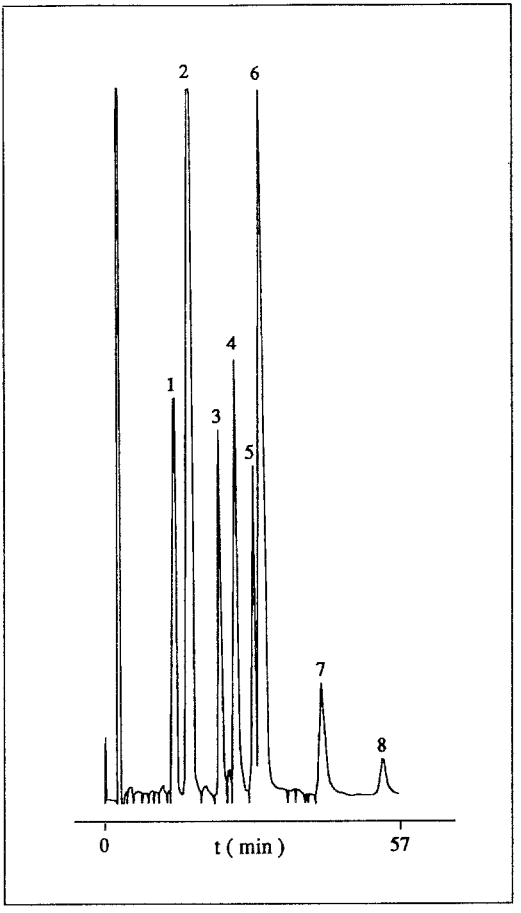

FIG. 7. Reverse-phase HPLC chromatogram of *Cistus ladanifer* exudate using a C_{18} Nucleosil column. The column was eluted with H_2O plus 0.5% phosphoric acid–methanol–acetonitrile–tetrahydrofuran (56:16:6:22 v/v) with a flow rate of 0.7 ml/min, and a detection wavelength of 350 nm has been used. Peaks 1–8 were identified using purified flavonoids run under the same experimental conditions and correspond to: (1) apigenin; (2) kaempferol-3-(*O*)methyl; (3) apigenin-4′-(*O*)methyl; (4) apigenin-7-(*O*)methyl; (5) kaempferol-3,4′-di(*O*)methyl; (6) kaempferol-3,7-di(*O*)methyl; (7) apigenin-7,4′-di(*O*)methyl, and (8) kaempferol-3,7,4′-tri(*O*)methyl.

ural sunlight-protecting filter, because its absorption wavelength range largely reduces the UV–visible radiation that rapidly destroys such as plant pigments chlorophylls, while it does not significantly absorb between 450 and 700 nm, i.e., in the wavelength range of absorption of pigments involved in plant pho-

TABLE 1. SEASONAL VARIATION OF COMPOSITION OF FLAVONOIDS OF *Cistus ladanifer* EXUDATE[a]

Flavonoid	Percent		
	March	May	August
Apigenin	0.141	0.060	0.037
Kaempferol-3-(*O*)methyl	0.575	0.534	0.406
Apigenin-4′-(*O*)methyl	0.114	0.065	0.045
Apigenin-7-(*O*)methyl	0.075	0.090	0.108
Kampferol-3-4′-di(*O*)methyl	0.026	0.055	0.057
Kaempferol-3-7-di(*O*)methyl	0.050	0.140	0.240
Apigenin-7-4′-di(*O*)methyl	0.019	0.041	0.059
Kaempferol-3-7-4′-tri(*O*)methyl		0.015	0.047

[a] Flavonoids were separated by reverse-phase HPLC using a C_{18} Nucleosil column, eluted with H_2O plus 0.5% phosphoric acid–methanol–acetonotrile–tetrahydrofuran (56:16:622 v/v) and a flow rate of 0.7 ml/min. The content of each flavonoid was calculated from the area under the corresponding HPLC peak (see chromatogram of Figure 7), with a detection wavelength of 350 nm. The data are presented as percentage of total flavonoids.

tosystems, PSI and PSII (Rawn, 1989). In addition, the physical–chemical characteristics of this exudate (highly viscous and low water solubility) serve to prevent its excessive loss by an occasional summer storm or rainfall and likely should be useful as well in protecting against dehydration of leaves and photosynthetic stems.

Among the reported components of extracts of whole labdanum leaves are a wide variety of flavonoids (J. De Pascual et al., 1974; Proksch and Gülz, 1984). From the comparison of their spectral properties (Markham, 1982, and this study) with those of the extracts of labdanum exudate, we suspected that they could be components of these extracts and, moreover, that they could be the major components responsible for the absorption above 350 nm. The HPLC results reported here fully confirm this hypothesis and, in addition, show that kaempferol-3-(*O*)methyl, kaempferol-3,7-di(*O*)methyl, and apigenin-4′-(*O*)methyl are highly enriched in the labdanum exudate. In addition to flavonoids, other components found in the extracts of whole *C. ladanifer* leaves (Ohno et al., 1981), are likely to be present in the extracts of labdanum exudate, such as cinnamic acid, hydrocinnamic acid, diethyl phthalate, and benzyl alcohol. However, their low extinction coefficients, in particular above 280 nm, and the low area with absorbance at 260 nm of the HPLC peak of R_t of ca. 3 min (where these nonflavonoid components elute) with respect to that of peaks containing flavonoids (e.g., peaks of R_t of 7 and 13 min) show that they are of secondary importance with respect to the flavonoids as physiological UV-visible

filters. In addition, some of them are metabolic intermediates in the biosynthesis of flavonoids (e.g., cinnamic, hydrocinnamic, and ethyl hydrocinnamic acids), and cosecretion with flavonoids could merely indicate a compartmentalized metabolic pathway functionally coupled to the secretion process.

A priori, the seasonal variations of the potency of the labdanum exudate as a UV–visible filter can be rationalized by a large change of its chemical composition or as a result of a change in the rate of secretion. The results reported in this study clearly favor the latter hypothesis. Although changes in the chemical composition of the labdanum exudate are shown to occur as well (Table 1), these changes appears to be of secondary importance with regard to the potency of the labdanum exudate as a UV–visible filter, due to the similar absorption spectra of kaempferols and apigenins.

Because these variations are not detected in labdanum extracts prepared from senescent leaves, it is unlikely that they are due to a slow chemical transformation of the components of the 7-min peak into components of the 13-min peak after secretion, and this favors the hypothesis that they reflect a seasonal change of the metabolic flux of the biosynthetic pathways leading to the production of different flavonoids in the exudate. Light has been demonstrated to stimulate several of the key enzymes controlling the biosynthesis of flavonoids (Hrazdina and Parsons, 1982). However, the enzymes known to be modulated by light catalyze the initial steps of this metabolic pathway (Schmelzer et al., 1988), which are common to the biosynthesis of all flavonoids found in the exudate. Work is now in progress to establish whether the seasonal changes in the apigenins to kaempferols ratio in the exudate are also produced by light modulation (sunlight irradiance) of converting enzymes or by another physical–chemical variables, such as temperature or relative humidity, which also show seasonal variations. Moreover, as shown in Figure 6, the absorption ratio A_{345}/A_{280} steadily increases from late spring to summer, while near-UV radiation (260–280 nm) is more harmful to plant cells than radiation of wavelengths higher than 300 nm (particularly to DNA and to many photosynthetic pigments). This suggests that, in addition to behaving as a near-UV–visible filter, the labdanum exudate may also play other ecophysiological roles, for example, to achieve better protection against dehydration or to deter hervibores. Although it is outside the scope of this paper, work is now in progress using *C. ladanifer* grown in different and "closed" habitats to test these hypotheses experimentally.

In conclusion, the flavonoids present in the labdanum exudate are the components responsible for most of its UV–visible filtering potency, and the exudate production shows seasonal variation, reaching a maximum value during midsummer. In addition, during summer the ratio of kaempferols to apigenins of the exudate rises mostly due to the large increase in content of methylated kaempferol derivatives.

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CHEMOATTRACTION OF *Biomphalaria glabrata* (GASTROPODA: PLANORBIDAE) TO LIPID STANDARDS AND LIPOPHILIC FACTORS IN LEAF LETTUCE AND TETRAMIN

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Abstract—Chemoattraction of individual *Biomphalaria glabrata* snails for lipid standards and lipophilic fractions of leaf lettuce and Tetramin were studied in a Petri dish bioassay. Snails were more significantly attracted to a whole Tetramin lipophilic fraction than that of leaf lettuce. Thin-layer chromatography showed that major neutral lipid fractions in Tetramin were triacylglycerols, free fatty acids, and free sterols, and in leaf lettuce were free fatty acids and a mixed free sterol–chlorophyll fraction. Snails were significantly attracted to both the free fatty acid and free sterol fractions from Tetramin, but only to the free fatty acid fraction from leaf lettuce. Snails were significantly attracted to a mixed lipid standard containing equal amounts of phosphatidylcholine, cholesterol, oleic acid, triolein, and cholesteryl oleate. Of four individual neutral lipid standards tested, i.e., cholesterol, oleic acid, triolein, and cholesteryl oleate, snails were only attracted to cholesteryl oleate.

Key Words—Chemoattraction, *Biomphalaria glabrata*, Gastropoda, Planorbidae, lipids, iceberg leaf lettuce, *Lactuca sativa*, Tetramin.

INTRODUCTION

Studies on the medically important planorbid snail, *Biomphalaria glabrata*, have examined chemoattraction and dietary preferences of this snail in a Petri dish bioassay (Masterson and Fried, 1992). Snails were attracted to lipophilic fractions of various foods, but detailed analyses of individual lipid classes in the

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foods as possible chemoattractants were not done (Masterson and Fried, 1992). The purpose of the present study was to examine neutral lipid classes in leaf lettuce and Tetramin, which may serve as chemoattractants for *B. glabrata* snails. We also examined the role of certain lipid standards as possible chemoattractants for this snail.

METHODS AND MATERIALS

Stock cultures of *B. glabrata* were maintained in the laboratory on a mixed diet of iceberg leaf lettuce (*Lactuca sativa*) and Tetramin (TetraWerke, Melle, Germany) (Duncan et al., 1987). The bioassay chamber for the chemoattraction studies consisted of 100 × 15-mm Petri dishes (Masterson and Fried, 1992). Two parallel lines were drawn 2.8 cm apart on the bottom of each Petri dish to produce three zones (A, B, C). The side zones (A and C) each had an area of 14.1 cm^2, and the middle zone (B) had an area of 20.0 cm^2 (Masterson and Fried, 1992). Each Petri dish was filled with 55 ml of artificial spring water (Cohen et al., 1980). The dishes were maintained at 22 ± 1°C on a level workbench under overhead diffuse fluorescent light. Ten Petri dishes were used for each trial.

Snails were 8 ± 1 mm in shell diameter and were maintained without food for approximately 2 hr prior to each trial. A single snail was placed in the center of zone B, whereas controls (blank 1-cm^2 pieces of filter paper impregnated with 25 μl of chloroform) and food extracts (1 cm^2 of filter paper impregnated with 25 μl of the chloroform–methanol extract of the food) were placed at the edges of zones A and C, respectively. The pieces of filter paper were allowed to air dry prior to introducing them into the Petri dish bioassay and were held in place in the dish by paper clips. The snails were placed in the dishes 10 min after the food extracts to allow the potential chemoattractants to diffuse.

Lipophilic extracts were prepared by homogenizing 1 g of iceberg leaf lettuce with 2 ml of chloroform–methanol (2:1), filtering the homogenate through glass wool, and then adding 0.5 ml of the Folch wash (0.88% KCl) to the filtrate. The hydrophilic fraction was discarded. The lipophilic Tetramin extracts were prepared as described for lettuce using 1 g of Tetramin, 4 ml of chloroform–methanol (2:1), and 1 ml of Folch wash.

For 50 min, at intervals of 5 min, the zone in which the snail was located was recorded (a total of 10 observations per snail).

A control group was used in each trial. It consisted of blanks in place of food extracts in order to observe the random movements of the snails.

Baker-Flex IB2 20 × 20-cm silica gel sheets were used for analytical thin-layer chromatography (TLC) to determine major lipid fractions in Tetramin and leaf lettuce. Material from leaf lettuce- and Tetramin-lipophilic fractions was

spotted in 5-, 10-, and 15-μl aliquots, as was a 1 mg/ml mixed lipid standard, 18-5A (Nuchek Prep Inc., Elysian, Minnesota). This standard contains equal amounts of phosphatidylcholine, cholesterol, oleic acid, triolein, and cholesteryl oleate. The plate was placed into a paper-lined glass TLC chamber containing petroleum ether–diethyl ether–acetic acid (80:20:1). The lipids were detected by spraying the plate with 5% ethanolic phosphomolybdic acid (PMA) and heating the plate for 15 min at 115°C.

Preparative TLC was used to isolate the neutral lipid fractions (Fried and Sherma, 1986). Baker-flex IB2 silica gel plates were developed in petroleum ether–diethyl ether–acetic acid (80:20:1). The neutral lipid bands were identified through comigration of neutral lipid standards on guide strips (Fried and Sherma, 1986). Each major neutral lipid band was scraped off the plate and eluted with 500–1000 μl of chloroform through a disposable glass pipet plugged with glass wool. Twenty-five microliters of the isolated neutral lipid fractions was placed on 1-cm^2 pieces of filter paper; control pieces of filter paper received equal aliquots of chloroform. The filter paper was air dried and introduced into the Petri dish bioassay as described previously.

Additionally, 25 μl of 1 mg/ml individual neutral lipid standards of cholesterol, oleic acid, triolein, and cholesteryl oleate (Matreya Inc., Pleasant Gap, Pennsylvania) and 25 μl of the 18-5A lipid standard were tested.

Random observations based on blank experiments were used as the expected values to calculate the chi-square value in each experimental design. A P value of <0.05 was considered significant. Each experiment was done 10 times for every group.

RESULTS

The results of chemoattraction to whole lipophilic fractions of Tetramin and leaf lettuce are presented in Table 1. Snails were more significantly attracted to the lipophilic fraction of Tetramin than that of lettuce.

Analytical and preparative TLC analyses showed that the major neutral lipids in the Tetramin extracts were triacylglycerols ($R_f = 0.31$), free fatty acids ($R_f = 0.23$), and free sterols ($R_f = 0.14$), along with lesser amounts of sterol esters ($R_f = 0.61$). The major neutral lipid present in the leaf lettuce fraction was free fatty acids ($R_f = 0.24$) along with lesser amounts of sterol esters ($R_f = 0.61$), triacylglycerols ($R_f = 0.31$), and free sterols ($R_f = 0.14$). A chlorophyll zone ($R_f = 0.10$) was also present in leaf lettuce.

When Tetramin lipophilic fractions were eluted from the silica gel plates, placed on filter paper squares, and tested in the Petri dish bioassay, only free fatty acids and free sterols showed chemoattraction (Table 2). When leaf lettuce fractions were eluted from the silica gel plates and tested as described for Tetramin, only the sterol ester fraction showed chemoattraction (Table 3).

TABLE 1. CHEMOATTRACTION OF SNAILS TO LIPOPHILIC EXTRACTS OF TETRAMIN AND LEAF LETTUCE[a]

Group	Experiment	Percentage of snails in zones			chi-square	*P*
		A	B	C		
A	Bl vs Bl	34	29	37		
Tetramin lipophilic						
B	Bl vs TL	15	17	68	52.04	0.00001
Lettuce lipophilic						
C	Bl vs LL	22	22	56	22.01	0.00002

[a]Bl = blank; TL Tetramin lipophilic; LL = lettuce lipophilic.

TABLE 2. CHEMOATTRACTION OF SNAILS TO TETRAMIN LIPID FRACTIONS ELUTED WITH CHLOROFORM[a]

Group	Experiment	Percentage of snails in zones			chi-square	*P*
		A	B	C		
A	Bl vs Bl	43	25	32		
Sterol esters						
B	Bl vs SE	37	33	30	3.522	0.172
Triacylglycerols						
C	Bl vs TG	39	21	40	3.012	0.222
Free fatty acids						
D	Bl vs FFA	36	20	44	6.639	0.036
Free sterols						
E	Bl vs FS	28	25	37	6.014	0.049

[a]Bl = blank; SE = sterol esters; TG = triacylglycerols; FFA = free fatty acids; FS = free sterols.

As seen in Table 4, the mixed lipid standard, 18-5A, was significantly attractive. The individual neutral lipid standard cholesterol oleate was chemoattractive, whereas triolein, cholesterol, and oleic acid were not.

DISCUSSION

Individual *B. glabrata* snails were significantly attracted to the lipophilic fractions of both leaf lettuce and Tetramin, a finding in accord with a previous study by Masterson and Fried (1992). Our results differ from those of Uhazy et

TABLE 3. CHEMOATTRACTION OF SNAILS TO LEAF LETTUCE LIPID FRACTION ELUTED WITH CHLOROFORM[a]

Group	Experiment	Percentage of snails in zones			chi-square	*P*
		A	B	C		
A	Bl vs Bl	42	20	38		
Sterol esters						
B	Bl vs SE	30	20	50	7.218	0.027
Triacylglycerols						
C	Bl vs TG	43	28	29	5.355	0.069
Free fatty acids						
D	Bl vs FFA	35	16	49	5.151	0.076
Free sterols and chlorophyll						
E	Bl vs FS + C	32	28	40	5.686	0.058

[a]Bl = blank; SE = sterol esters; TG = triacylglycerols; FFA = free fatty acids; FS + C = free sterols and chlorophyll.

TABLE 4. CHEMOATTRACTION OF SNAILS TO INDIVIDUAL LIPID STANDARDS AND MIXED LIPID STANDARD 18-5A[a]

Group	Experiment	Percentage of snails in zones			chi-square	*P*
		A	B	C		
Lipid standard 18-5A						
A	Bl vs Bl	37	17	46		
B	Bl vs LS	22	28	50	13.55	0.0011
Cholesterol						
C	Bl vs CH	38	25	37	5.553	0.062
Oleic acid						
D	Bl vs OA	33	24	43	3.510	0.173
Triolein						
E	Bl vs TR	38	22	40	2.280	0.320
Cholesteryl oleate						
F	Bl vs CO	28	34	38	20.58	0.00004

[a]Bl = blank; LS = lipid standard 18-5A; CH = cholesterol; OA = oleic acid; TR = triolein; CO = cholesteryl oleate.

al. (1978), in which *B. glabrata* was not significantly attracted to the lipophilic fraction of romaine leaf lettuce. The design of Uhazy et al. (1978) was different than ours in that they examined the response of 10 snails per chamber in the presence of snail conditioned water. When numerous snails are used in the same chamber, there are probably pheromonal and tactile interactions between them that may affect the migration to a food stimulus.

The finding that individual snails were attracted to 25 μg of cholesteryl oleate provides additional information on the role of lipids as chemoattractants. Although dosage–response data were not obtained for either standards or natural products, snails were able to detect 25 μg or less of the lipid constituents tested in our Petri dish bioassay.

The most consistent lipophilic chemoattractant in both Tetramin and leaf lettuce was the free fatty acid fraction. Since this fraction was eluted by preparative thin-layer chromatography with no additional cleanup steps, there is no guarantee of the purity of this fraction. However, our findings do suggest that free fatty acids should be added to the list of food chemoattractants for *B. glabrata* and related planorbids. Food chemoattractants for this snail already include amino acids and organic acids (Uhazy et al., 1978; Thomas, 1982; Thomas and Assefa, 1979) and various carbohydrates for the related bulinid snails (Kpikpi and Thomas, 1992).

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CHEMICAL DISCRIMINATION OF PREY BY NAIVE NEONATE GOULD'S MONITORS *Varanus gouldii*

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Abstract—Previous studies have demonstrated that actively foraging autarchoglossan lizards rely in part on chemoreception to detect and locate prey. In one of two experiments, neonate Gould's monitors *Varanus gouldii* were studied to determine whether they were able to discriminate between multiple prey odors and control odors by tongue-flicking. Responses of lizards to deionized water, a pungency control (cologne), mouse, gecko, and cricket odors on cotton-tipped applicators were studied in experiments using repeated-measures designs and using the tongue-flick attack score (TFAS) as the primary measure of response strength. The TFAS was greater in response to cricket odors than to other prey odors or to either of the control stimuli, and there was no statistically significant difference in response between control stimuli. Range of tongue-flicks elicited by cricket odor were greater than those for other prey odors and control stimuli. Only applicators bearing cricket odor were bitten. In the second experiment, lizards were tested to determine whether they respond differently to chemical stimuli taken from the exoskeleton vs. internal fluids of crickets. TFAS were slightly higher for chemical stimuli taken from internal fluids, but not significantly so. Lizards bit applicators in both conditions. Details of responses to experimental trials are discussed in relation to the feeding behavior of this species.

Key Words—*Varanus gouldii*, lizard, Sauria, Varanidae, chemoreception, vomerolfaction, prey discrimination.

INTRODUCTION

Response to chemical cues sampled by tongue-flicking has been established in various lizard species and is known to be important in numerous ecologically

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important contexts (Simon, 1983; Auffenberg, 1984; Cooper, 1989, 1990a,b; 1991). Varanid lizards are opportunistic predators that tongue-flick frequently while active. Chemical cues from prey are important stimuli for varanid lizards and may facilitate the location and identification of prey items (Auffenburg, 1981, 1984). Varanid tongue morphology is very snakelike (McDowell, 1972). In snakes, other lizards, and presumably varanids, molecules sampled by the tongue when it is protruded are thought to be transferred to the vomeronasal ducts and then to the vomeronasal epithelia, the site of the chemoreceptor cells (Halpern and Fruman, 1979; Halpern, 1992). The ability to detect chemical prey odors and discriminate them from nonodorous and biologically irrelevant odorous controls has been demonstrated in a variety of autarchoglossan lizards (Burghardt, 1973; Cooper and Vitt, 1989; Cooper, 1990a).

Gould's monitor lizard (*Varanus gouldii*) is found in a wide variety of habitats throughout most of Australia (Mertens, 1958; Pianka, 1972; King and Green, 1979). This species forages widely, often covering nearly two square kilometers per day (Pianka, 1970). The diet of wild *V. gouldii* consists mainly of mole crickets, grasshoppers, beetles, spiders, and frogs, most often encountered while the lizards are burrowing (Pianka, 1970; Pengilly, 1981).

This paper describes two experiments: experiment 1 is designed to determine whether naive (no previous exposure to food or prey chemicals) neonate *Varanus gouldii* can detect prey chemicals and discriminate chemically between prey, odorless stimuli, and odorous, but irrelevant stimuli; experiment 2 is designed to determine whether lizards respond differently to chemical stimuli taken from the exoskeleton vs. internal fluids of a cricket. The experimental techniques have been applied to a wide variety of lizard taxa in studies of prey chemical discrimination (Burghardt 1973; Cooper, 1989, 1990a; Cooper and Vitt, 1989; Achen and Rakestraw, 1984).

METHODS AND MATERIALS

Seven Gould's monitors (sex unknown) were hatched from a single clutch at the Dallas Zoo in November 1992. The lizards were housed individually in glass aquaria (50 × 25 × 29 cm) with metal screen tops. Blank newsprint served as substrate. Each lizard was provided with a 75-W incandescent lamp, a 20-W fluorescent ultraviolet lamp (Sylvania BL 350) and an ABS plastic hidebox. Water was available ad libitum. Lizards were acclimated to laboratory conditions for at least three days and not tested until the umbilicus retracted and retained yolk was visibly absorbed. These experiments were conducted between November 17, and December 10, 1992, between 1300 and 1500 hr. One trial per day was conducted for each lizard. The ambient temperature was ca. 29°C and the photoperiod was ca. 10:14 hr light–dark.

Experiment 1 Design. To detect ability to discriminate between prey chemicals and other chemical stimuli, responses of *V. gouldii* were tested with cricket (*Acheta domestica*), gecko (*Hemidactylus frenatus*), and mouse (*Mus musculus*) chemicals. Cologne (Spice Bracer) and distilled water were used as controls. Each of the five stimuli was presented to each lizard once in a randomized block design. All stimuli were first prepared by immersing a cotton-tipped applicator in distilled water. To add other chemical cues, the wet applicator was dipped in cologne, or rolled over the dorsal and ventral aspect of a gecko or mouse. The invertebrate stimuli were applied by pressing a wet applicator onto a mashed cricket. Trials were initiated by slowly approaching a lizard's enclosure and gently removing the top. The cotton-tipped applicator was slowly moved to within 2 cm of the lizard's snout. Beginning with the first tongue-flick, tongue-flicks directed at the applicator were counted for 60 sec or until the lizard bit the swab, whichever occurred first. For lizards that bit the swab, latency to biting in seconds and number of tongue-flicks before biting were recorded. Tongue-flicks directed away from the applicator were not counted.

The measure of predatory response strength to a stimulus condition was the tongue-flick attack score (TFAS), a composite variable. When the stimulus does not elicit biting, it is the number of tongue-flicks. When biting occurs, it is the maximum number of tongue-flicks emitted by any individual in any stimulus condition plus (60 minus the latency to bite in seconds). This measure, developed by Burghardt (1967, 1969, 1970), assumes that increasing numbers of tongue-flicks represents increasing chemosensory examination of the stimulus and that biting represents a predatory attack (Cooper and Burghardt, 1990). A bite is thus weighted more heavily than any number of tongue-flicks.

Experiment 2 Design. Methods used to detect ability to discriminate between chemical stimuli taken from the exoskeleton vs. internal fluids of a cricket, followed that of experiment 1.

Analysis. Data are presented as means $\pm$ 1 SE of the mean. Analysis was conducted using procedures for single-factor experiments having repeated-measures designs. Bartlett's tests were conducted to determine homogeneity of variances among conditions. Raw data or homoscedastic transformed data were subjected to ANOVA. Significance for the two-tailed alpha was accepted at $P < 0.05$. Kruskal-Wallis tests and paired t tests were used for multiple comparisons.

RESULTS

Experiment 1. The lizards tongue-flicked applicators in all test conditions; four lizards bit applicators bearing cricket odors (Table 1). Because variances in the TFAS were heterogeneous ($F = 7.26$; $df = 4$; $P < 0.001$), a 3rd root

TABLE 1. TONGUE-FLICK RESPONSE (TFAS) TO TEST CONDITIONS BY NAIVE *Varanus gouldii*

	Cologne	Cricket	Gecko	Mouse	Water
Mean TFAS	6.43	38.00	7.58	12.71	4.14
Range of TFAS	1–14	2–63	1–13	2–24	1–14
SE of TFAS	1.85	9.74	1.91	3.36	1.79
No. of attacks	0	4	0	0	0
X latency to attack (sec)		13.25			

transformation was performed that yielded homogeneous variances ($F = 1.63$; $df = 4$; $P > 0.05$). The main stimulus condition effect was highly significant ($F = 4.96$; $df = 4$; $P < 0.003$). Multiple comparisons revealed a significantly higher TFAS for cricket stimuli than for other prey chemicals, cologne, and water (H corrected for ties, $P < 0.045$). The TFAS for water, cologne, gecko, and mouse odors did not differ significantly (Table 1).

For all seven monitors, the highest TFAS was emitted in response to cricket odors. The mean TFAS was 38.0, with a range of 2–63; for the remaining conditions number of tongue-flicks and the TFAS were identical. Greater numbers of tongue-flicks were elicited by cricket odors than by either control treatment, and there was no significant difference in response to the two control conditions ($t = 0.84$, $df = 6$, $P = 0.4334$).

Four individuals bit applicators bearing cricket chemicals within 18 sec, and no bites occurred in the other conditions. A binomial test revealed that lizards were significantly more likely to bite in the cricket condition than in the others (two-tailed binomial $P = 0.0004$).

Experiment 2. Lizards tongue-flicked applicators in both test conditions; four bit applicators bearing cricket chemicals from internal fluids, while only one lizard bit an applicator bearing chemical stimuli from the exoskeleton (Table 2).

Variance in tongue-flick rate was homogeneous (approximate $F = 1.83$; $df = 1$; $P > 0.05$). Variance in the TFAS were homogeneous (approximate $F = 0.23$; $df = 1$; $P > 0.05$). A paired t test revealed that the difference in response to the two conditions was not significant ($t = 1.69$; $df = 6$; $P = 0.1427$).

DISCUSSION

Neonate reptiles of many species exhibit well-developed behavioral repertoires immediately after parturition or hatching (Burghardt, 1977; Fuchs and Burghardt, 1971). Naive *V. gouldii* in this study readily distinguished cricket

TABLE 2. TONGUE-FLICK RESPONSES (TFAS) TO TEST CONDITIONS BY NEONATE *Varanus gouldii*

	Chemical stimuli from	
	Exoskeleton	Internal fluids
Mean TFAS	26.14	41.14
Range of TFAS	12-60	15-62
SE of TFAS	6.58	8.11
No. of attacks	1	5
X latency to attack (sec)	7.0	10.0

odors from controls, demonstrating that chemoreception and prey chemical discrimination are innate functions that are well developed at birth and that previous exposure to prey chemicals is not necessary to elicit a significant response (see Burghardt, 1973). While visual cues may be the primary means of prey location for most varanids (Auffenberg, 1984), chemosensory trailing may occur in some species (Auffenberg, 1981). It is possible that burrowing species such as *V. gouldii* can detect residual chemical cues or trails left on substrata by invertebrates, thus facilitating the detection of subterranean prey.

The geckkonid species used as a source of prey chemical stimuli in this experiment was selected because neonate *V. gouldii* may encounter this species in the wild, even considering that this species is predominantly nonterrestrial. However, as results indicate, responses were not significantly different from controls. The TFAS for neonatal mouse stimuli were greater than that to stimuli other than cricket stimuli. Hence, it was not surprising that lizards readily accepted neonatal mice at ca. one week after the termination of these trials.

In the first experiment, response to cricket odors was consistent with the invertebrate diet of *V. gouldii*, but we could not specify whether the effective chemical cues were of external or internal origin. However, the results of experiment 2 led us to conclude that responses to cricket stimuli were based on both classes of chemical cues (see Chiszar et al., 1993).

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INFLUENCE OF NONPROTEIN NITROGEN ON ESTIMATION OF PROTEIN FROM TOTAL NITROGEN IN FLESHY FRUITS

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Abstract—The protein content of pulps of 26 fleshy fruit species from east Mediterranean habitats in Israel were estimated using two different methods: (1) the Kjeldahl procedure in which the total recovered nitrogen is multiplied by 6.25 to estimate total proteins, and (2) amino acid analysis by amino acid analyzer. The average protein content obtained by the Kjeldahl procedure was 5.75% (dry weight) while it was only 3.90% when amino acids were analyzed. The higher value of protein content by the Kjeldahl procedure is most likely the result of a relatively high proportion of nonprotein nitrogen compounds (31%) in these pulps. Therefore the 6.25 factor is not valid and a 4.05 factor may be more accurate for assessing the true protein content of these fleshy fruits. The data also suggest that the more accurate estimate of true protein (Y) from Kjeldahl total nitrogen (X) should be based on the highly significant linear regression between these two variables: $Y = 4.885X - 0.6$.

Key Words—Frugivory, seed dispersal, amino acids, Kjeldahl, protein, fleshy fruit, nutrition, secondary compounds, plant–animal interactions.

INTRODUCTION

One of the most frequently used methods for determining dietary crude protein is the Kjeldahl procedure, published nearly a century ago, which is based on total nitrogen content of food items (Robbins, 1983). Although this method determines nitrogen, the results are converted to crude protein by multiplying N × 6.25 (Maynard and Loosli, 1969; Bondi, 1987). The factor 6.25 is derived from the average analysis of protein, being 16% nitrogen. However, the nitrogen content of proteins from different food types varies, depending on the amount

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of nonprotein nitrogen compounds. Therefore, the true protein content of many food items calculated by the Kjeldahl method is probably overestimated because crude protein is based on an analysis of total nitrogen and not on protein nitrogen (e.g., Hansen, 1970).

The traditional 6.25 factor is widely used for estimating the crude protein content of fleshy pulps of fruits (e.g., Piper, 1986; Debussche et al., 1987; Herrera, 1987; Sakai and Carpenter, 1990). In several studies this factor was used to estimate the nutritional value of the fruit for frugivorous animals (e.g., Foster, 1978; Poddar and Lederer, 1982; Calvert, 1985; Herrera, 1989; Izhaki and Safriel, 1989; Rogers et al., 1990; Izhaki, 1992; Kool, 1992). Because pulps may contain appreciable amounts of nonprotein nitrogen compounds, the 6.25 factor may overestimate the protein content of these fruits. Fruits may contain different sources of nonprotein nitrogen such as inorganic nitrogen, low-molecular-weight peptides, nucleic acids, free amino acids, ammonium salts, and secondary compounds (Maynard and Loosli, 1969; Lyttleton, 1973). Alkaloids are the best known of the nitrogen-containing secondary metabolites of plants, but nonprotein amino acids, cyanogenic glycosides, and glucosinolates also occur in plants (Harborne, 1991).

Milton and Dintzis (1981) suggested that the appropriate nitrogen-to-protein conversion factor for leaves, flowers, and fruit from eight tropical woody species was 4.4. Their data showed that the proteins in these samples averaged 19% nitrogen, and an average of 20% of the total nitrogen in these samples was nonproteinaceous. However, their study was mainly based on leaves, and included only one species of fleshy fruit (*Ficus insipida*).

Here I report the amino acid content of the largest sample of noncultivated fleshy fruit species analyzed to date. For each species, the total protein content was calculated by summing the amino acid content and was compared to the estimate of crude protein by Kjeldahl method.

METHODS AND MATERIALS

Research Species. Twenty-six plant species that produce fleshy fruits in the northern part of Israel were studied. These species belong to 17 families and may be categorized into four life forms: 11 trees, seven shrubs, seven climbers, and one herbaceous plant. Most of these species are bird-dispersed plants (Izhaki, 1986; Izhaki and Safriel, 1985; Barnea et al., 1991; Izhaki et al., 1991), but at least two of them (*Ziziphus spina-christi*, *Styrax officinalis*) are mainly mammal-dispersed. There is some evidence that frugivorous birds are unable to subsist on a diet comprised of one or several of these fruits (Izhaki, 1992; Izhaki and Safriel, 1989). The nonexclusive suggested explanations of this phenomenon include the low protein content of the pulp (Berthold, 1976; Levey and Karasov,

1989; Sedinger, 1990), shortage of specific amino acids (Parrish and Martin, 1977; Mack, 1990; Sedinger, 1990), and secondary compounds that interfere with protein metabolism (Izhaki and Safriel, 1989).

Methods. Fresh ripe fruits were collected in the field, the seeds removed, and the pulp oven dried at 40°C to constant mass. The dried pulp was ground to powder, and the sample was divided into two fractions for two different analyses. The total nitrogen content of the pulp was determined by the Kjeldahl technique (AOAC, 1984) using a Kjeltec Auto 1030 Analyzer. In this method, the pulp sample was boiled in a concentrated sulfuric acid-catalyst solution until all organic matter was destroyed and the nitrogen was converted to ammonium sulfate. The ammonia was volatilized by sodium hydroxide (AOAC, 1984). Amino acid composition and released ammonia were determined according to the procedure described by Elkin and Griffith (1985). The pulp powders were oxidized with performic acid prior to hydrolysis, and their amino acid contents were determined by cation exchange chromatography using a Biotronic LC 5000 Amino Acid Analyzer. HCl was used to destroy excess performic acid (Elkin and Griffith, 1985). Released ammonia and 17 common amino acids were measured (Table 1). No analysis was made for tryptophan, but this should not alter protein values or conversion factors significantly (Milton and Dintzis, 1981).

RESULTS AND DISCUSSION

The amino acid composition varied greatly among the species (Table 1). *Ephedra aphylla* and *Withania somnifera* both have similar, relatively high, total protein estimates (14.5% and 14.2%, respectively), yet *E. aphylla* is rich in phenylalanine and valine, whereas *W. somnifera* is rich in aspartic acid, glutamic acid, and arginine (Table 1). Differences in amino acid composition were also detected in congeneric species. Although the general patterns of amino acid composition and total protein were relatively similar for the two studied species of *Rhamnus*, *R. lycioides* pulp was twice as rich in aspartic acid as *R. alaternus* (Table 1). Protein quality, expressed by amino acid composition, may have more relevance to patterns of frugivory than total protein per se (Parrish and Martin, 1977; Mack, 1990).

The total nitrogen recovered by the Kjeldahl determination was significantly higher than the total nitrogen recovered by the amino acid analysis for all studied species (paired t test on arcsin square-root transformed values, $T = 11.6$, $df = 25$, $P < 0.0001$ Table 2). The average difference between these two results is 31% and may be considered as nonproteinaceous nitrogen content of these fleshy fruits (Table 2). This average is larger than the value (20%) reported by Milton and Dintzis (1981) for tropical plants. In three plant species, *Osyris alba*, *Rhamnus lycioides*, and *Tamus orientalis*, 50% or more of their pulp nitrogen may

TABLE 1. TOTAL PULP AMINO ACID COMPOSITION OF 26 FRUIT SPECIES FROM EAST MEDITERRANEAN HABITATS (% Dry Weight)

Plant species	Cysteine	Aspartic acid	Methionine	Threonine	Serine	Glutamic acid	Proline
Ephedra aphylla (Ephedraceae)	0.232	0.853	0.178	0.461	0.393	2.068	1.116
Osyris alba (Santalaceae)	0.045	0.293	0.050	0.111	0.124	0.252	0.174
Laurus nobilis (Lauraceae)	0.040	0.390	0.123	0.163	0.162	0.352	0.264
Rosa canina (Rosaceae)	0.055	0.471	0.045	0.126	0.148	0.271	0.396
Crataegus monogyna (Rosaceae)	0.021	0.186	0.027	0.068	0.073	0.106	0.090
Pistacia lentiscus (Anacardiaceae)	0.074	0.362	0.131	0.174	0.206	0.364	0.464
Pistacia atlantica (Anacardiaceae)	0.067	0.469	0.143	0.199	0.250	0.391	0.482
Pistacia palaestina (Anacardiaceae)	0.059	0.363	0.100	0.151	0.188	0.348	0.300
Rhus coriaria (Anacardiaceae)	0.035	0.204	0.042	0.095	0.106	0.197	0.117
Rhamnus alaternus (Rhamnaceae)	0.031	0.186	0.024	0.062	0.074	0.152	0.070
Rhamnus lycioides (Rhamnaceae)	0.023	0.333	0.025	0.068	0.063	0.130	0.096
Ziziphus spina-christi (Rhamnaceae)	0.032	0.737	0.026	0.078	0.102	0.239	0.080
Myrtus communis (Myrtaceae)	0.031	0.150	0.032	0.066	0.073	0.167	0.087
Arbutus andrachne (Ericaceae)	0.014	0.074	0.013	0.036	0.040	0.092	0.041
Styrax officinalis (Styracaceae)	0.023	0.157	0.039	0.051	0.073	0.248	0.043
Phillyrea latifolia (Oleaceae)	0.056	0.319	0.048	0.140	0.169	0.330	0.163
Withania somnifera (Solanaceae)	0.415	1.698	0.253	0.612	0.707	1.792	1.178
Rubia tenuifolia (Rubiaceae)	0.030	0.256	0.032	0.075	0.091	0.175	0.221
Viburnum tinus (Caprifoliaceae)	0.044	0.238	0.062	0.114	0.125	0.238	0.136
Lonicera etrusca (Caprifoliaceae)	0.028	0.228	0.038	0.095	0.104	0.236	0.091
Bryonia spp. (Cucurbitaceae)	0.195	1.521	0.189	0.573	0.818	0.956	0.730
Asparagus aphyllus (Liliaceae)	0.167	0.523	0.081	0.250	0.269	0.521	1.090
Ruscus aculeatus (Liliaceae)	0.056	0.335	0.039	0.113	0.143	0.378	0.223
Smilax aspera (Liliaceae)	0.076	0.356	0.049	0.131	0.149	0.290	0.171
Tamus orientalis (Dioscoreaceae)	0.038	0.259	0.042	0.111	0.127	0.332	0.126
Arum dioscoridis (Araceae)	0.083	0.406	0.073	0.174	0.179	0.874	0.209

be attributable to nonprotein compounds. Using the traditional 6.25 factor yielded an average of 5.75% crude protein in the analyzed fruits while the average protein content based on the total amino acid content was only 3.90% (paired t test on arcsin square-root transformed values, $T = 11.0$, $df = 25$, $P < 0.0001$, Table 2). These data suggest that levels of protein in fruit pulp are even lower than previously estimated. This would lend support to the suggestion that protein

TABLE 1. CONTINUED

Glycine	Alanine	Valine	Isoleucine	Leucine	Tyrosine	Phenyl-alanine	Histidine	Lysine	Arginine	Ammonia
0.652	0.768	1.250	0.478	0.850	0.549	2.910	0.226	0.430	0.302	0.793
0.140	0.135	0.147	0.110	0.194	0.095	0.127	0.064	0.116	0.193	0.045
0.174	0.204	0.216	0.163	0.279	0.144	0.161	0.083	0.238	0.181	0.073
0.127	0.128	0.145	0.107	0.166	0.117	0.116	0.081	0.128	0.209	0.061
0.067	0.072	0.088	0.057	0.103	0.057	0.063	0.033	0.085	0.070	0.048
0.202	0.220	0.222	0.176	0.301	0.188	0.205	0.116	0.275	0.246	0.104
0.255	0.271	0.298	0.220	0.356	0.289	0.245	0.147	0.378	0.374	0.080
0.193	0.201	0.234	0.151	0.247	0.186	0.170	0.094	0.238	0.195	0.082
0.109	0.114	0.121	0.101	0.173	0.097	0.117	0.045	0.136	0.104	0.076
0.085	0.076	0.083	0.069	0.119	0.053	0.070	0.034	0.056	0.064	0.122
0.077	0.064	0.093	0.050	0.117	0.057	0.065	0.032	0.068	0.066	0.051
0.100	0.128	0.097	0.072	0.143	0.077	0.075	0.046	0.109	0.541	0.096
0.094	0.084	0.083	0.063	0.117	0.069	0.071	0.039	0.086	0.079	0.041
0.042	0.044	0.050	0.037	0.057	0.024	0.034	0.016	0.037	0.041	0.043
0.061	0.102	0.069	0.034	0.080	0.039	0.048	0.032	0.051	0.056	0.041
0.172	0.161	0.178	0.155	0.247	0.135	0.156	0.052	0.032	0.166	0.080
0.709	0.755	0.663	0.584	0.866	0.573	0.559	0.389	0.911	1.213	0.326
0.141	0.107	0.108	0.084	0.122	0.077	0.084	0.037	0.059	0.089	0.047
0.151	0.129	0.140	0.110	0.197	0.094	0.127	0.050	0.141	0.131	0.093
0.112	0.110	0.110	0.107	0.159	0.087	0.103	0.033	0.052	0.108	0.064
0.654	0.723	0.841	0.663	0.868	0.596	0.719	0.208	0.576	0.892	0.264
0.422	0.321	0.327	0.268	0.395	0.293	0.277	0.115	0.265	0.299	0.103
0.168	0.182	0.157	0.128	0.150	0.109	0.111	0.069	0.155	0.263	0.111
0.240	0.184	0.168	0.129	0.214	0.216	0.130	0.071	0.161	0.191	0.094
0.146	0.189	0.143	0.110	0.178	0.114	0.116	0.044	0.084	0.179	0.070
0.241	0.338	0.276	0.169	0.285	0.149	0.217	0.073	0.114	0.279	0.078

limitation is one reason for the inadequacy of a strict fruit pulp diet (Levey and Karasov, 1989; Sedinger, 1990).

The average ratio between total amino acids and total amino acid nitrogen was 6.32 (Table 2). Therefore, the amino acids present in these fruits averaged 15.8% nitrogen (100/6.32) which is close to the expected value of 16%, but since these fruits contained nonprotein nitrogen as discussed before, one should reevaluate the valid conversion factor for the Kjeldahl procedure. The 4.05

TABLE 2. ANALYSIS OF NITROGEN, PROTEIN, AND CONVERSION FACTORS OF PULPS OF 26 FLESHY FRUIT SPECIES FROM EAST MEDITERRANEAN HABITATS (Values are Percentage Dry-Weight)

	Kjeldahl determination		Amino acid analysis		Evaluation of the regression equation		Conversion factors		
Plant species	Total nitrogen (%)	Estimated protein (N × 6.25) (%)	Nitrogen (amino acid N + ammonia) (%)	Protein (total amino acids) (%)	Protein predicted (%)[a]	Residuals (%)[b]	Total amino acids/total amino acid N	Total amino acids/total Kjeldahl N	Nonproteinous N[c]
Ephedra aphylla (Ephedraceae)	3.25	20.3	2.29	14.5	15.3	0.8	6.34	4.47	0.30
Osyris alba (Santalaceae)	0.99	6.2	0.37	2.4	4.2	1.8	6.54	2.43	0.63
Laurus nobilis (Lauraceae)	0.69	4.3	0.51	3.4	2.8	−0.6	6.66	4.96	0.26
Rosa canina (Rosaceae)	0.77	4.8	0.44	2.9	3.2	0.3	6.56	3.77	0.42
Crataegus monogyna (Rosaceae)	0.34	2.1	0.21	1.3	1.1	−0.2	6.21	3.57	0.42
Pistacia lentiscus (Anacardiaceae)	0.82	5.1	0.63	4.0	3.4	−0.6	6.41	4.94	0.23
Pistacia atlantica (Anacardiaceae)	1.28	8.0	0.75	4.9	5.7	0.8	6.54	3.84	0.41
Pistacia palaestina (Anacardiaceae)	0.98	6.1	0.53	3.5	4.2	0.7	6.54	3.59	0.45
Rhus coriaria (Anacardiaceae)	0.48	3.0	0.32	2.0	1.7	−0.3	6.19	4.14	0.33
Rhamnus alaternus (Rhamnaceae)	0.45	2.8	0.27	1.4	1.6	0.2	5.21	3.19	0.39

Rhamnus lycioides (Rhamnaceae)	0.48	3.0	0.23	1.5	1.7	0.2	6.46	3.07	0.52
Ziziphus spina-christi (Rhamnaceae)	0.86	5.4	0.51	2.8	3.6	0.8	5.44	3.22	0.41
Myrtus communis (Myrtaceae)	0.34	2.1	0.22	1.4	1.1	−0.3	6.40	4.26	0.33
Arbutus andrachne (Ericaceae)	0.18	1.1	0.13	0.7	0.3	−0.4	5.68	4.18	0.26
Styrax officinalis (Styracaceae)	0.37	2.3	0.19	1.2	1.2	0.0	6.48	3.39	0.48
Phillyrea latifolia (Oleaceae)	0.56	3.5	0.42	2.8	2.1	−0.7	6.58	4.93	0.25
Withania somnifera (Solanaceae)	2.82	17.6	2.25	14.2	13.2	−1.0	6.33	5.04	0.20
Rubia tenuifolia (Rubiaceae)	0.40	2.5	0.28	1.8	1.4	−0.4	6.61	4.59	0.31
Viburnum tinus (Caprifoliaceae)	0.46	2.9	0.38	2.3	1.6	−0.7	6.10	5.00	0.18
Lonicera etrusca (Caprifoliaceae)	0.53	3.3	0.29	1.9	2.0	0.1	6.41	3.53	0.45
Bryonia spp. (Cucurbitaceae)	2.37	14.8	1.83	12.0	11.0	−0.1	6.54	5.06	0.23
Asparagus aphyllus (Liliaceae)	1.07	6.7	0.88	6.0	4.6	−1.4	6.82	5.58	0.18
Ruscus aculeatus (Liliaceae)	0.93	5.8	0.49	2.9	3.9	1.0	5.88	3.11	0.47
Smilax aspera (Liliaceae)	0.72	4.5	0.48	3.0	2.9	−0.1	6.27	4.19	0.33
Tamus orientalis (Dioscoreaceae)	0.75	4.7	0.38	2.4	3.1	0.7	6.35	3.20	0.50
Arum dioscoridis (Araceae)	1.06	6.6	0.62	4.2	4.6	0.4	6.83	3.99	0.42
	0.92 ± 0.76	5.75 ± 4.74	0.61 ± 0.59	3.90 ± 3.78			6.32 ± 0.38	4.05 ± 0.79	0.37 ± 0.11

[a] Estimate based on $Y = (4.885X) - 0.6$.
[b] % protein (total amino acids) − % protein predicted.
[c] (% total Kjeldahl N − % total amino acid N)/% total kjeldahl N.

average factor for all species is one approach to converting Kjeldahl total nitrogen to protein content (Table 2). This factor is slightly lower than the 4.4 factor suggested by Milton and Dintzis (1981) for tropical plants but higher than the average factor of 3.55 detected by Herbst (1986) for four tropical fruit species.

A significant linear equation $Y = 4.885X - 0.6$ was detected between protein content and total Kjeldahl N (Figure 1). This equation may be more accurate for estimating protein content than the 4.05 factor because of the positive intercept on the x axis (Figure 1). Despite the relatively good estimation of true protein on total Kjeldahl N based on this regression, nonprotein N estimates range from less than 20% to more than 60% for this set of species. It is possible that nonprotein N may vary even more for other species. Further, analysis of residuals of the regression (Table 2) reveals that estimated total protein from total N can be relatively inaccurate for particular species. For instance, protein is underestimated by 133% for *Arbutus andrachne* and overestimated by 43% for *Osyris alba* upon applying the regression equation (Table 2). Typical errors for other species tend to be in the 10–30% range. In addition, the proportional error tends to be greater with decreasing total N; nevertheless, most fruit pulps tend to be low in N. Thus, the regression of total protein on total N would seem to be adequate only for broad-scale estimates where positive and negative errors would tend to cancel. Such a case is the estimation of total protein intake for animals on a mixed-fruit diet. In contrast, any estimate of protein content derived from total N would be inadequate when accurate interspecific comparisons are

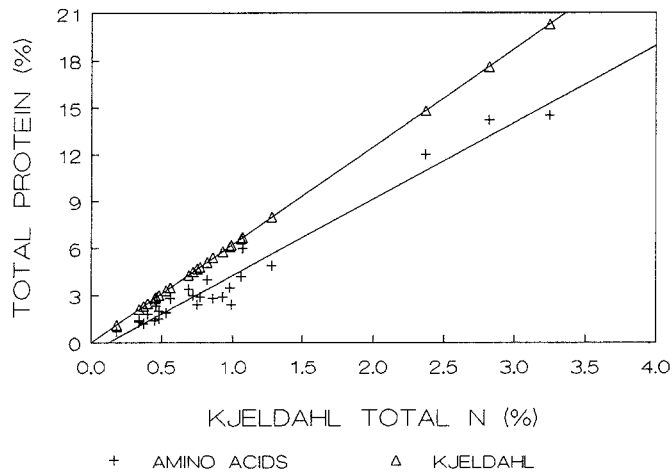

FIG. 1. The crude total protein content (calculated by multiplying total N by the traditional 6.25 factor) and the true protein content (using the total amino acid content) of 26 fruit species plotted against Kjeldahl total N. The linear equation for calculating true protein as a function of Kjeldahl total N is $Y = 4.885X - 0.6$, ($R^2 = 0.96$, $P < 0.0001$).

needed. Thus, while the use of the regression is indeed better than the use of the 6.25 conversion factor, the data suggest that it may be risky to use total N to estimate protein in fruit pulps, especially of those species with unusually high or low levels of nonprotein N.

The relatively large differences in nitrogen content estimated by the Kjeldahl procedure and the one calculated by the amino acid analysis (Table 2) were probably not an artifact effect of these methods. As discussed earlier by Milton and Dintzis (1981), it is possible that the HCl hydrolysis in the amino acid analysis does not release all the nitrogen from compounds that are tightly bonded as plant-cell material, while the sulfuric acid in the Kjeldahl method releases this nitrogen. However, most plant protein is found within the cytoplasm of the cell, with only small amounts associated with the cell wall (Lyttleton, 1973; Albersheim, 1975). Further, if such nitrogen exists, it is probably not digestible by animals. Therefore, it seems that most of the differences in nitrogen content between the two methods is due to the high levels of nonprotein nitrogen in wild fruits.

There is little information on the nonprotein nitrogen compounds in fruits. The presence of alkaloids and glycosides is frequent among European and Mediterranean species even in ripe fruits (Jordano, 1988; Ehrlén and Eriksson, 1993, and references therein) and is also reported in other ecosystems (e.g., Calvert, 1985; Potter and Kimmerer, 1986; Gargiullo and Stiles, 1991). Therefore, these compounds may constitute a major fraction of the nonprotein nitrogen compounds in fleshy fruits. In order to confirm this assumption, a direct analysis of secondary compounds in these fruits should be carried out. The significance of such compounds to the evolution of mutual relations between plants that produce fleshy fruits and their frugivorous seed dispersers is still not clear (see Jordano, 1991, and references therein).

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EFFECT OF DESIGN OF A SEX-PHEROMONE-BAITED DELTA TRAP ON BEHAVIOR AND CATCH OF MALE *Epiphyas postvittana* (WALKER)

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Abstract—Through field trials and wind-tunnel studies, we have demonstrated that certain structural features of a sex-pheromone-baited delta trap affect catch of light-brown apple moth, *Epiphyas postvittana*, males, by influencing behaviors used to enter and exit the trap. Field catch of males was dependent upon length (and width) of the trap, with increases in length yielding linear increases in catch. In the wind tunnel, similar numbers of males entered the two traps, but significantly fewer males exited the longer trap within 1 min after entering it. Although males landed on the sticky surface at similar distances from the downwind entrances of the traps, they were stuck farther upwind on the longer trap. Thus, it is probable that the increase in field catch with increase in trap length relates to the increase in distance (and hence time) that males walk on the sticky surface, towards the pheromone source, before attempting to exit. The bottom barriers (as well as additional barriers at the top and sides) at the entrances of the trap also significantly influenced trap catch. The barriers apparently influence trap catch in two ways. Firstly, they hinder the exit of males from the trap, thus diverting males back into the trap and increasing their chance of being caught. Secondly, they influence where the male lands on the sticky surface; with higher barriers, males land farther upwind (i.e., nearer the source), and thus farther from an exit. Finally, as the source was suspended higher above a horizontal surface, greater numbers of males landed on the source. This result shows that the position where a male *E. postvittana* lands is influenced by the relationship of the source to the surface and suggests that trap catch of males may similarly be influenced (i.e., by inducing males to land farther from the exits).

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Key Words—Sex pheromones, trapping, landing behavior, *Epiphyas postvittana*, Lepidoptera: Tortricidae.

INTRODUCTION

In essence, a trap is a contrivance for catching animals that contact or enter it. For a trap to be effective there must be, firstly, a reasonably high probability that the animal of interest will actually encounter and contact or enter the trap, and, secondly, the trap must have some mechanism for retaining the animal once it has contacted or entered the trap. A high probability of encountering the trap can be achieved by appropriate placement of the trap or by use of a lure that entices the animal to come to that position. Retention of the animal upon contact with the trap is usually achieved by either immobilization or encapsulation.

Trapping insects by using synthetic versions of their own sex pheromones incorporates the two essential features outlined above. The insect is lured into the trap by the use of the sex pheromone and is immobilized or captured by insecticide, electricity, shape of the trap, or a sticky surface (Cardé and Elkington, 1984). A wide variety of designs of traps utilizing sticky surfaces have been tried, and although the rationale behind the designs is not always clear, the *modus operandi* is the same: lure the insect into the trap and hope it makes contact with, and remains caught on, the sticky surface. Most probably, the insect is caught as a consequence of controlled landing on the sticky surface.

In addition to the considerable efforts to identify blends of sex pheromone chemicals that yield the best trap catches of insects (Arn et al., 1986), there have been numerous studies determining the most effective placement or type of trap for increasing catches of a particular species (Cardé and Elkington, 1984). Although it is generally recognized that trap catches are a consequence of the appropriate behaviors exhibited by the insects, there have been few studies on the influence of the design of a trap on insect behavior (e.g., Foster et al., 1991; Phillips and Wyatt, 1992; Quartey and Coaker, 1992). More particularly, the behavior of pheromone-mediated landing has rarely, if ever, been considered when designing a suitable trap for a particular insect. Recently, we have begun studying the various factors that influence the landing behavior of male light-brown apple moth, *Epiphyas postvittana* (Walker), exhibiting pheromone-mediated flight, and have found that visual stimuli (along with the olfactory stimulus) can significantly influence landing (Foster and Harris, 1992).

In this report, we have studied the effect of some features of a delta trap on field catches of male *E. postvittana*. Furthermore, using a wind tunnel, we

have shown how the differences in field catch that result from these changes in the trap are, at least in part, due to the effect of these features on the landing and exiting behaviors used by males.

METHODS AND MATERIALS

Insects. E. postvittana used in the wind-tunnel experiments were from a laboratory colony maintained at HortResearch in Auckland. Larvae were fed on synthetic diet (Singh, 1974). Male pupae were placed in an incubator at 20 ± 1°C and a 16:8 hr light–dark photoperiod. Male moths were collected within 24 hr of emergence and placed in a plastic container with moist vermiculite on the bottom, and a 10% honey solution.

Field-Trapping Experiments. Field trials were conducted using either standard-size (HortResearch, Auckland) (see Foster et al., 1991, for a more detailed description of these traps) or different-size delta traps, constructed of white corrugated plastic. The bases of the various traps were coated with a sticky glue (Product #633, Davis Gelatin Ltd., Christchurch, New Zealand). All traps were baited with a red rubber septum (Thomas Scientific Inc., Philadelphia, Pennsylvania), loaded with a mixture of 100 μg (*E*)-11-tetradecenyl acetate (*E*11-14:OAc) and 5 μg (*E*,*E*)-9,11-tetradecadienyl acetate (*E*,*E*-9,11-14:OAc) (Bellas et al., 1983). Field trials were conducted either at Mt. Albert Research Centre (Auckland) or at a Massey University Fruit Crops Unit orchard and in a home garden in Palmerston North. Traps were hung on apple or shelter (pine or poplar) trees, at least 10 m apart, with replicates of series at least 40 m apart. The traps were checked, insects removed, and the positions of the traps rerandomized once every one or two weeks.

The following field-trapping experiments were conducted:

1. A factorial design of different delta traps, of the same height (10 cm), but of all possible combinations of length (length is the distance between the entrances of the traps), 36, 18, 9 cm, and width (dimension horizontally perpendicular to length), 36, 18, 9 cm, for the sticky base, was used. The traps had no bottom barriers (see Foster et al., 1991) at the entrances. Four replicates of each trap were tested at Auckland.

2. Four replicates of delta traps, with bottom barriers, of regular width (18 cm) and height (10 cm), but of different lengths of sticky base (18, 36, 45, 54 cm) were tested at Auckland.

3. Any effects on catch observed in experiments 2 and experiment 1 could be due to either changes in dimension (length) or differential changes in the ability of the different length sticky surfaces to catch insects over time, e.g., the lesser sticky surface area of the shorter traps could saturate with debris more quickly than the longer traps, and hence become less efficient at trapping insects. To distinguish between these two possibilities, an experiment of factorial design

testing regular-length (18-cm) and longer (36-cm) traps, for which the sticky surface was either left unchanged or changed regularly over 14 weeks, was conducted. Four replicates of each trap were tested at Palmerston North.

4. Four replicates of regular (18 × 18-cm sticky base) delta traps, with or without bottom barriers, were tested at Auckland.

5. Four regular (18 × 18-cm sticky base) delta traps with additional (to the bottom) barriers were compared: a trap without additional barriers (regular), a trap with an additional barrier extending 4 cm down from its apex (top), a trap with additional barriers extending 4.5 cm in from the bottom corner of either side (side), and a trap with both additional top and side barriers. Four replicates of each trap were tested at Auckland.

6. Four long (36-cm base) delta traps with the same combinations (and number of replicates) of additional barriers as in experiment 5 were tested at Auckland.

Wind-Tunnel Experiments. E. postvittana males were flown in a 1.4-m-long wind tunnel based on the design of Miller and Roelofs (1978). The tunnel was illuminated by nine 15-W tungsten bulbs (Osram Co., Auckland) regulated to a light level of 1 lux. Individual males were released into the tunnel between the second and fourth hours of the scotophase (Muggleston and Foster, 1989). The pheromone source was identical to those used for the field-trapping experiments. When the source was not in use, it was stored in a 20-ml glass vial at −15°C.

Three experiments were conducted:

1. The first experiment compared two different length delta traps, the regular length (i.e., with a 18 × 18-cm sticky base) and the other of twice the length (i.e., 36 cm). A trap was placed on a 10 × 10 × 10-cm wire stand and aligned so that its length was parallel with the wind. The traps had clear Mylar sides, so that the insect could be observed after it had entered the trap. The source was placed on the base in the middle of a trap. For each moth the following were recorded: (a) whether it entered the trap, (b) whether it exited the trap within 1 min after entering, and, if so, roughly at what position (top = approximately within a region 4 cm down from the apex; side = approximately within a region extending 4.5 cm laterally inwards from the bottom corners; bottom = anywhere else), (c) where it initially landed on the sticky surface (the surface was divided into 2-cm rows along its length, the most downwind designated A, the next row upwind, B, and so on; for analysis, moths that landed in row A were assumed to land 1 cm from the downwind edge, in row B, as 3 cm from the downwind edge and so on), (d) how many moths were on the sticky surface at the end of each experimental session, and (e) the distance from the downwind edge that these moths were located.

2. In the second experiment, an 18 × 18-cm horizontal, white corrugated plastic surface was placed atop the 10-cm-high stand. Attached to the downwind

edge of the surface was a 1.4-, 3.0-, or 5.0-cm-high × 18-cm-wide white, corrugated plastic vertical barrier. The horizontal surface was divided into a grid of 2 × 2-cm squares, with the downwind row (rows were perpendicular to the wind) labeled A, the next row upwind B, etc., and the columns (along the axis of the wind) labeled 1–9 from the right-hand side (looking upwind in the tunnel). The source was in the middle of the horizontal surface (i.e., at position E5). The initial landing position of the moth was recorded as barrier when it either landed on, or flew over, the barrier before landing on the surface, or as side when it flew around (to the side of) the barrier and landed on the surface. The grid position on which the male first landed was recorded.

3. In the third experiment, five treatments were compared. Three treatments consisted of a 18 × 18-cm horizontal surface, with the source on the surface (at position E5) or 1.5 or 2.5 cm above the surface, atop a thin (100 gauge) wire support at position E5. The other two treatments consisted of a 18 × 36-cm-long surface (length aligned with the wind direction), with the source at position I5 either on the surface or 1.5-cm atop the wire support. The position where the moth landed on the surface was recorded; if the moth landed on the source, the position was recorded as E5 or I5, as appropriate.

Chemicals. The *E*11–14:OAc (Sigma Chemical Company, St. Louis, Missouri) was >99% isomerically pure as determined by capillary gas chromatography, while the *E*,*E*-9,11–14:OAc (gift from Shin Etsu Chemical Company, Tokyo, Japan) was approximately 94% isomerically pure.

Statistical Analyses. Data from the field-trapping experiments were analyzed by two-way ANOVA, and means compared by Fisher's least significant difference test. For the first and third experiments, factorial analyses of the width and length, and of the length and changing of sticky bases, respectively, were performed using the program JMP 2.0 (1991, SAS Institute, Cary, North Carolina), to test for significant interactions between these parameters. For the second experiment, data were also analyzed by linear regression of trap length versus catch, again using the program JMP 2.0.

For the wind-tunnel experiments, the number of males that landed in the cells of a particular row or column (if applicable) for a treatment were summed, and the distributions of the landing positions in the different rows or columns between treatments compared by χ^2 tests. If significant differences within experiments were found, then pairs of treatments were also compared by χ^2 tests. For experiment 1, the distances from the downwind edge that the males landed or were stuck on the surface were analyzed by one-way ANOVA. Unless stated, all differences are reported to $P < 0.05$.

RESULTS

Field-Trapping Trial 1. The results showed a trend of larger traps catching greater numbers of males (Table 1). The greatest numbers of males were caught in the two traps of largest surface area of length 36 cm. The largest trap (36 ×

TABLE 1. FIELD-TRIAL CATCHES OF *Epiphyas postvittana* IN DELTA TRAPS OF VARIOUS WIDTHS AND LENGTHS[a]

Width (cm)	Length (cm)	Mean catch/trap/week
Trial 1		
36	36	5.43 a
36	18	2.4 b
36	9	0.31 c
18	36	3.57 a
18	18	2.00 b
18	9	0.17 c
9	36	0.97 c
9	18	0.60 c
9	9	0.071 c
Trial 2		
18	18	7.64 c
18	36	10.2 b
18	45	12.6 ab
18	54	14.5 a

[a] Trial 1 conducted at Auckland, from January 16 to March 15, 1991. Trial 2 conducted at Auckland, from March 25 to April 29, 1991. Means followed by the same letter are not significantly different at $P < 0.05$, ANOVA, and comparison of means by Fisher's protected least significant difference test.

36 cm), for example, caught more than twice as many moths as the regular-size (18 × 18-cm) trap. Traps of length 9 cm caught few moths. Analysis of the data showed significant effects due to both length ($F_{2,242} = 24.14$, $P < 0.0001$) and width ($F_{2,242} = 11.18$, $P < 0.0001$). There was a significant interaction between these two parameters ($F_{4,242} = 2.90$).

Field-Trapping Trial 2. The greatest numbers of moths were caught in the two longest traps (Table 1). There was a significant ($P < 0.01$) linear relationship between mean catch/trap/week and length of the trap: mean catch/trap/week = 3.93 (±0.735) + 0.192 (±0.018)L; $F_{1,2} = 111.48$; where L = length of trap in cm, and values in parentheses are standard errors.

Field-Trapping Trial 3. The greatest number (significantly greater than for all other treatments) of males was caught in the 36-cm trap with its base changed regularly (Table 2). The 36-cm trap without its base changed and the 18-cm trap with its base changed caught similar numbers of moths after 14 weeks; both caught significantly greater numbers of moths than the 18-cm trap without its base changed. Factorial analysis of the data revealed significant effects due to changing the base ($F_{1,156} = 6.33$, $P < 0.01$) and to length of the trap ($F_{1,156} = 9.82$, $P < 0.0001$). There was no significant interaction ($F_{1,156} = 0.012$) between these two effects. If the weekly catches of the traps without bases

changed are compared with those of the traps with bases changed (Figure 1), it can be seen that catches in the traps without bases changed generally decreased throughout the experiment, whereas the catches in the traps with bases changed increased late in the experiment.

Field-Trapping Trial 4. Traps with the bottom barrier caught over 2.4 times (significantly greater, $P < 0.0001$) as many males than traps without the barrier (Table 3).

TABLE 2. FIELD-TRIAL CATCHES OF MALE *Epiphyas postvittana* IN TWO DELTA TRAPS OF DIFFERENT LENGTH, WITH THEIR BASES CHANGED REGULARLY OR NOT AT ALL[a]

Treatment	Mean catch/trap/week
Trial 3	
18-cm trap, base not changed	1.57 c
18-cm trap, base changed regularly	2.71 b
36-cm trap, base not changed	2.57 b
36-cm trap, base changed regularly	4.29 a

[a]Trial conducted at a Massey University Fruit Crops Unit orchard and at a home orchard in Palmerston North from February 4 to May 14, 1992. Means followed by the same letter are not significantly different at $P < 0.05$.

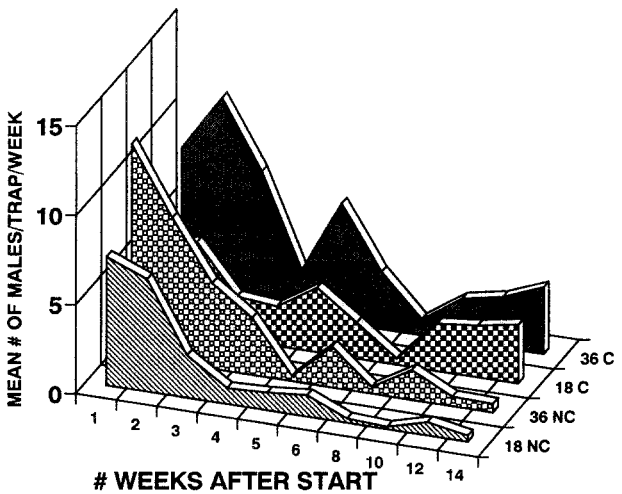

FIG. 1. Mean weekly catches of *Epiphyas postvittana* in delta traps over 14 weeks; 18 NC = 18-cm-long base that was not changed, 36 NC = 36-cm-long base that was not changed, 18 C = 18-cm-long base changed every one or two weeks, 36 C = 36-cm-long base changed every one or two weeks.

TABLE 3. FIELD-TRIAL CATCHES OF MALE *Epiphyas postvittana* IN DELTA TRAPS WITH OR WITHOUT VARIOUS BARRIERS AT ENTRANCES OF TRAPS[a]

Treatment	Mean catch/trap/week
Trial 4	
Trap with bottom barriers	6.07 a
Trap without bottom barriers	2.5 b
Trial 5	
Trap with bottom barriers	5.40 c
Trap with bottom and top barriers	9.04 ab
Trap with bottom and side barriers	8.60 b
Trap with bottom, top and side barriers	10.9 a
Trial 6	
Long trap with bottom barriers	4.03 a
Long trap with bottom and top barriers	3.30 a
Long trap with bottom and side barriers	3.44 a
Long trap with bottom, top and side barriers	3.74 a

[a] Trial 4 conducted at Auckland, from January 11 to March 1, 1991. Trial 5 conducted at Auckland, from May 8 to June 12, 1991. Trial 6 conducted at Auckland, from September 30, 1991, to January 20, 1992. Trap = 18 × 18-cm base; long trap = 18 × 36-cm-long base; barriers are described in the text. Means followed by the same letter are not significantly different at $P < 0.05$.

Field-Trapping Trial 5. The greatest number of males was caught in the trap with the additional top and side barriers, although this was not different from the number of males caught in the trap with just the additional top barrier (Table 3). In turn, the mean of the latter was not different from that of the trap with just the side barriers. However, all traps with additional barriers caught significantly more moths than the regular trap (with just the bottom barrier).

Field-Trapping Trial 6. There were no significant differences between the number of males caught in any of the 36-cm traps with or without additional barriers (Table 3).

Wind-Tunnel Experiment 1. Very high percentages of the males released (>93% for both treatments) flew upwind and entered the 18-cm or 36-cm traps. Of the males that entered the 18-cm trap, a relatively high proportion (19/46; 41%) exited the downwind end of the trap within 1 min of entering. Of the 19 males that exited the 18-cm trap, eight exited from the top, six from the side, and five from the bottom. In contrast, a significantly ($P < 0.001$; χ^2 analysis of distributions) lower proportion (3/42; 7%) of the males that entered the 36-cm trap exited it within 1 min; the three males that exited the 36-cm trap were evenly divided across the three categories.

The initial landing positions on the sticky surfaces for both traps are shown in Figure 2. Although males were able to land farther upwind on the 36-cm

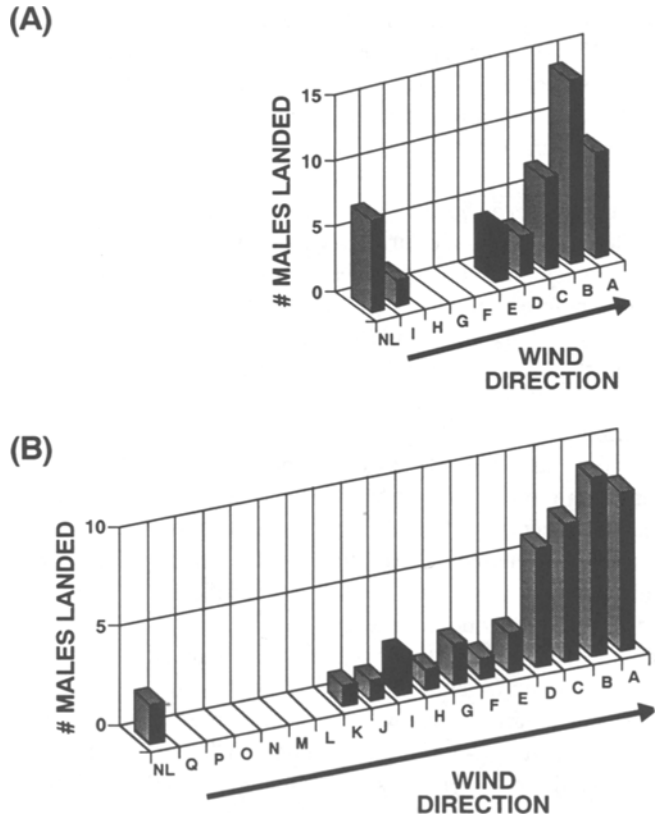

FIG. 2. Distribution of landing positions of male *Epiphyas postvittana* on the sticky surfaces of (A) a regular (18-cm base) delta trap, and (B) a long (36-cm base) delta trap. The source (position in black) was in the middle of each trap at position E (18-cm base) or position I (36-cm base). The letters refer to 2-cm rows of the base (see text); NL = insects that were not observed to land.

than on the 18-cm trap, there was no statistical difference between the landing patterns. This was predominantly because of the very high and similar percentages of males that landed on one of the first four rows (71% and 70%, respectively, for the 18-cm and 36-cm traps) for both traps. The mean distances from the downwind edge that males landed on the sticky surface were: 4.74 $\pm$ 0.75 cm (for the 18-cm trap) and 6.40 $\pm$ 0.75 cm (for the 36-cm trap). The proportion of males that entered the traps and were not observed to land on the sticky surface was not different between the two traps.

Of the 27 males that did not exit the 18-cm trap within 1 min after entering it, 24 (52% of the males that entered) were later found stuck on the base. In

comparison, a significantly greater ($P < 0.01$; χ^2 analysis of distributions) proportion (33/42; 79%) of the males that entered the longer trap were later found stuck on the base. The mean distances from the downwind edge that males were stuck on the surfaces were: 6.52 ± 1.25 cm (for the 18-cm trap) and 11.27 ± 1.07 cm (for the 36-cm trap). These distances were significantly different ($P < 0.01$).

Wind-Tunnel Experiment 2. There were no differences in the percentages of males that landed on the three surfaces with barriers of different height (>92% for all three surfaces). For all three treatments, most males landed on or flew over the barrier before landing on the horizontal surface. However, a significantly (χ^2 analysis of distributions) higher proportion (13/38; 43%) of males flew around (to the side of) the 5.0-cm barrier compared to the 3.0-cm (4/38; 11%) and 1.4-cm (1/38; 3%) barriers.

The distributions of landing positions of males on the surfaces with the barriers are shown in Figure 3. With the increase in height of barrier, males tended to land in a more dispersed fashion, particularly landing farther upwind. For all three treatments, a relatively high proportion of males landed to either side of the source (compared to just a horizontal surface; see Foster and Harris, 1992, and also wind-tunnel experiment 3). A significantly higher proportion of males landed farther upwind on the surfaces with the 5.0- and 3.0-cm-high barriers than on the surface with the 1.4-cm-high barrier.

Wind-Tunnel Experiment 3. For the 18 × 18-cm surface, the proportion of males that landed farther upwind increased as the source was raised higher above the surface (Figure 4). The distribution of landing in rows (i.e., along the axis of the wind direction) was significantly ($P < 0.001$) different when the source was 2.5 cm above the surface than when it was 1.5 cm above the surface, which in turn was significantly ($P < 0.001$) different than when the source was on the surface. These results reflect, in large part, the greater proportion of males that landed on the source as it was raised higher above the surface: 0% of the males landed on the source when it was on the surface, 25% when it was 1.5 cm above the surface, and 77% when it was 2.5 cm above the surface.

The pattern of landing on the 36-cm-long surface (Figure 5A) was apparently not different from that on the 18-cm surface, when the source was on the surface. That is, the vast majority of males (36/42; 86%) landed on the downwind row; 32 of these males landed in line with, or one grid position either side of, the column (i.e., 5) with the source. When the source was raised 1.5 cm above the surface, males landed significantly ($P < 0.0001$) farther upwind than when the source was on the surface (Figure 5). In comparison with when the source was on the surface, relatively few (8/33; 24%) of the males landed on the most downwind row, while a larger proportion (15/33; 48%) landed on the source. When the source was 1.5 cm above the surface, a significantly greater proportion of males landed upwind of the downwind row on the 36-cm surface

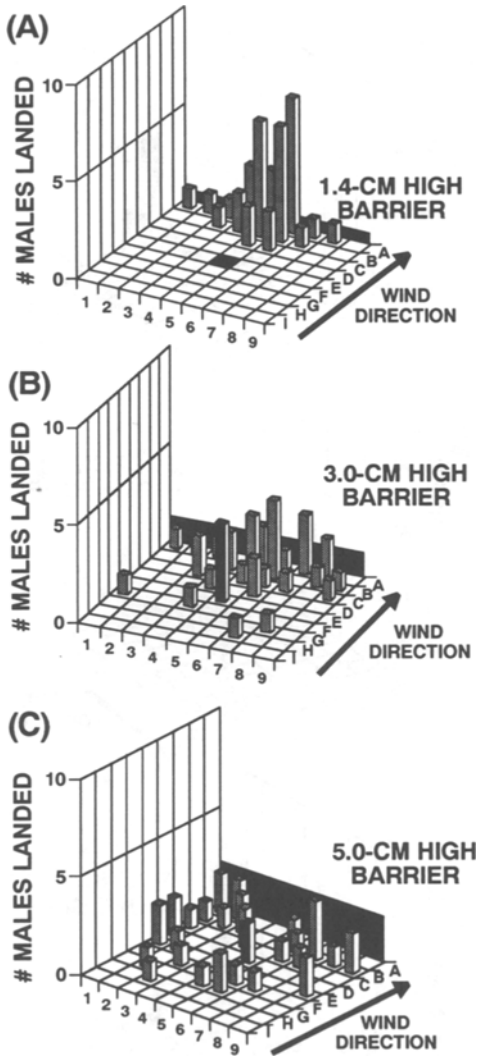

FIG. 3. Distribution of landing positions of male *Epiphyas postvittana* on a 18 × 18-cm horizontal surface with (A) a 1.4-cm-high barrier at the downwind edge of the surface, (B) a 3.0-cm-high barrier at the downwind edge of the surface, and (C) a 5.0-cm-high barrier at the downwind edge of the surface. The source (position in black) was at position E5 for all three treatments.

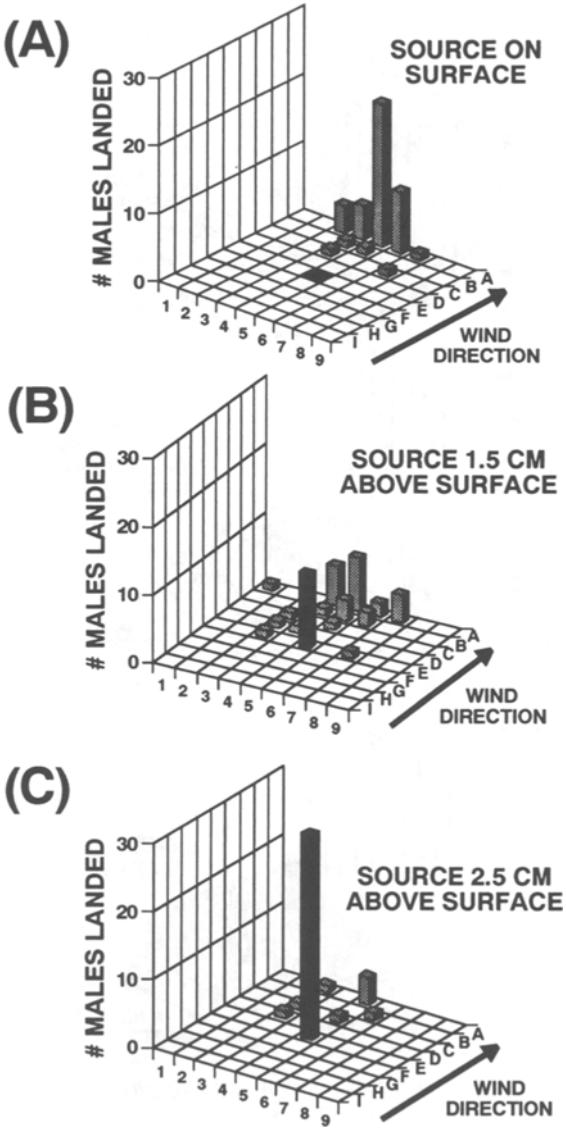

FIG. 4. Distribution of landing positions of male *Epiphyas postvittana* on an 18 × 18-cm horizontal surface with the source at position E5 (in black): (A) source on the surface, (B) source 1.5 cm above the surface (C) source 2.5 cm above the surface.

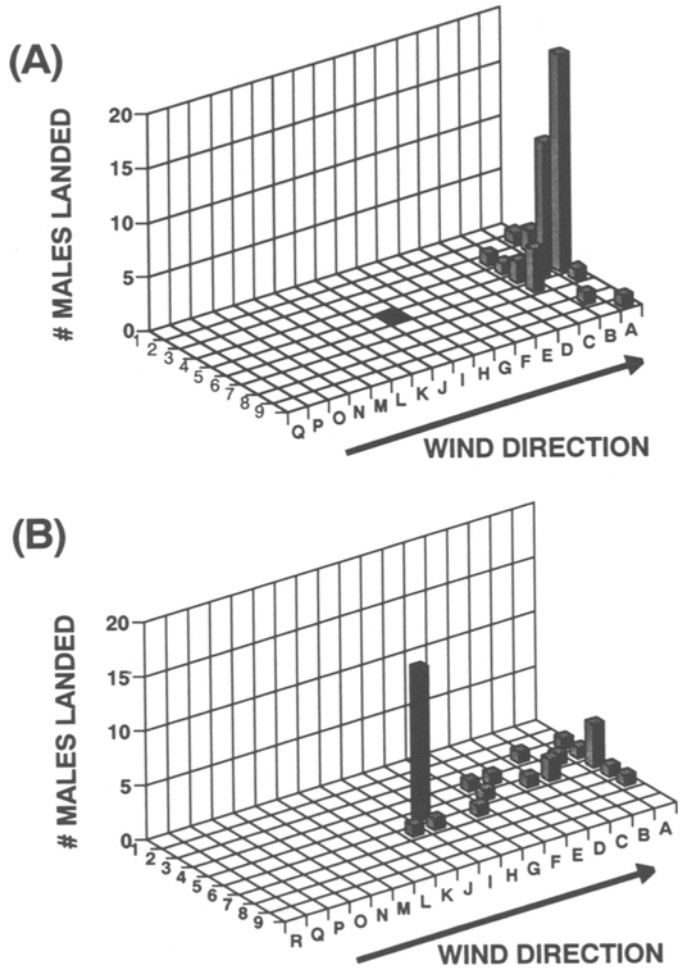

FIG. 5. Distribution of landing positions of male *Epiphyas postvittana* on a 18 × 36-cm-long surface with the source at position I5 (in black): (A) source on the surface, and (B) source 1.5 cm above the surface at position I5.

(25/33; 76%) compared with the 18-cm surface (23/44; 52%) (Figures 4 and 5).

DISCUSSION

The number of insects caught in a pheromone-baited trap is dependent upon a multitude of factors, including local population size, external factors, such as temperature or wind direction, as well as directly upon features of the trap itself.

Disregarding population size, the effect that the other factors have on the numbers of insects caught in the trap inevitably relates to their effect on the behavior of the insect. For example, changes in the direction of the wind can affect the structure of the pheromone plume emanating from the trap, which in turn can have a profound influence on the flight and landing behavior of the insect, resulting in fewer insects landing on or entering the trap (Lewis and Macauley, 1976; Foster et al., 1991).

In this study, features of a delta trap that influence the behavior of male *E. postvittana*, and consequently affect the number of individuals caught, have been investigated. The first field-trapping experiment showed that both width and length of the trap had important effects upon field catch. The second field trial confirmed that length was an important factor, with increases in length of the sticky base of the trap of up to three times the regular length resulting in increases in catch of male *E. postvittana*. The results from the third field trial showed that the effect of trap length on field catch was distinct from the effect of changing the sticky surface regularly (i.e., from changes over time in the efficiency of the glue to catch insects). Thus the length of a delta trap directly influences field catch of male *E. postvittana*.

In the first wind-tunnel experiment we determined how length of the delta trap influenced behavior. As expected, similar proportions of males entered the regular-size (18-cm) and longer (36-cm) traps. However, whereas a relatively large proportion of the moths exited the regular trap within 1 min of entering, very few moths did so in the longer trap. The relative numbers of moths left in the traps at the end of each run of the experiment (i.e., 0–2 hr later) reflected the proportions that had exited the trap within 1 min of entering. This difference in numbers of moths remaining in the regular and longer traps, particularly after 1 min of entering it, corresponded with the difference in field-trap catch between the regular and longer traps.

Males landed on the sticky surface, inside the two traps, at similar distances from the downwind entrances. Of the males that remained stuck in the two traps, males on the longer surface were located significantly farther upwind from the downwind entrance of the trap than males on the regular-length surface. On average, males stuck on the longer surface were almost twice as far from the downwind edge compared to where they landed. After landing, male *E. postvittana* walk over a sticky surface towards the pheromone source (Foster et al., 1991). Thus it is likely that the increase in trap catch in the longer trap is a consequence of the greater distance that males walk, or move, towards (and then away from) the pheromone source; the greater distance (and hence time) that males walk on the longer surfaces increases their chance of sticking to the surface.

In field trial 4, removal of the end barriers from the regular trap resulted in a large decrease in the catch of males relative to traps with the barriers. In

field trial 5, additional barriers, on the sides and top of the entrances (exits) of the trap resulted in further increases in catches of males. The addition of the same barriers to a longer (36-cm) trap did not, however, yield any increases in trap catch over a trap with just the bottom barrier (field trial 6), indicating that there was no additional increase in trapping efficiency of the longer trap by addition of the extra barriers. The wind-tunnel data indicate that the barriers in the delta traps influence the behavior of male *E. postvittana* in two different ways. Firstly, as observed in wind-tunnel experiment 1, a relatively high proportion of males exit the regular-length trap after entering. In this experiment, males were observed exiting the trap at positions near the top, sides, and bottom of the entrances (exits). It is probable that the additional barriers (i.e., at the top or the sides) on the regular-size trap in field trial 5 would have impeded the exit of moths at this position, thus forcing the moths to remain in the trap for a longer period, and consequently increasing the chance of their being caught. Similarly, the bottom barrier in a regular trap would impede the exit of moths, thus increasing the trap catch. As fewer moths exited the longer trap in the wind tunnel, the additional barriers would not be expected to affect trap catch, as was observed in field trial 5.

The second way that the entrance (bottom) barrier probably affects trap catch is by influencing where the insect lands on the sticky surface of the trap. Thus, in the second wind-tunnel experiment (and observed in the first also), males landed first on the barrier, then flew (or in some cases, walked) to the horizontal surface; the higher the barrier, the more likely males landed farther upwind on the surface. This latter result is probably a consequence of both the takeoff velocity from the barrier and a limited rate of controlled descent onto the surface by the males, i.e., the higher the takeoff point, the farther upwind males must land. As the barriers induce males to land farther upwind and nearer the source, than they would in the absence of barriers, there is, consequently, a greater distance for the males to exit the trap. Thus, as for the longer traps, there is a greater chance of males becoming stuck before they exit.

Thus, the number of *E. postvittana* caught in a delta trap is not solely dependent upon the number that enter the trap combined with a constant ability of a glue to catch the moth upon contact, but also upon features of the trap that elicit the male to land farther inside the trap, as well as features that hinder the moth exiting the trap. These features increase the chance of the moth sticking to the glue. The effects of these features on trap catch will likely be more apparent with relatively inefficient glues or when the efficiency of a glue decreases over time.

In the final wind-tunnel experiment we did not test any effect we had observed in the field, but rather tested whether the height of the source above the surface influenced the landing position of male *E. postvittana*. Sources are often suspended above the trapping surface or container in a pheromone-baited

trap (e.g., Quartey and Coaker, 1992). Suspension of the source above the surface strongly influenced the landing position of males, even when the source was suspended only 1.5 cm above the surface. Suspension of the source elicited males to fly past the downwind edge of the surface (the usual landing place when the source is on the surface) and to land on the suspended source. This effect was more pronounced the higher the source was suspended above the surface. This result is consistent with previous results (Foster and Harris, 1992) where it was shown that the landing position of male *E. postvittana* is affected by discontinuities between the pheromone source and a surface (i.e., males usually land on a surface connected with the source). In experiment 3, it is possible that raising the source above the surface resulted in the lower boundary of the pheromone plume being sufficiently above the downwind edge of the surface for the males to lose contact with the plume near the edge, and consequently males were not induced to land. This would not, however, explain the greater proportion of males that landed on the source when it was suspended 1.5 cm above the 36-cm- compared to the 18-cm-long surface, since the lower boundary of the plume should be the same or lower at the downwind edge of the longer surface, because of diffusion over the greater distance (Murlis et al., 1992). Further experiments will be aimed at determining how such differences in the olfactory and visual stimuli elicit changes in where male *E. postvittana* land.

From a practical viewpoint, these experiments have shown that improvements in trap catch of *E. postvittana* can be made by simply increasing the length of the trap, adding additional barriers to the entrances of the traps, or changing the sticky surface more frequently. Additionally, by suspending the source at an appropriate height above the sticky surface, it may be possible to increase further trap catch by inducing a larger proportion of males to land farther inside the trap. Whether this actually improves trap catch will, however, be dependent upon whether males that land on the suspended source eventually contact the sticky surface farther inside the trap. We intend to test whether this effect actually influences field catch. It is hoped that further study on flight and landing behavior, combined with a better understanding of the ecology of *E. postvittana*, will improve control of this, and other, lepidopterous pests.

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XANTHINE TOXICITY TO CATERPILLARS SYNERGIZED BY ALLOPURINOL, A XANTHINE DEHYDROGENASE/OXIDASE INHIBITOR

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Abstract—Xanthine (2,6-dioxypurine), which occurs in certain legumes and other plants, was fed in artificial diet to larvae of two noctuid moth species, a legume specialist, *Anticarsia gemmatalis*, and a generalist, *Spodoptera frugiperda*. In addition, diets either lacked or contained allopurinol (4-hydroxypyrazolo(3,4-*d*)-pyrimidine), an inhibitor of xanthine dehydrogenase and oxidase, enzymes that convert xanthine to uric acid. Xanthine alone (up to 2% fresh mass, fm) had little deleterious effect on either species, whereas allopurinol alone (up to 1% fm) had moderate but significant effects, increasing mortality, slowing development, and reducing insect biomass. At 0.5% fm allopurinol, the decrease in biomass-relative growth rate (RGR) was associated with reductions in the efficiency of conversion to biomass of digested food (ECD; both species) and in the biomass-relative consumption rate (RCR; *A. gemmatalis*). In addition, pupae of each species from allopurinol-fed larvae had increased water retention (i.e., lower percentage dry mass) compared with insects consuming control diet. When fed diet containing both compounds (1% fm xanthine + 0.5% fm allopurinol), no *A. gemmatalis* and only 40% of *S. frugiperda* larvae reached the prepupal stage; additionally for the latter species, there was a substantial slowing of growth and reductions in final biomass, RGR, RCR, and ECD. These results indicate a synergistic interaction, in which the effects of xanthine and allopurinol combined in the diet were significantly greater than the additive effects of each compound tested separately. Presumably, the inhibition of xanthine dehydrogenase by allopurinol prevented the absorbed xanthine from being converted to uric acid and excreted. In addition, this study expands the phenomenon of phytochemical detoxification by insects to include xanthine dehydrogenase, an enzyme generally not considered within this context.

Key Words—Allelochemical, allopurinol, *Anticarsia gemmatalis*, detoxifi-

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cation, dose–response, consumption rate, food utilization, Lepidoptera, Noctuidae, purine, *Spodoptera frugiperda*, synergism, uric acid, xanthine.

INTRODUCTION

Polysubstrate monooxygenases, hydrolases, and group transferases, which typically increase the polarity and thus presumably the excretability of xenobiotics, are generally considered the primary enzyme systems responsible for the detoxification of allelochemicals by plant-feeding insects (Lindroth, 1991; Brattsten, 1992). However, other enzymes, including those involved in the formation of nitrogenous excretory compounds, may also contribute to allelochemical detoxification. For example, bruchid beetles specialized to feed on legume seeds containing the nonprotein amino acid canavanine metabolize it by arginase to urea, which in turn is metabolized by urease to ammonia (Rosenthal et al., 1978). In this case, rather than being excreted, the ammonia is used in the synthesis of protein amino acids (Rosenthal et al., 1982).

Other nitrogen-containing allelochemicals, especially alkaloids, also may be detoxified by conversion to nitrogenous excretory compounds. For example, the naturally occurring purines, including hypoxanthine (6-oxypurine) in *Lupinus* and *Solanum*, xanthine (2,6-dioxypurine) in *Beta*, *Medicago*, *Vicia*, and *Coffea*, caffeine (1,3,7-trimethyl-2,6-dioxypurine) in *Coffea*, and zeatin (6-[4-hydroxy-3-methyl-2-butenylamino]purine) in *Zea* (Gibbs, 1974; Duke, 1981; Robinson, 1983), have the same basic structure as uric acid (2,6,8-trioxypurine; Figure 1), the main nitrogenous excretory compound of most terrestrial insects (Cochran, 1985), and thus are likely candidates for detoxification and excretion as urates. However, there appear to be few published studies examining the effect on insect performance of dietary purines other than caffeine and related methylxanthines (Chovnick et al., 1980; Nathanson, 1984; Saul, 1984; Slansky and Wheeler, 1992 and references therein). Furthermore, although the metabolism of endogenous purines (e.g., adenine, guanine, hypoxanthine, and xanthine) is reasonably well understood (Cochran, 1985), little is known about the detoxification by insects of purines occurring in their food. I am aware of only one relevant study, that by Yu (1987) on larvae of *Spodoptera frugiperda* (J.E. Smith), showing a low rate of caffeine metabolism by microsomal oxidation in vitro.

A key enzyme involved in the metabolism of nitrogenous compounds is xanthine oxidase (EC 1.1.3.22), although xanthine dehydrogenase (EC 1.1.1.204) is the common in vivo form of this enzyme in vertebrates as well as insects (the latter name will be used here) (Cochran, 1975; Hochstein et al., 1984; Parks and Granger, 1986). This enzyme oxidizes certain purines, including hypoxanthine to xanthine and xanthine to uric acid (Bergmann and Dikstein,

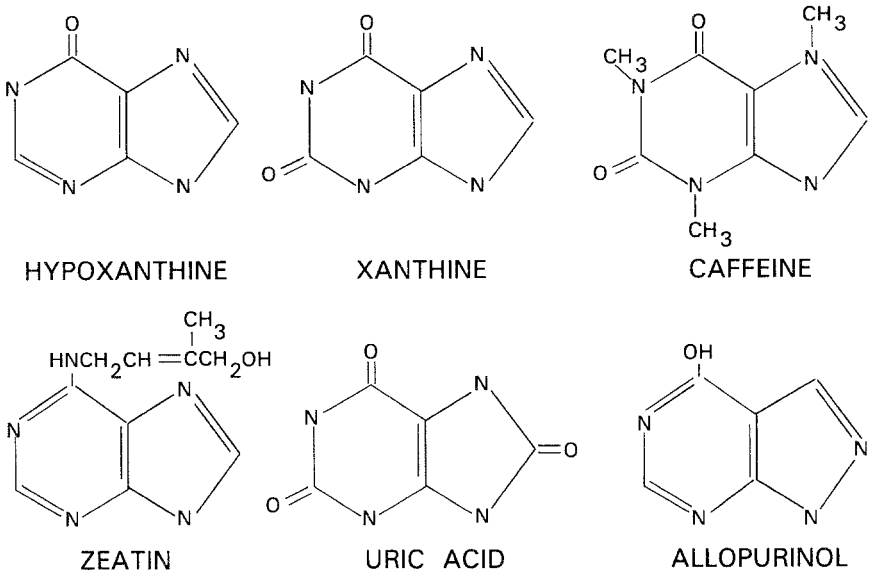

FIG. 1. Chemical structures of some naturally occurring purines and of allopurinol, an inhibitor of the enzymes (xanthine dehydrogenase and oxidase) that convert hypoxanthine to xanthine and xanthine to uric acid.

1956; Cochran, 1985; Elion, 1989). An effective inhibitor of xanthine dehydrogenase, the hypoxanthine analog allopurinol [4-hydroxypyrazolo(3,4-*d*) - pyrimidine; Figure 1] (Elion, 1966; Williams and Bray, 1981), has been used in studies of purine metabolism in vertebrates (e.g., Lohmann and Miech, 1976; Aldridge et al., 1977; Elion, 1989), as well as clinically to treat gout by preventing the buildup of urate crystals (Bartels, 1966). However, there are few studies examining allopurinol's effects on insects, although it has been used to investigate the role of stored urate in cockroaches (Engebretson and Mullins, 1986 and references therein to other allopurinol/insect studies; Suiter et al., 1992). This inhibitor should also make it possible to assess, in vivo, the involvement of xanthine dehydrogenase in allelochemical detoxification and excretion. Thus, in this study I examined whether the toxic effects of dietary xanthine could be synergized by allopurinol in larvae of two species of noctuid moths. I studied the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, which feeds on several legumes (Herzog and Todd, 1980), and the more generalized fall armyworm, *S. frugiperda*, which feeds on a variety of grasses as well as plants in over 20 other families (Tietz, 1972).

METHODS AND MATERIALS

Experimental Protocol. Both species were obtained as eggs from laboratory colonies maintained at the Insect Attractants, Behavior, and Basic Biology Research Laboratory, ARS/USDA, Gainesville, Florida. Caterpillars were reared in groups on an artificial diet following Greene et al. (1976), except for the omission of formalin, methylparaben, and tetracycline. At the start of each experiment, individual late third–early fourth instars (15–26 mg fresh mass, fm) were weighed and placed in inverted 30-ml clear plastic cups capped with tightly fitting flexible plastic tops; a weighed portion of diet was then added to each cup. Fifteen caterpillars were set up in each treatment. Experimental diets were prepared by vigorously stirring each test compound along with the other dietary ingredients into the hot (ca. 65°C) water–agar solution. Diets were allowed to cool and gel prior to slicing into portions fed to the larvae. Insects were maintained at 27 ± 1°C and 50 ± 15% relative humidity, with a photoperiod of 14 : 10 hr light–dark.

Chemical Tests. Initial experiments tested the separate effects on caterpillar growth and survival of dietary xanthine and allopurinol (both obtained from Sigma Chemical Co., St. Louis, Missouri) over a range of concentrations. Based on the results of these experiments, the diets for the synergism experiments were formulated with the concentrations of xanthine and allopurinol that had limited effects on caterpillar performance when tested separately (see Results). In the synergism experiments, survival, developmental time, biomass gain, and the consumption and utilization of food were assessed (see below).

Quantifying Food Consumption and Utilization. Each caterpillar was fed a weighed quantity of diet sufficient to last the entire experiment, beginning with late third–early fourth instars and ending with pupation or death. A gravimetric technique was used to determine food consumption, frass egestion, and biomass gain, all based on oven-dry (60–65°C) mass (dm), which allowed calculation of the following growth and consumption rates and food utilization efficiencies (Waldbauer, 1968; Slansky and Scriber, 1985): body mass-relative growth rate [RGR, (milligrams biomass gained) (milligram mean body mass × day)$^{-1}$]; body mass-relative consumption rate [RCR, (milligrams food consumed) (milligram mean body mass × day)$^{-1}$]; approximate digestibility [AD, 100 (food consumed − frass) (food consumed)$^{-1}$]; and efficiency of conversion to biomass of digested (absorbed) food [ECD, 100 (biomass gained) (food consumed − frass)$^{-1}$]. In addition to the RCR based on dry mass of food, the RCR of xanthine was calculated [RCR_{xan}, mg xanthine ingested (gram mean body mass × day)$^{-1}$]. Following Gordon (1968), biomass-relative consumption and growth rates were calculated based on a caterpillar's exponential mean biomass during the experiment, except that dry rather than live biomass was used.

When larvae were fed, an additional 10 diet samples and 10 larvae were

weighed fresh, oven-dried (larvae were frozen prior to drying), and reweighed, providing fresh to dry mass conversion factors that allowed calculation of the dry mass of food provided to each larva and the initial dry mass of the experimental larvae, respectively. Frass was removed from the rearing cups after the first two days of an experiment and daily thereafter, oven-dried, and weighed. Developmental time was measured as the number of days from the start of an experiment until a larva reached the prepupal stage, when feeding stopped. Final dry mass was based on 1-day-old pupae, or, for larvae that reached the prepupal stage but failed to pupate, on prepupal mass. Most larvae reached the prepupal stage in approximately six days; any larvae remaining after 12 days were frozen and oven-dried (duration of development for these larvae was considered to be 12 days and their dry mass at this time was used as their final dry mass). Percentage dry mass of the insects was calculated only for 1-day-old pupae by dividing their dry mass by their fresh mass. All weighing occurred on electronic balances with 0.1 mg precision.

Statistical Analysis. Performance data for the dosage experiments were analyzed using PC/SAS (SAS Institute, 1987) with one-way ANOVA (general linear models). If the F statistic was significant ($P \leq 0.05$), Dunnett's test was used to compare each treatment mean with the control mean. In the synergism experiments, performance values for larvae fed each test diet were compared to values for control larvae using a two-tailed t test (PC/SAS; SAS Institute, 1987). To test for synergistic effects, performance values for individual larvae in the three treatments (i.e., xanthine alone, allopurinol alone, and the xanthine + allopurinol combination diet) were subtracted from the appropriate mean values for control larvae, and means of these differences were calculated for each treatment. Then, for each performance measure, the mean difference for larvae fed the combination diet was compared (using a one-tailed t test) with the sum of the mean differences for larvae fed each compound separately. If the mean difference for larvae fed the combination diet (compared with control larvae) was significantly different from the sum of the mean differences due to each compound separately, then a synergistic effect was indicated (i.e., the effect of the two compounds combined in the same diet was greater than that of the sum of each compound tested separately). If either or both of the compounds separately altered performance compared with control larvae, but no significant difference occurred between the mean differences for larvae fed the combination diet (compared with control larvae) and the sum of the mean differences for larvae fed each compound separately, then an additive effect was indicated (i.e., the effect of the two compounds combined in the same diet was equivalent to the sum of the effect of each compound tested separately). In all experiments, mortality to the prepupal stage was analyzed with a G test of independence (Zar, 1984).

RESULTS

Dose–Response to Xanthine and Allopurinol. Xanthine at dietary concentrations up to 2% fresh mass (fm) had little deleterious effect on *S. frugiperda* (Table 1). Survival on the xanthine diets was comparable to that on the control diet (no added xanthine) and neither developmental time, RGR, nor percentage dry mass of the pupae was affected significantly by dietary xanthine. Although the ANOVA for final dry mass was significant, the Dunnett's test indicated that none of the values differed significantly from the control value ($P > 0.05$).

Performance of *A. gemmatalis* also was generally unaffected by diets containing up to 1% fm xanthine (Table 1). Survival was equivalent on all three diets, and there was no significant effect of xanthine on either developmental

TABLE 1. MEAN (± SEM) DURATION OF DEVELOPMENT, FINAL DRY MASS, BIOMASS-RELATIVE GROWTH RATE, AND % DRY MASS FOR LARVAE OF *S. frugiperda* AND *A. gemmatalis* FED ARTIFICIAL DIET CONTAINING DIFFERENT CONCENTRATIONS OF XANTHINE[a]

Xanthine conc. (% fm) *(*N*)	Duration (days)	Final dry mass (mg)	RGR (mg/mg/day)	Dry mass (%)
S. frugiperda				
0 (12)	6.8 ± 0.3	40.5 ± 3.0	0.44 ± 0.03	26.2 ± 0.4
0.5 (15)	6.7 ± 0.2	45.6 ± 1.3	0.45 ± 0.02	27.2 ± 0.9
1.0 (10)	6.9 ± 0.2	36.4 ± 2.4	0.40 ± 0.01	26.3 ± 1.0
2.0 (15)	7.1 ± 0.2	38.4 ± 1.9	0.40 ± 0.02	26.5 ± 0.5
$G = 0.17$[b]	$F_{3,48} = 0.80$	= 3.52	= 2.10	= 0.34
$P > 0.50$	$P = 0.50$	= 0.02	= 0.11	= 0.79[c]
A. gemmatalis				
0 (13)[d]	6.8 ± 0.2	66.2 ± 2.8	0.48 ± 0.01	23.5 ± 0.6
0.5 (14)	6.3 ± 0.2	57.5 ± 1.9*	0.50 ± 0.01	23.9 ± 0.3
1.0 (14)	6.4 ± 0.2	66.0 ± 2.3	0.52 ± 0.01	24.9 ± 0.6
	$F_{2,38} = 2.11$	= 4.55	= 1.69	= 2.06
	$P = 0.14$	= 0.02	= 0.20	= 0.15[c]

[a] *N* = number of larvae surviving to the prepupal stage out of 15 initial larvae per treatment level. Asterisk indicates that a treatment mean was significantly different from the respective control mean based on Dunnett's test.

[b] *G* test of independence with Yates correction (Zar, 1984) comparing mortality on the control and 1.0% xanthine diets.

[c] This *P* value is based on $F_{3,30}$ (for *S. frugiperda*) and $F_{2,21}$ (for *A. gemmatalis*) because % dry mass was calculated for 1-day-old pupae and not all larvae reaching the prepupal stage pupated (*N* = 6, 7, 8, 13 for *S. frugiperda*, and 6, 10, 8 for *A. gemmatalis*, for the control and increasing xanthine concentration diets, respectively).

[d] *G* test was not performed because mortality was the same in each treatment (i.e., one larva died). For the control diet, data for one developmentally abnormal larva were omitted from the analysis.

time, RGR, or percentage dry mass. The only significant reduction occurred for final dry mass on the 0.5% xanthine diet. In a separate study examining the interaction of dietary water and xanthine, larvae of *A. gemmatalis* fed the same diet formulation used in the present study but containing 2% fm xanthine had their developmental time prolonged about one day and final dry mass reduced about 10% compared with control larvae, resulting in a significant reduction in RGR ($t_{26} = 3.39$, $P = 0.002$; Slansky, unpublished data); percentage dry mass was not affected significantly, and survival was identical to that of larvae fed control diet.

Allopurinol was tested at 0.5 and 1% fm. At these concentrations, survival of *S. frugiperda* declined significantly, developmental time was prolonged (statistically significant only on the 0.5% diet), final dry mass was reduced (significant only on the 1% diet), RGR declined significantly on both diets, and percentage dry mass was reduced (significant only on the 0.5% diet) compared with larvae fed the control diet (Table 2). Allopurinol had similar effects on *A. gemmatalis*, causing significant reductions in survival, final dry mass, RGR, and percentage dry mass, and significantly prolonging duration of development at each concentration tested compared with control larvae (Table 2).

Synergism between Allopurinol and Xanthine. In these experiments, *S. frugiperda* and *A. gemmatalis* were fed diets containing either 1% xanthine (no significant deleterious effects on performance in the dose–response experiments; Table 1), 0.5% allopurinol (the lowest concentration tested in the dose–response experiments, but with deleterious effects on performance; Table 2), a combination of the two compounds at these concentrations, or a control diet with neither compound added. All *S. frugiperda* survived to the prepupal stage on the control, xanthine, and allopurinol diets (Table 3). Xanthine alone did not significantly affect duration of development or final dry mass, whereas allopurinol prolonged development and reduced final dry mass significantly. Addition of either compound to the diet significantly reduced percentage dry mass (Table 3).

When *S. frugiperda* was fed the combination diet containing both xanthine and allopurinol, synergistic effects occurred. Only 40% of the larvae fed this diet formed prepupae ($G = 11.74$, $P < 0.001$, compared with control larvae); 20% died and 40% remained as larvae when the experiment was stopped after 12 days (Table 3). Developmental time of the *S. frugiperda* larvae fed the combination diet was prolonged and their final dry mass was reduced compared with larvae fed the control diet, and in addition, these changes were significantly greater than the additive effects for larvae fed diets containing the compounds separately ($t_{40} = 4.44$, $P < 0.0005$ and $t_{40} = 4.78$, $P < 0.0005$, respectively; Table 3). The percentage dry mass for larvae fed the combination diet also was reduced significantly compared with control larvae, but the effect was additive rather than synergistic (i.e., the reduction on the combination diet was not

TABLE 2. MEAN (± SEM) DURATION OF DEVELOPMENT, FINAL DRY MASS, BIOMASS-RELATIVE GROWTH RATE, AND % DRY MASS FOR LARVAE OF *S. frugiperda* AND *A. gemmatalis* FED ARTIFICIAL DIET CONTAINING DIFFERENT CONCENTRATIONS OF ALLOPURINOL[a]

Allopurinol conc. (% fm) (*N*)	Duration (days)	Final dry mass (mg)	RGR (mg/mg/day)	Dry mass (%)
S. frugiperda				
0 (15)	7.3 ± 0.3	39.7 ± 2.5	0.38 ± 0.02	27.3 ± 0.5
0.5 (10)[b]	8.8 ± 0.4*	33.7 ± 2.1	0.31 ± 0.02*	24.8 ± 0.7*
1.0 (9)[b]	8.3 ± 0.3	31.9 ± 1.8*	0.31 ± 0.01*	25.8 ± 0.5
$G = 4.61$[c]	$F_{2,31} = 6.23$	= 3.28	= 6.45	= 5.07
$P < 0.05$	$P = 0.005$	= 0.051	= 0.005	= 0.02[d]
A. gemmatalis				
0 (15)	5.5 ± 0.2	57.3 ± 2.5	0.57 ± 0.02	23.4 ± 0.4
0.5 (11)	6.6 ± 0.5*	32.7 ± 2.5*	0.41 ± 0.03*	21.3 ± 0.6*
1.0 (5)	7.2 ± 0.5*	30.3 ± 3.5*	0.33 ± 0.03*	20.9 ± 1.1*
$G = 10.14$[c]	$F_{2,28} = 5.65$	= 30.8	= 24.4	= 7.07
$P < 0.005$	$P = 0.009$	= 0.0001	= 0.0001	= 0.004[d]

[a] *N* = number of larvae surviving to the prepupal stage out of 15 initial larvae per treatment level. Asterisk indicates that a treatment mean was significantly different from the respective control mean based on Dunnett's test.

[b] Data for two abnormally developing larvae fed the 0.5% allopurinol diet and one fed the 1% diet were omitted from the analysis.

[c] *G* test of independence with Yates correction (Zar, 1984) comparing mortality on the control and 0.5% + 1.0% allopurinol diets.

[d] This *P* value is based on $F_{2,21}$ for *S. frugiperda* and $F_{2,24}$ for *A. gemmatalis* because % dry mass was calculated for 1-day-old pupae and not all larvae reaching the prepupal stage pupated (*N* = 9, 9, 6 for *S. frugiperda* and 14, 9, 4 for *A. gemmatalis*, for the control, 0.5% and 1.0% allopurinol diets, respectively).

significantly different from the sum of the reductions due to the two compounds when tested separately; $t_{30} = 1.23$, $P > 0.10$; Table 3).

For *S. frugiperda*, xanthine alone did not significantly affect RGR or any of its components (i.e., RCR, AD, and ECD; Figure 2). However, dietary allopurinol caused a significant reduction in RGR, associated with a significantly lower ECD; neither RCR nor AD were reduced significantly (Figure 2). RGR, RCR, and ECD for larvae fed the combination diet were all significantly less than the values for larvae fed control diet, and in addition, synergistic effects were evident. For larvae fed the combination diet, the reductions in RGR, RCR, and ECD (compared with control larvae) were all significantly greater than the additive reductions from the xanthine and allopurinol diets tested separately (Figure 2). The AD of *S. frugiperda* larvae fed the combination diet was not altered significantly compared with that of control larvae.

TABLE 3. MEAN (± SEM) DURATION OF DEVELOPMENT, FINAL DRY MASS, AND % DRY MASS FOR LARVAE OF *S. frugiperda* AND *A. gemmatalis* FED CONTROL ARTIFICIAL DIET OR ONE CONTAINING EITHER XANTHINE (1% FM), ALLOPURINOL (0.5% FM), OR BOTH AT THESE CONCENTRATIONS[a]

Diet (*N*)	Duration (days)	Final dry mass (mg)	Dry mass (%)
S. frugiperda			
Control (15)	7.1 ± 0.1	44.9 ± 2.4	28.5 ± 0.4[b]
Xanthine (15)	6.8 ± 0.2	42.1 ± 1.7	26.3 ± 0.5*
Allopurinol (15)	7.7 ± 0.2*	38.0 ± 2.0*	26.0 ± 0.9*
Xanthine + allopurinol (12)[c]	10.7 ± 0.5*†	26.0 ± 2.1*†	24.7 ± 0.6*
A. gemmatalis			
Control (13)	6.5 ± 0.1	52.5 ± 2.4	22.2 ± 1.0[b]
Xanthine (14)	6.9 ± 0.3	47.1 ± 2.9	22.8 ± 0.4
Allopurinol (9)[d]	7.8 ± 0.4*	36.2 ± 1.4*[e]	20.5 ± 0.8
Xanthine + allopurinol (1)[f]			

[a] *N* = number of larvae surviving to the prepupal stage out of 15 initial larvae in each treatment. Asterisk indicates that a treatment mean was significantly different from the respective control mean, based on a two-tailed *t*-test, and † indicates that the effect of the two compounds combined in the same diet, compared with larvae fed the control diet, was significantly greater than that of the sum of the effects of each compound tested separately, based on a one-tailed *t* test ($P < 0.05$). See text for outcome of *G* tests.

[b] Because % dry mass was calculated for 1-day-old pupae and not all larvae reaching the prepupal stage pupated, these means are based on the following sample sizes: *N* = 15, 14, 13, 5 for *S. frugiperda* and 12, 10, 6, 0 for *A. gemmatalis*, respectively.

[c] This number includes six larvae that had not reached the prepupal stage by day 12 when the experiment was stopped.

[d] Data for two developmentally abnormal larvae fed this diet were omitted from the analysis.

[e] This mean is based on *N* = 8 because one pupa was lost during processing.

[f] Only one *A. gemmatalis* larva fed the combination diet did not die prior to the prepupal stage, precluding collection of meaningful data for this treatment. The one surviving insect remained in the larval stage on day 12 when the experiment was stopped.

For *A. gemmatalis*, survival on the xanthine diet was not reduced compared with that on the control diet and, although survival declined on the allopurinol diet, the reduction was not statistically significant ($G = 0.44$, $P > 0.25$; Table 3). Xanthine alone had no effect on any of the measures of larval performance, whereas allopurinol alone prolonged development and caused significant changes in all other performance measures (reductions in final dry mass, RGR, RCR, and ECD, and an increase in AD) except percentage dry mass (Table 3 and Figure 3). When allopurinol and xanthine were combined in the same diet, 14 of 15 *A. gemmatalis* larvae died prior to reaching the prepupal stage, preventing collection of performance data for insects in this treatment. The one remaining larva had not formed a prepupa by day 12 when the experiment was stopped.

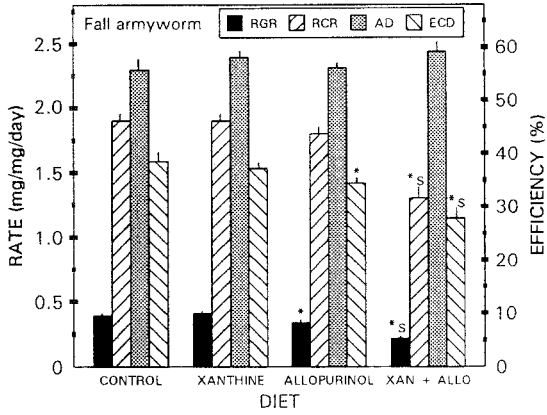

FIG. 2. Mean (+SEM) relative growth rate (RGR) and its three components [relative consumption rate (RCR), approximate digestibility (AD), and efficiency of conversion to biomass of digested food (ECD); see METHODS AND MATERIALS for formulae and Table 3 for N values] for larvae of the fall armyworm (*S. frugiperda*) fed a control artificial diet or diets containing xanthine (1.0% fm), allopurinol (0.5% fm), or the combination of these two compounds at the concentrations indicated. An asterisk indicates a significant difference from the control value based on a two-tailed t test and an "s" indicates a synergistic interaction in which the effect of the two compounds combined in the same diet was significantly greater than that of the sum of the effects of each compound tested separately, based on a one-tailed t test ($P < 0.05$).

DISCUSSION

The results of this study clearly demonstrate a synergistic interaction between xanthine and allopurinol for both *S. frugiperda* and *A. gemmatalis*, although the latter species appeared to be more sensitive in terms of greater mortality when fed the combination diet. The deleterious effects of these two compounds when combined in the diet generally were much greater than the additive influence when each compound was tested separately. This synergism is particularly dramatic in that, for both species, xanthine alone (up to 2% fm or 10% dm) had little or no effect on performance and allopurinol alone (at 0.5% fm or 2.5% dm) had only moderate (albeit statistically significant) deleterious effects. Several studies of plant-feeding insects have demonstrated synergism of deleterious allelochemical activity using inhibitors of the enzymes traditionally considered to detoxify xenobiotics, such as polysubstrate monooxygenases and hydrolases (esterases) (reviewed in Lindroth, 1991; Brattsten, 1992; and Wheeler et al., 1993). The present study expands the phenomenon of phytochemical detoxification by insects, as demonstrated by synergism through

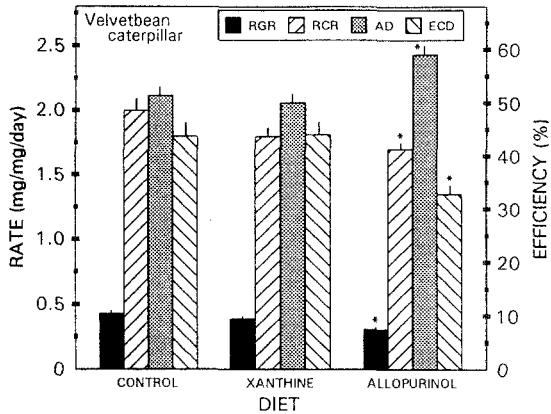

FIG. 3. Mean (+SEM) quantitative performance measures for larvae of the velvetbean caterpillar (*A. gemmatalis*) fed a control artificial diet or diets containing xanthine (1.0% fm) or allopurinol (0.5% fm); all but one larva died when fed diet containing the combination of these two compounds at the concentrations indicated. An asterisk indicates a significant difference from the control value based on a two-tailed *t* test (see Figure 2 for additional information).

use of an enzyme inhibitor, to include xanthine dehydrogenase, an enzyme generally not considered within the context of xenobiotic detoxification.

Xanthine alone does not appear to be a very potent defensive allelochemical against these caterpillars. In the experiments in which food consumption was measured, larvae of these two species ingested xanthine (dietary concentration = 1% fm) at a biomass-relative consumption rate (RCR_{xan}) as high as 82 mg (539 μmol) $(g \times d)^{-1}$, and likely at twice this rate on the 2% xanthine diet in the experiments in which ingestion was not measured, with little reduction in performance. In terms of comparative toxicity to *S. frugiperda*, xanthine is similar to other allelochemicals such as phytic acid, oxalic acid, atropine, and chlorogenic acid (unpublished data). Other phytochemicals, including flavone (Wheeler et al., 1993), rotenone (G.S. Wheeler, unpublished data), benzaldehyde, caffeine, indole 3-acetonitrile, indole 3-carbinol, and certain simple coumarins (unpublished data) are more toxic, causing significant reductions in growth and/or survival at dietary concentrations lower than 1% fm. Less information is available on the sensitivity of *A. gemmatalis* larvae to allelochemicals, but caffeine (Slansky and Wheeler, 1992), flavone, indole 3-acetonitrile, indole 3-carbinol, and certain simple coumarins (unpublished data) all reduce performance at dietary concentrations less than 1% fm.

One reason why increased concentrations of dietary xanthine have little effect on larval performance may be that a greater rate of absorption of xanthine

is accommodated by an increased rate of conversion to and excretion of uric acid. Enhanced uric acid excretion has been demonstrated in various insects when they were fed diets containing high levels of, or poor quality, protein, and diets lacking certain amino acids or the vitamin pyridoxine (Horie and Inokuchi, 1978; Horie and Watanabe, 1983a,b; Cochran, 1985). For example, when dietary protein (soybean meal) was increased from 20 to 60% dm, silkworms (*Bombyx mori* L.) absorbed about fourfold more nitrogen and excreted almost 18-fold more uric acid (Horie and Watanabe, 1983a; see also Kamioka et al., 1971), associated with induction of xanthine dehydrogenase activity (Ito and Mukaiyama, 1964). Similarly, larvae of *Manduca sexta* (L.) produced 2.5-fold more uric acid when fed a low water (65% fm) versus high water (82% fm) diet despite similar biomass-relative consumption rates of nitrogen (Van't Hof and Martin, 1989). In that study, caterpillar growth was apparently limited by reduced water intake on the low-water diet, resulting in an excess of absorbed nitrogen that required excretion.

If greater xanthine dehydrogenase activity and uric acid synthesis occurred in the present experiments, as is likely when larvae were fed increased concentrations of xanthine, then the lack of decline in ECD (efficiency of conversion to biomass of digested food) for either species fed diet supplemented with this purine suggests that there was no significant metabolic cost to these activities. This apparent lack of cost is supported by the results of studies on increased allelochemical processing by caterpillars, e.g., through greater detoxification enzyme activity (Neal, 1987; Appel and Martin, 1992). However, increased metabolic costs may not be reflected in a declining ECD when calculated based on dry mass (as used here); more appropriate measures include ECD based on energy values of insect, food, and frass and on direct measurement of metabolic rate (Slansky, 1985; Appel and Martin, 1992).

Addition of up to 1% fm (5% dm) of allopurinol to the standard diet consistently reduced the RGR (biomass-relative growth rate) and ECD of both species, suggesting that the presumed inhibition of xanthine dehydrogenase and consequent disruption of normal synthesis and excretion of uric acid exerted deleterious effects, although certain other enzymes also may have been inhibited (Glassman and Mitchell, 1959; Kelley and Beardmore, 1970; Jones et al., 1978; Chovnick et al., 1980). The reduction in percentage dry mass (i.e., increase in percentage of body water) for each species when fed diet containing allopurinol is consistent with this interpretation that normal excretion was disrupted, in that excretory activity and osmoregulation are interrelated (Cochran, 1985). Significant synergistic effects on the consumption and utilization of food (assessed only for *S. frugiperda* because only one *A. gemmatalis* larva fed the combination diet survived to the end of the experiment) included reductions in RCR, ECD, and RGR, associated with prolonged development and a decrease in final pupal mass; AD was not affected. It is evident that the deleterious effects of allopurinol

absorption were magnified when larvae consumed the combination diet, most likely because the dietary xanthine absorbed could not be converted to uric acid and excreted. Why *A. gemmatalis* appeared to be more sensitive than *S. frugiperda* to the dietary combination of xanthine and allopurinol, despite apparently similar ingested doses of these compounds, is unknown.

Xanthine (1.0% fm) and allopurinol (0.5% fm) were used in both the dosage and synergism experiments. Most of the results for these compounds were consistent between experiments, showing either similar statistically significant differences or at least similar trends. Some variation in the outcome of a treatment when tested with larvae from different generations, even from the same laboratory colony, is to be expected and is likely associated with intergenerational changes in the "quality" of the insects (unpublished data; see also Clancy, 1991).

Xanthine has been identified in certain plant species (see Introduction). Some of these are reported as food plants of the noctuid species studied here (Tietz, 1972), although there appear to be few published studies that have evaluated growth and survival of larvae of either species on such plants (e.g., Slansky, 1989). The results reported here suggest that this purine, by itself, in these plants would be basically innocuous, but that there could be substantial deleterious effects if it cooccurred with an inhibitor of xanthine dehydrogenase. To my knowledge, such an interaction involving a naturally occurring purine and an inhibitor of xanthine dehydrogenase has not been identified, although it is likely to occur, given the variety of modes of action already demonstrated for allelochemicals, e.g., digestive and detoxification enzyme inhibitors, nutrient absorption blockers, antivitamins, nutrient analogs, antihormones, and hormone analogs (Slansky, 1992). Studies on vertebrates indicate that xanthine dehydrogenase has a relatively broad range of substrates (Bergmann and Dikstein, 1956; Lohmann and Miech, 1976; Parks and Granger, 1986). Thus, this enzyme may be involved in the detoxification and excretion by insects of other allelochemicals in addition to xanthine, although further research is required to investigate this possibility.

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INHIBITORY EFFECT OF PARTHENIUM (*Parthenium hysterophorus* L.) RESIDUE ON GROWTH OF WATER HYACINTH (*Eichhornia crassipes* MART SOLMS.) I. EFFECT OF LEAF RESIDUE

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Abstract—The allelopathic effect of parthenium (*Parthenium hysterophorus* L.) leaf residue (dry leaf powder, DLP) on water hyacinth (*Eichhornia crassipes* Mart Solms.) was studied. The treatment caused wilting starting from the margins of the older leaves and desiccation of above-water plant parts (shoot). Appearance, persistence, and disappearance of symptoms depended on the level and duration of the treatment and recovery of the treated plants, if it occurred. The treatment drastically reduced the number of healthy leaves (HLN) and the plant biomass at 0.25% (w/v) DLP; the treated plants recovered in about one month. At and above 0.50% (w/v) DLP, the plants were killed in about one month, resulting in sinking of the dead mass in water. Physiological effects of the treatment included deterioration of membrane integrity, loss of dehydrogenase activity with concurrent drastic reduction or total failure of water absorption by the roots, and reduction of chlorophyll contents in the leaves. The results indicate that the inhibitors leached out of the DLP affected the water hyacinth plants through changes in macromolecules: protein, lipid, and nucleic acid, resulting in root dysfunction and other inhibitory activities both in the root and shoot. Phenolic and other inhibitors including those found in the parthenium plant (except sesquiterpene lactones which have not been tested) at 50 ppm, except *p*-hydroxybenzoic acid, did not affect the treated plants. Such a high concentration of the allelochemicals is unlikely to be present in the medium at the lethal dose (0.50% w/v) of the DLP. Even with *p*-hydroxybenzoic acid, the plants recovered subsequently and grew normally. Thus, it appears that other allelochemicals including sesquiterpene lactones were mainly responsible for the inhibitory activity of the DLP on water hyacinth plants.

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Key Words—Allelopathy, *Parthenium hysterophorus* leaf residue, *Eichhornia crassipes* growth inhibition, membrane integrity, dehydrogenase activity, chlorophyll, water absorption, phenolic acids, inhibitors.

INTRODUCTION

Parthenium (*Parthenium hysterophorus* L.) is an obnoxious, aggressive tropical weed of the Asteraceae, endemic to the Americas, and having found its way to Africa, Australia, and Asia (Towers et al., 1977). The weed has spread throughout India, posing serious threats to the environment. The inhibitors identified in parthenium plant parts include sesquiterpene lactones and phenolics (Towers et al., 1977; Picman, 1986; Kanchan and Jayachandra, 1979a,b). Biological activity of these inhibitors is well known (Picman, 1986; Kanchan and Jayachandra, 1980). Their range of biological activity affects various plants, some economically important, through allelopathy (Kanchan, 1975; Kanchan and Jayachandra, 1979a,b; Sharma et al., 1976; Mersie and Singh, 1987, 1988). The allelopathy is caused by germination and growth inhibitors that are released from aerial and underground parts of the plants and their residue into the soil environment (Kanchan and Jayachandra, 1979a,b). Since, the inhibitors are water soluble, they may find their way or get washed by rains into bodies of water. Information on the effect of parthenium plant residue or the inhibitors released from it into an aqueous environment on aquatic weeds such as water hyacinth (*Eichhornia crassipes*) seems to be rare in the literature. It remains to be determined whether such leaching could be of a magnitude sufficient to influence the population dynamics of the weed. The present study was undertaken to determine the effect of parthenium leaf residue on the growth of water hyacinth, to study associated physiological effects, and to pinpoint the class of compounds responsible for the inhibitory activity.

METHODS AND MATERIALS

Collection of Water Hyacinths. Water hyacinth (*Eichhornia crassipes* Mart Solms.) plants were collected from the natural habitat having a pH of 6.8–7.4, electrical conductivity (EC) of 2.53–2.55 mS/cm, osmotic potential of 0.828–0.936, bars, and water potential (Ψ_w) about 897×10^{-4} MPa. The plants were washed before use in the experiments.

Collection and Preparation of Parthenium Leaf Residue. Parthenium (*Parthenium hysterophorus* L.) plants were collected from a natural stand of the weed around the National Research Centre for Weed Science, Jabalpur (M.P.), India. The plants at the flowering stage were uprooted and washed quickly to remove dust and soil. The leaves were dried in the sun for two days and sub-

sequently at about 70°C for 48 hr in an oven. The dry matter was ground in a mixer–grinder to pass through an 80-mesh sieve. The dry leaf powder (DLP) was then stored in 400-gauge polyethylene bags at ambient temperature for use in the experiments.

Phytotoxicity of Parthenium Leaf Residue. An appropriate quantity of the DLP was dispersed in 20 liters of tap water (EC 0.745 mS/cm) in each of five plastic tubs to make 0.25, 0.50, 0.75, 1.00, and 1.25% (w/v) suspensions. The tap water was used in the experiments for two reasons: feasibility, for a large quantity was needed, and to see whether at any stage the plant constituents in the DLP serve as a source of nutrients when the level of the inhibitors in the DLP is insufficient to suppress growth of the water hyacinth. Twenty preweighed uniform plants each with four fully opened leaves were placed on the water in the tubs. The plants were allowed to grow under natural outdoor conditions during March–June 1992. The level of water in the tubs was kept constant by regularly replenishing the water lost due to evapotranspiration. Biomass and healthy leaf number (HLN) were monitored. A leaf that did not show wilting or drying of the margins or desiccation was considered to be a healthy leaf.

Analyses of the Medium. Twenty-four hours after suspending the DLP, the medium was stirred and samples were drawn, filtered through Whatman No. 1 filter paper disks and analyzed for pH using a digital pH meter and EC using a digital conductivity meter. Osmotic potential of the samples was calculated by multiplying the EC in mmho/cm by 0.36. The water potential (Ψ_w) was calculated using the osmotic potential. In simple systems at constant temperatures, the Ψ_w results from the combined but opposing actions of pressure (P), and osmotic (π) potentials, or $\Psi_P + \Psi_\pi = \Psi_w$; since by convention $P = 0$ at atmospheric pressure, $\Psi_w = \psi_\pi$ (Salisbury and Ross, 1984). Total phenolic acids were measured colorimetrically using the Folin Denis reagent method (Swain and Hillis, 1959). An optical density at 215 and 340 nm, corresponding to absorption maxima of parthenin (a major sesquiterpene lactone in the parthenium plant), was measured in the samples using tap water as a blank. The samples were tested for inhibitory activity using a wheat (*Triticum aestivum* L. var. Sujata) coleoptile growth bioassay. For this, 50 seeds were placed on top of Whatman No. 1 filter paper disks laid on 8 ml of the aqueous test solution in 9-cm-diameter Petri dishes and allowed to germinate at 30 ± 1°C for 48 hr. Thereafter, coleoptile lengths of the seedlings were measured. Seeds germinated in water served as a control.

Absorption of Water and Change in Fresh Weight Over a Brief Period. Preweighed plants were placed in a measured quantity of water or the aqueous media containing different levels of DLP and allowed to stand for 24 hr. Water was similarly kept for measuring evaporative loss from the free open surface. After the treatment, the plants were weighed and the volume of aqueous medium

was measured. The quantity of water absorbed by the plants was calculated by subtracting the evaporative loss.

Solute Leakage from Roots. The roots (about 1 g) were washed with distilled water, blotter-dried, weighed, and steeped in 100 ml distilled water at 30°C for 4 hr. Thereafter, the roots were removed, the steep water was filtered through Whatman No. 1 filter paper, and OD at 264 nm was measured. An OD of 0.01 was considered as one unit of UV-absorbing substance and was expressed on a per gram root (fresh weight) basis.

Dehydrogenase Activity in Roots. The method used for measuring the activity of dehydrogenase has been described previously (Pandey, 1989) and was modified to suit the experiments. The roots (about 0.5 g) were washed, blotter-dried, weighed, and soaked in 5 ml of 1% (w/v) 2,3,5-triphenyl tetrazolium chloride in distilled water in darkness at 30 ± 1°C for 4 hr. Thereafter, the roots were washed with distilled water, blotter-dried, and ground in 10 ml of ethanol with a mortar and pestle. The extract was placed in a water bath at 80°C for 20 min, cooled to ambient temperature, and filtered through Whatman No. 1 filter paper. The volume was made up to 10 ml and OD of the formazan extract was read at 520 nm. An enzyme unit was defined as 0.01 OD at 520 nm and was expressed on a per gram root (fresh weight) basis.

Determination of Chlorophyll a, b, *and Total Chlorophyll.* Fresh leaf disks (about 5 cm^2) were ground with a pinch of acid washed silica sand in 25 ml of 80% (v/v) acetone using a mortar and pestle. The extract was filtered through Whatman No. 1 filter paper. The volume was made up to 25 ml with filtered 80% (v/v) acetone and the OD at 645, 652, and 663 nm was measured. Chlorophyll *a*, *b*, and total chlorophyll were calculated considering equal area equal weight by the method of Arnon (1949).

Effect of Standard Inhibitors. Phenolics and other growth inhibitors, including all those major constituents reported in parthenium plant parts, viz., caffeic acid, ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, tannic acid, cinnamic acid, gallic acid, vanillic acid, anisic acid, fumaric acid, salicylic acid, and chlorogenic acid at 10, 25, and 50 ppm were, wherever necessary, dissolved in a small quantity of acetone or ethanol, then diluted with tap water or directly prepared in the tap water and preweighed water hyacinth plants were placed in the medium. The small quantity of acetone or ethanol used for dissolving the inhibitors did not affect the growth of water hyacinth. The biomass and HLN were monitored as in the DLP experiment.

The plants grown in tap water served as untreated controls in all comparisons. All experiments and determinations were repeated at least three times. The data were statistically analyzed for indices of significance (LSD) using completely randomized block design.

RESULTS

Analyses of the medium revealed that addition of DLP to water did not change the pH much to either side of neutral (range 6.9 $\pm$ 0.5). Electrical conductivity (Figure 1a) increased with an increase in the DLP. The method employed for calculation of Ψ_w was tentative, and comparison was made presuming that the contributions of nonelectrolytic solutes to the growing medium in nature and in the DLP were not much different. The Ψ_w of the medium (Figure 1b) decreased with an increase in the DLP. Total phenolic acids (Figure 1c) and OD at 215 nm (Figure 1d) and 340 nm (Figure 1e), probably indicating the presence of parthenin, increased with an increase in the DLP. Bioassay of

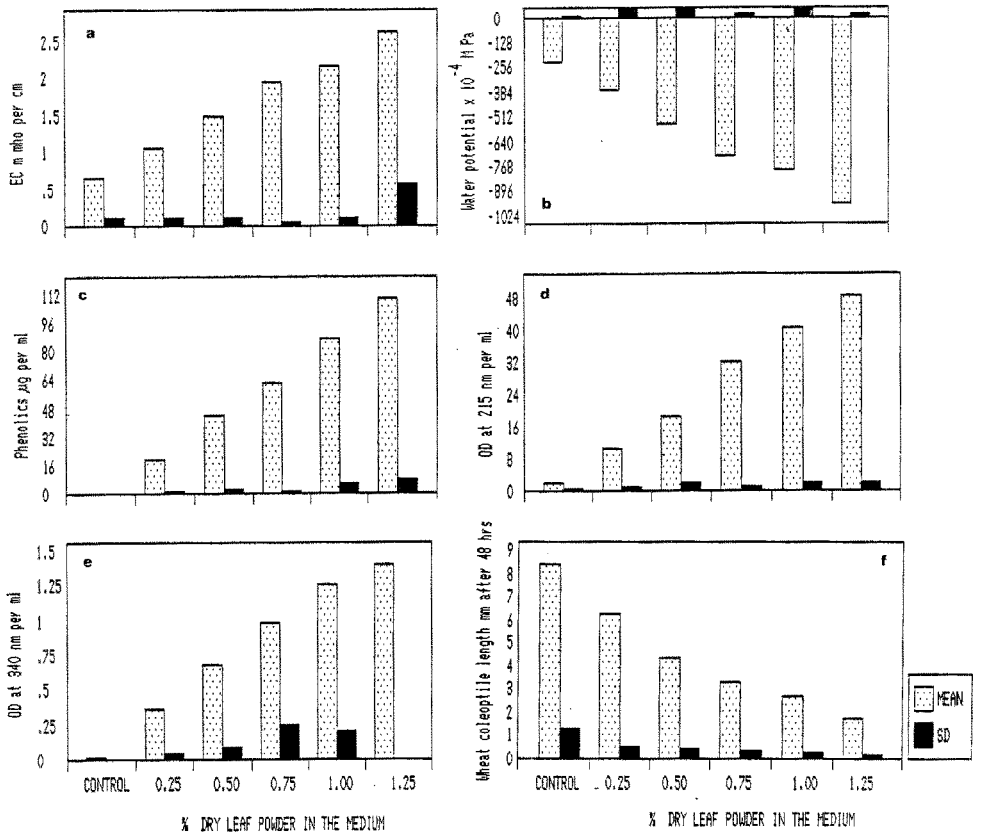

FIG. 1. Analyses of the medium 24 hr after suspending parthenium leaf residue (DLP) in water: (a) conductivity (EC), (b) water potential (Ψ_w), (c) phenolics, (d) OD at 215 nm, (e) OD at 340 nm, and (f) bioassay for inhibitors using wheat var. Sujata.

the medium using wheat coleoptile length as a parameter showed a consistent rise in inhibitory activity of the medium with an increase in the DLP (Figure 1f). It was clear that the DLP at 0.25–1.25% (w/v) did not result in unfavorable pH, salinity, or water stress, when compared with the values of these parameters in the natural growing medium of the water hyacinth. Thus, the inhibitory activity in the medium containing DLP could be attributed mainly to the phenolic acids and sesquiterpene lactones.

In the control, the biomass (Figure 2a) of the water hyacinth plants increased sharply with time. At 0.25% (w/v) DLP, compared to original biomass, it declined in the beginning but recovered subsequently to show an increase over the original level. At and above 0.50% (w/v) DLP, the treatments resulted in consistent loss of biomass, and the plants were unable to recover. The roots became flaccid and further growth of new roots was completely checked in contrast to the DLP treatment at 0.25% (w/v) in which sustained growth of roots was conspicuous after five days. The plants died in about a month and sank in the water. The number of healthy leaves (HLN) (Figure 2b) followed almost the same trend. In the control, the HLN increased dramatically. This was due to new leaf growth. At 0.25% (w/v) DLP, there was a rapid recovery in the HLN after 25 days, showing an appreciable increase in new growth after 30 days. At higher levels, the more the DLP in the medium, the quicker was

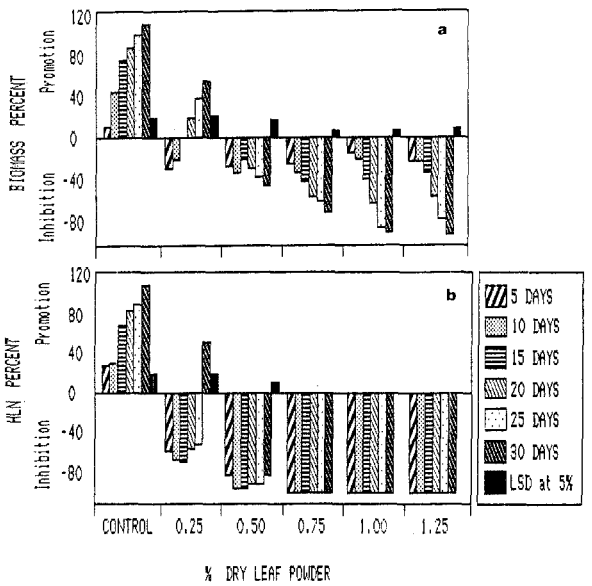

FIG. 2. Effect of parthenium leaf residue (DLP) on biomass (a) and number of healthy leaves (HLN) (b) of water hyacinth.

the wilting, desiccation, and eventual death and resultant sinking of the leaves and plants in the medium. The primary symptoms appearing on the leaves were wilting and drying from the margins. The older leaves usually showed the symptoms before the younger ones. Leaves of healthy plants submerged in the medium at all levels of DLP retained a lush green color and turgidity, except at certain places where dull green patches developed before initiation of rotting. The plants submerged in the medium did not show net reduction in biomass.

Water absorption over a short period (24 hr) of treatment (Figure 3a) was maximum in the control plants. It declined to about half at 0.25% (w/v) and to about one sixth or lower at and above 0.50% (w/v) DLP. Thus, loss of water absorption and initiation of wilting and drying of leaf margins were among the earliest visible symptoms of the treatment. At and above 0.75% (w/v) DLP, there was no further reduction in water absorption. Fresh weight of the plants over original weight (Figure 3b) showed an increase in control, probably to a large extent due to excessive absorption and the relatively lower rate of transpiration. Meanwhile, the weight of plants at 0.25% (w/v) DLP did not show any change due, probably, to water absorption and loss in transpiration being equal. At and above 0.50% (w/v) DLP, there was a drastic loss of fresh weight over the original weight with no obvious effect of the higher levels of DLP in the medium.

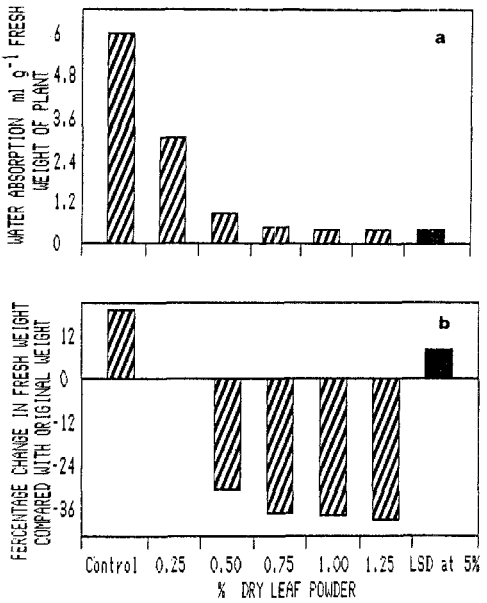

FIG. 3. Effect of parthenium leaf residue (DLP) on water absorption (a) and fresh weight (b) of water hyacinth over 24 hr.

Leakage of solutes (Figure 4a), as evidenced by OD at 264 nm (corresponding to nucleotides, amino acids, polypetides, etc.), showed an increase, both with the levels of DLP in the medium and with the duration of the treatment. Solute leakage from the roots due to DLP treatment for 24 hr or more was either doubled or increased still more.

Dehydrogenase activity in the roots (Figure 4b) was drastically reduced by the treatment in about 4 hr at 0.25% (w/v) DLP. There was little further decline

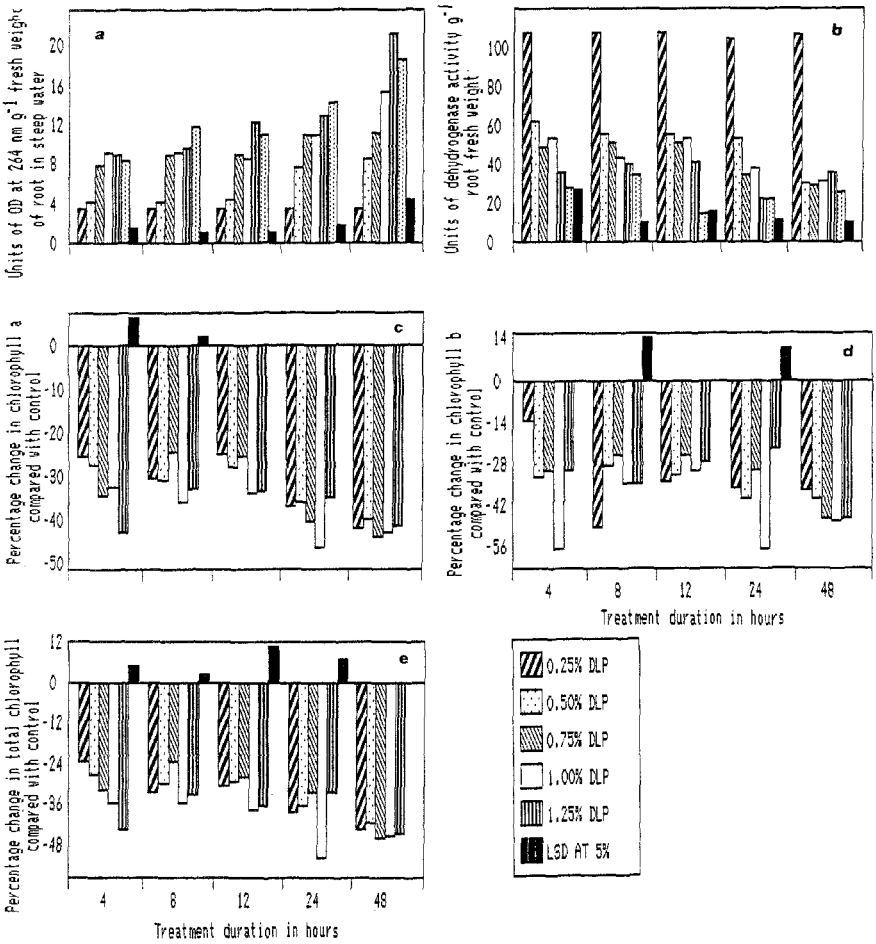

FIG. 4. Effect of parthenium leaf residue (DLP) on solute leakage (OD at 264 nm) from roots (a) and dehydrogenase activity in roots, (b) and change in chlorophyll *a* (c), chlorophyll *b* (d), and total chlorophyll (e) in the leaves of water hyacinth.

in the activity of the enzyme either at higher levels of DLP or with the duration of treatment.

The DLP treatment considerably reduced chlorophyll *a*, *b*, and total chlorophyll compared with the control (Figure 4c–e).

The maximum level of phenolic acids in the medium at 0.50% (w/v) DLP was below 50 ppm. At this concentration of DLP, the water hyacinth plants were killed. However, individual standards of allelochemicals, including those found in the parthenium plant (except sesquiterpene lactones, which have not been tested) at 50 ppm, did not affect the treated plants except for *p*-hydroxybenzoic acid (Table 1). Although *p*-hydroxybenzoic acid at 50 ppm reduced growth of the treated plants, the plants recovered and grew normally. Such a high concentration of allelochemicals is unlikely to be present in the medium at the lethal dose (0.50%, w/v) of the DLP.

DISCUSSION

The pH, EC, and Ψ_w of the medium at different levels of DLP did not differ much from the range of values of these parameters in the natural habitat of the water hyacinth plants. The increase in inhibitory activity with DLP was evident from the results of the bioassay for inhibitory activity and was due to

TABLE 1. EFFECT OF DIFFERENT PHENOLIC AND OTHER INHIBITORS AT 50 PPM ON BIOMASS AND HEALTHY LEAF NUMBER (HLN) OF WATER HYACINTH PLANTS[a]

Treatments	Biomass after (days)		HLN after (days)	
	5	10	5	10
Control	47.1	118.7	75.6	95.0
Caffeic acid	41.3	81.4	87.6	94.3
Coumaric acid	50.4	107.5	91.0	129.6
Ferulic acid	57.0	103.7	62.0	106.3
Tannic acid	55.2	101.7	49.6	105.0
Cinnamic acid	44.8	98.1	78.6	117.6
Gallic acid	49.5	106.1	70.0	100.0
Vanillic acid	52.9	115.3	48.6	94.3
p-hydroxybenzoic acid	−15.3	−33.5	−100.0	−51.6
Fumaric acid	57.1	110.1	60.6	144.0
Salicylic acid	53.9	119.9	68.6	94.3
Chlorogenic acid	46.1	120.0	87.6	125.3
Anisic acid	42.5	64.5	48.6	117.3
LSD at 5% (df 38)	17.68	39.42	48.10	31.26

[a]Data are expressed as percent change over the original values.

the rise in phenolic acids and probably in the sesquiterpene lactones, as these substances are the dominant class of inhibitors in the residue of the parthenium leaf (Kanchan and Jayachandra, 1980; Picman, 1986).

Biomass of the water hyacinth plants in the control doubled in about one month. This was due to new growth accompanied by doubling of the number of leaves. The inhibitors in the leaf residue at 0.25% (w/v) initially reduced the biomass and caused wilting of more than half of the total leaves, probably due to both sustained transpiration and reduced water absorption by the roots. Subsequently, the plants recovered, showing an increase in the biomass and new leaf growth. Higher doses of DLP (0.50%, w/v, or more) consistently reduced both the biomass and HLN with no recovery, killing the treated plants due to the effects of phytotoxins, including those causing failure of roots to keep sufficient absorption of water and sustained evapotranspiration through the aerial plant parts.

An immediate effect of the DLP treatment appears to be a drastic effect on water absorption by the roots. This, together with a sustained high rate of transpiration from the aerial parts of the plants and the inhibitory activities of the allelochemicals, stalled an increase in biomass at 0.25% (w/v) DLP. This caused wilting of leaves to varying degrees depending on the DLP concentration and treatment durations. Interestingly, drenching and spraying of water hyacinth plants, except the root portion, with 48-hr soak leachates of DLP at 0.25–25% (w/v) did not affect the water hyacinth plants in any manner (unpublished data). Thus, roots appear to be one of the primary sites of action of the inhibitors.

Increased solute leakage with the treatment showed that membranes of the root cells were deteriorated by the inhibitors leaking out of the DLP. The dehydrogenase activity in the roots was reduced to about half or more, depending somewhat on the level of DLP and the duration of the treatment. Similarly, chlorophyll *a*, *b*, and total chlorophyll contents in the leaves of treated plants decreased considerably when compared with untreated controls. Membranes play important roles in living systems and are essential for the maintenance of structure and function. Apart from serving as a permeability barrier, they play vital roles in compartmentalization of cellular components. Moreover, cooperative enzymes of a metabolic pathway may be linked together in association with, or integrated into, membrane structures. Thus, in the present case, the contribution of the membrane integrity deterioration caused by the inhibitors leached out of the DLP into the medium in inhibition of growth of water hyacinth plants is obvious. Similarly, the dehydrogenase activity loss may indicate loss of respiration (Mackay, 1972). Deterioration of membrane integrity, loss of dehydrogenase activity in roots, and reduced chlorophyll content in the leaves show that the inhibitors may have acted through affecting the macromolecules—proteins, lipids and nucleic acids.

The allelochemicals, other than phenolic and organic acids (Table 1),

including the sesquiterpene lactones, appear to be contributing more substantially to the inhibitory activity of the leaf residue. This is clear from the fact that individual phenolic and organic acids, at a higher concentration than the total phenolic acid contents detected in the aqueous growth medium or of the organic acid (fumaric acid) at a higher concentration than could be envisaged in the medium containing 0.50% (w/v) DLP, failed to affect the growth of water hyacinth plants. Only *p*-hydroxybenzoic acid affected the growth (in terms of decline in biomass and HLN) of the treated plants, causing wilting and growth inhibition initially; subsequently the plants recovered and grew normally (unpublished data).

Under natural conditions, buildup of enough residue or an equivalent level of inhibitors as is released into the aqueous medium at 0.50% (w/v) DLP may be required to inhibit growth of water hyacinth plants. Considering autotoxicity of parthenium and its possible role in control of its own population (Picman and Picman, 1984), building up of such a high concentration of residue or inhibitors equivalent to those available at 0.50% (w/v) DLP appears to be a remote possibility. Further, the inhibitory activity of the DLP in an aqueous medium showed a decline with time that was apparent after a period of a month when DLP at 0.25% (w/v) was unable to show any inhibitory activity on water hyacinth plants. By contrast, it promoted growth of the treated plants when compared with untreated controls in tap water. Thus, DLP acted as a source of nutrients (unpublished data). This further strengthens the unlikelihood of such a possibility in water bodies where the weed grows naturally.

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INHIBITORY EFFECT OF PARTHENIUM (*Parthenium hysterophorus* L.) RESIDUE ON GROWTH OF WATER HYACINTH (*Eichhornia crassipes* MART SOLMS.) II. RELATIVE EFFECT OF FLOWER, LEAF, STEM, AND ROOT RESIDUE

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Abstract—The relative effect of residue of leaf, flower, stem, and root of parthenium (*Parthenium hysterophorus* L.) on growth of water hyacinth was studied. The inhibitory activity of the residue as shown by its effect on biomass and healthy leaf number (HLN) of treated plants was in the order: leaf and flower > stem > root. Total phenolic acids in the medium after 72 hr of suspending the plant part residue were maximum in flower followed by leaf, root, and stem, successively. The dry leaf powder (DLP) and dry flower powder (DFP) at and above 0.50% (w/v) and dry stem powder (DSP) at 1.00% (w/v) killed water hyacinth in about one month. Dry root powder (DRP) at the highest dose (1.25% w/v) reduced the growth of the treated plants drastically, but the plants recovered after about one month. The DSP at 0.50% (w/v) and DRP at 0.25-0.75% (w/v) supported growth of treated plants, probably due to lower levels of inhibitors, allowing utilization of constituents of the residue as nutrients. Using wheat seedlings as a reference material, it was observed that in aquaculture at different levels of parthenium plant parts residue, water hyacinth plants were much more sensitive to inhibitory activity. Thus, water hyacinth is suggested as a material for bioassay of inhibitory activity of the parthenium plant residue.

Key Words—*Parthenium hysterophorus*, *Eichhornia crassipes*, *Triticum aestivum*, biomass, inhibitory activity.

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INTRODUCTION

Parthenium (*Parthenium hysterophorus* L.) is an annual Asteraceae weed endemic to the Americas, now spread to Africa, Australia, and Asia (Towers et al., 1977). Sesquiterpene lactones and phenolics form the main water-soluble inhibitory constituents in the plant parts (Kanchan and Jayachandra, 1980). Leaves and inflorescence have been reported to contain the maximum quantity of parthenin, followed by the stem and roots, successively, while the total phenolics were maximum in leaf followed by inflorescence, roots, and stem, successively (Kanchan and Jayachandra, 1980). A previous study (Pandey et al., 1993) showed that residue of parthenium leaf powder either inhibited growth (at lower doses) or killed the plants (at higher doses) of water hyacinth, resulting in clearing the water surface. While it implied that sesquiterpene lactones to a larger extent were responsible for the inhibitory activity, the relative inhibitory activity of residue of different parts of parthenium plant remains to be studied. The present study was conducted to determine the relative inhibitory activity of the residue of different parts of the parthenium plant on water hyacinth and to compare its relative sensitivity to wheat seedlings.

METHODS AND MATERIALS

Plant Material. Water hyacinth plants were collected from the natural habitat as has been described earlier (Pandey et al., 1993).

Parthenium Plant Part Residue. Parthenium leaves, flowers, stem, and roots were collected from plants growing near the National Research Centre for Weed Science, Jabalpur (M.P.). Each of the plant parts was pooled separately, washed quickly, blotter-dried, and, after initial drying in the sun for two days, further dried at 70°C for 48 hr in an oven. The dry matter was ground in a mixer–grinder to pass through an 80-mesh sieve. Then, the dry leaf (DLP), flower (DFP), stem (DSP), and root (DRP) powders were stored in 400-gauge polyethylene bags at ambient temperature for use in the experiments.

Effect of Residue of Plant Parts on Biomass and Leaves. Appropriate quantities of DLP, DFP, DSP, and DRP, were each dispersed in 20 liters of tap water in plastic tubs to make 0.25%, 0.5%, 0.75%, 1.00%, and 1.25% (w/v) solutions, following the method described earlier (Pandey et al., 1993). The tap water was used for two reasons: (1) feasibility—a large quantity was required and (2) to see whether at any stage constituents serve as a source of nutrients when the level of inhibitors in the residue of the plant parts is insufficient to suppress growth of water hyacinth plants, although distilled or deionized water would serve better than tap water to determine this. Twenty preweighed plants, each with four uniformly fully opened leaves, were placed on the water in the tubs. The plants were allowed to grow under natural outdoor conditions during

March–June 1992. The volume of water in the tubs was kept constant by regularly replenishing the water lost due to evapotranspiration. The biomass and healthy leaf number (HLN) were monitored throughout the period of the experiment up to 90 days. A leaf not showing wilting or drying of the margins or desiccation was considered to be healthy.

Analysis of Phenolic Acids in Medium. Samples of the medium were drawn 72 hr after suspending the plant residue in water, filtered through Whatman No. 1 filter paper, and the phenolic acids were determined using the method of Swain and Hillis (1959).

Inhibitory Activity of Medium to Wheat Seedlings. Wheat (*Triticum aestivum* L. var. Sujata) seeds were germinated on filter paper, following the method of the International Seed Testing Association (Anonymous, 1985), for 48 hr. The seedlings (20) with 2-cm radicles were placed on a perforated aluminum foil cover with the radicle protruding down into 100 ml of a selected medium or water (control) in 100-ml beakers. The seedlings were allowed to grow for 10 days. Subsequently, the seedlings were removed, washed with water, and seminal root length and fresh weight of root and shoot were measured on all seedlings in three replications.

All experiments were repeated three times. The data were subjected to analysis of variance and differences of means were estimated by calculating least significant differences.

RESULTS AND DISCUSSION

The effects of parthenium plant parts on water hyacinth biomass and HLN are shown in Figures 1–4. The DLP at 0.25% (w/v) reduced both biomass and HLN initially (Figure 1a and b). The plants treated with DLP showed conspicuous wilting symptoms in about 6–8 hr. Wilting appeared along the leaf margins of older leaves first, then spread to the younger ones. Wilting was followed by drying and development of necrotic regions in the leaf, if it failed to recover due to a high dose of the residue. However, subsequently the plants started recovering from the effects of inhibitors in the medium, showing a considerable increase after 30 days while recovery in HLN was dramatic after 30 days. At and above 0.50% (w/v) DLP biomass and HLN both declined dramatically over original values, and finally the plants died. The dead plants sank in the water, clearing the surface. The DFP at 0.25% (w/v) (Figure 2a and b) reduced the biomass and HLN as did the DLP. The recovery in biomass was immediate, while recovery of HLN soon followed. Subsequently, the plants recovered after about one month and grew normally. At and above 0.50% (w/v) DFP, HLN and biomass loss over original values was consistent with the treatment. Finally, the plants died in about one month or shortly after. The DSP up to 0.50% (w/v)

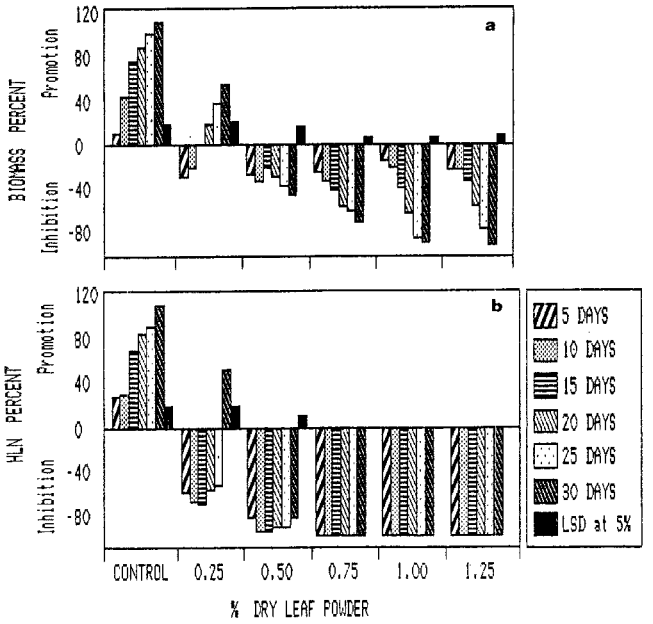

FIG. 1. Effect of parthenium leaf residue on biomass (a) and number of healthy leaves (HLN) (b) of water hyacinth.

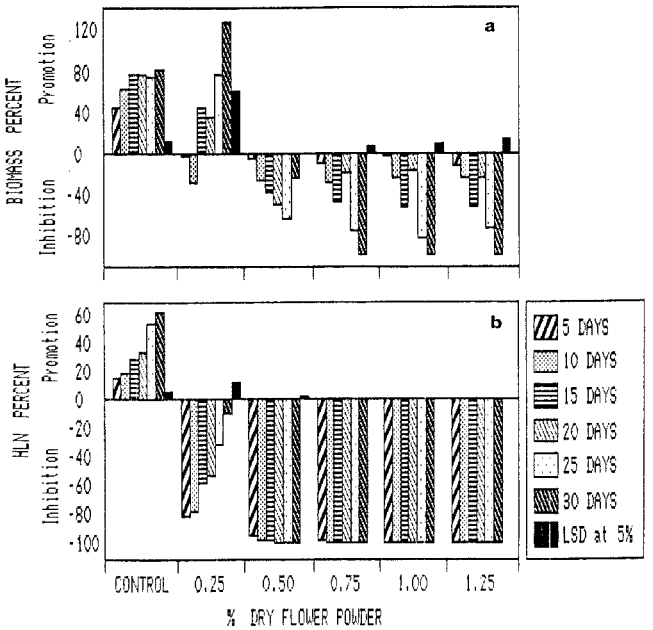

FIG. 2. Effect of parthenium flower residue on biomass (a) and number of healthy leaves (HLN) (b) of water hyacinth plants.

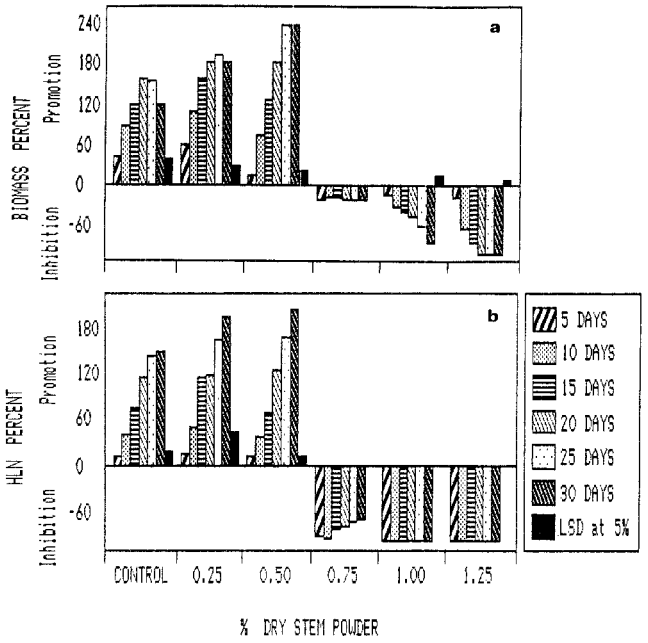

FIG. 3. Effect of parthenium stem residue on biomass (a) and number of healthy leaves (HLN) (b) of water hyacinth plants.

showed little increase in both biomass and HLN (Figure 3a and b). However, at 0.75% (w/v) the DSP reduced both biomass and HLN, and the plants started recovering in about 40 days. At and above 1.00% (w/v), the DLP reduced both biomass and HLN and the plants died. The DRP increased biomass and HLN up to 1.00% (w/v). At 0.25%, 0.50%, and 0.75% (w/v) DRP, the biomass and HLN both showed much higher values than the control. At 1.25% (w/v), it reduced both biomass and HLN up to 30 days. Subsequently, the plants recovered and grew normally.

Analysis of phenolic acids in the medium showed that total phenolic acids at 1.25% (w/v) residue were maximum in the medium containing DFP (222.5 ppm) followed by DLP (178 ppm), DRP (103 ppm), and DSP (72.0 ppm). This yield percent of residue was maximum at 1.78 $\pm$ 0.07 in DFP, followed by 1.425 $\pm$ 0.125 in DLP, 0.823 $\pm$ 0.025 in DRP, and 0.576 $\pm$ 0.020 in DSP.

The effects of parthenium plant part residues on wheat seedlings are shown in Figures 5 and 6. Seminal root length (Figure 5a) of wheat seedlings was substantially retarded by residue of all parts of the parthenium plant, more so at higher levels of DFP, DLP, DRP, and DSP. Fresh weight of root (Figure 5b) showed a decline at higher doses, especially at 1.25% (w/v) concentration.

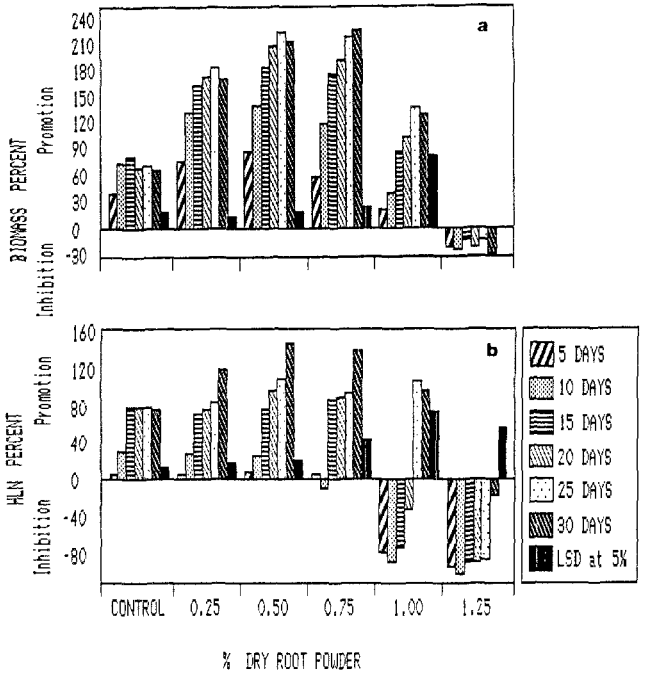

FIG. 4. Effect of parthenium root residue on biomass (a) and number of healthy leaves (HLN) (b) of water hyacinth plants.

The maximum inhibition of root length was at a 1.25% (w/v) concentration of DFP, DLP, and DRP. The maximum inhibition was caused by DLP. At 0.50% (w/v) DRP, the root fresh weight showed a marked increase over control. Shoot fresh weight (Figure 6), although decreased at 0.75–1.25% (w/v) DFP, 1.00–1.25% (w/v) DLP, and 1.25% (w/v) DRP, showed an increase over control at 0.25–0.50% (w/v) DLP and DRP. The results showed that the DLP and DFP at 0.50% (w/v) and DSP at 0.75% (w/v) killed water hyacinth plants, whereas the wheat seedlings survived and grew, although at higher doses of the residue marked reduction in growth over control was apparent.

The effectiveness of parthenium plant part residue in inhibiting growth of water hyacinth as inferred from the results of its effects on HLN and biomass was in the order: flower and leaf > stem > root. Total phenolic acid contents in the medium were maximum 72 hr after placing the residue in the water. The phenolic acid contents were maximum in flower followed by leaf, root, and stem, successively. This corresponds to the quantity of the major sesquiterpene lactone parthenin in the plant parts, which is known for its potent inhibitory activity (Kanchan and Jayachandra, 1980; Picman, 1986). Phenolic acids, ses-

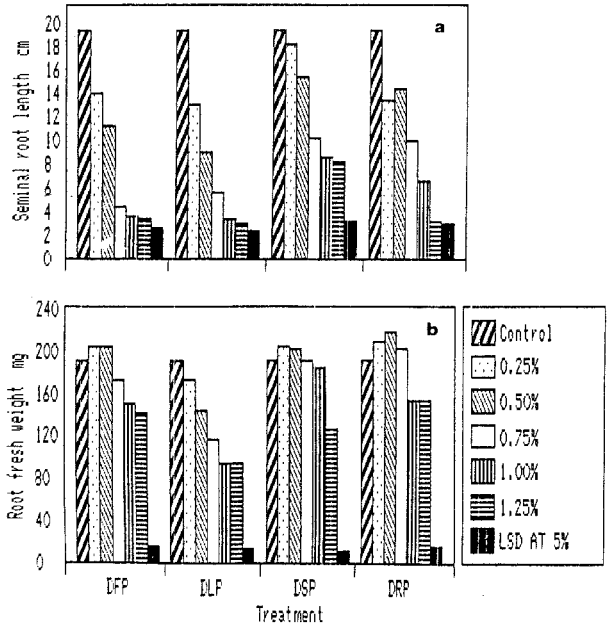

FIG. 5. Effect of parthenium plant part residue on seminal root length (a) and fresh weight (b) of wheat var. Sujata seedlings after 10 days of growth in an aquaculture test for inhibitory activity.

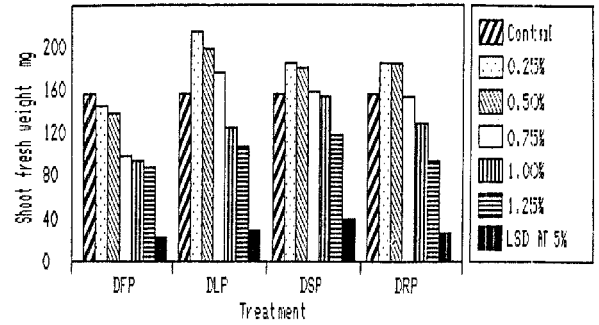

FIG. 6. Effect of parthenium plant part residue on shoot fresh weight of wheat var. Sujata seedlings after 10 days of growth in an aquaculture test for inhibitory activity.

quiterpene lactones, and other inhibitors have been identified in different parts of parthenium plants (Kanchan and Jayachandra, 1980). Quantities of these inhibitors, except the sesquiterpene lactone parthenin, at higher concentrations than could be expected (as calculated from the reported results of Kanchan and

Jayachandra, 1980) in DFP, DLP, DSP, and DRP levels effectively inhibiting growth or resulting in death, were unable to cause such inhibition or death in the treated plants (Pandey et al., 1993). This suggests that sesquiterpene lactones (probably mainly parthenin) are primarily responsible for the inhibitory activity of the parthenium plant part residue in causing growth inhibition and death in water hyacinth. The probable mechanism underlying the inhibitory activity has been discussed earlier (Pandey et al., 1993).

The increase in growth due to the treatments as in DSP at 0.25% and 0.50% (w/v) and in DRP at 0.25–0.75% (w/v) was probably the result of an inadequate level of inhibitors to suppress growth of the treated plants. The plants may use available nutrients in the residue of parthenium plant and/or may be stimulated to growth by allelochemicals present below the inhibitory concentration. Experiments (data not presented) have shown that when water hyacinth plants were placed in the medium containing DLP 0.25% (w/v) and kept for a month, the water hyacinth plants grew better than the controls. This suggested that the inhibitory activity of the residue declined with time.

In water culture, wheat seedlings were able to grow at all levels of DFP, DLP, DSP, and DRP and showed little reduction in growth at the level of residue at which water hyacinth plants were killed. Wheat seedlings were relatively much less sensitive to the inhibitory activity of the parthenium residue, as the wheat plants survived even at 1.25% (w/v) DFP and DLP, although growth inhibition was apparent. Due to its being more sensitive to the inhibitory activity of the parthenium plant residue, water hyacinth could be used as a bioassay material for inhibitory activity or perhaps parthenium could be used in biological control of water hyacinth.

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HOST ODOR AND VISUAL STIMULUS INTERACTION DURING INTRATREE HOST FINDING BEHAVIOR OF *Rhagoletis pomonella* FLIES

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Abstract—Responses of *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae) flies to host fruit visual stimuli (apples or models of apples) and chemical stimuli (synthetic apple volatile blend) were studied in semidwarf field-caged apple trees. Three different fruit or model densities (1, 4, or 16 fruit or models/tree) and two odor release rates [ca. 0.7 μg/hr (close to the natural release rate of a ripe apple) and ca. 500 μg/hr (amount of odor released by commercially sold apple maggot traps)] were tested. Individually released flies were followed as they moved within a tree for a maximum of 20 min. We recorded three-dimensional search paths followed by foraging flies and computed such variables as total relative distance traveled before alighting on a fruit or model, track length between individual alightment sites, and directness of flight to fruits or models. Effect of odor on propensity to alight on fruit or models and host-searching behavior prior to alighting on fruit or on models varied according to fruit or model color and density. If the fruit visual stimulus was strong (e.g., red color), odor did not increase the probability of finding fruit or fruit models. As the visual stimulus became progressively weaker (red to green to clear), odor (irrespective of concentration) appeared to aid flies during the fruit-finding process. As density of fruit or models increased, the probability of flies finding a fruit or model also increased (e.g., 50% of flies found a red fruit model at 1 model/tree while 90% found a red model at 16 models/tree; 4% of flies found a clear model with odor at 1 model/tree while 35% found a clear model with odor at 16 models/tree). Findings reported elsewhere indicate that *R. pomonella* flies are able to discover a point source of odor (an odor-bearing tree in a patch of trees) by flying upwind (in the tree patch) in response to intermittent exposure to odor. Findings here indicate that after arrival on a host tree (point source), flies discover individual apparent and

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abundant host fruit on the basis of vision. If fruit are less apparent or scarce, odor appears to interact with vision during the fruit-finding process.

Key Words—*Rhagoletis pomonella*, Diptera, Tephritidae, vision, olfaction, fruit volatiles, behavior, movement patterns.

INTRODUCTION

Recent studies have emphasized the importance of stimulus interaction during the process of host finding and host acceptance by insects (e.g., Robert, 1986; Torr, 1988, 1989; Judd, 1986; Todd et al., 1990; Brady et al., 1990; Harris and Miller, 1991; Gibson et al., 1991). Even though particular stimuli may play a singular role during certain stages of the host-finding process (e.g., short- and long-range orientation), it has become increasingly apparent that many insects respond to an array of host stimuli (or a "gestalt") during host-seeking bouts (Kogan, 1977; Prokopy, 1986). Harris and Miller (1983), in a series of classical experiments with the onion fly, *Delia antiqua* (Meigen), clearly demonstrated that host acceptance (oviposition) was contingent upon the insect being offered the appropriate combination of structural, visual, and chemical host characteristics. Even though individual onion flies responded positively to a single stimulus, combining these three sorts of stimuli had a pronounced synergistic (rather than additive) effect on oviposition. Judd (1986), working with the same insect, concluded that host-plant odor conditions mated females to respond to a particular visual stimulus. Saxena and Saxena (1975) demonstrated that *Empoasca devastans* (L.) responds to an amalgam of stimuli that includes humidity, host-plant color, and host-plant odor. Green (1986), working with *Glossina pallidipes* (Austen) and *G. morsitans morsitans* (Westwood), reported that the addition of odor (carbon dioxide and acetone) caused a fourfold increase in colored trap catches. When searching for a host tree, scolytid beetles respond to an array of stimuli, including tree silhouette, tree odor (acting as a possible arrestant), and pheromones released by conspecifics (Borden et al., 1986; Payne, 1986).

Research on the chemical and visual ecology of *Rhagoletis pomonella* (Walsh) flies (apple maggot flies) has thus far focused on understanding the role that individual stimuli (e.g., host-fruit volatiles and host-tree and host-fruit visual stimuli) play during host orientation behavior and on the development of traps that effectively mimic these stimuli.

After Prokopy et al. (1973) and Reissig (1974) provided evidence that *R. pomonella* flies respond to apple odor in the field, Fein et al. (1982) identified several volatiles emitted by stored Red Delicious and Red Astrachan apples and showed that a blend of six esters elicited positive behavioral responses in olfactometer and wind-tunnel studies. Averill et al. (1988) concluded that *R. pomonella* has a high degree of olfactory specificity and that maximum behavioral

response is contingent upon the following rules regarding size and structure of molecules. The ester must be a straight chain, be 10–11 carbons in length, and have an acid portion of six to eight carbons and an alcohol portion of three to five carbons.

R. pomonella flies are highly responsive to appropriate visual stimuli. Spectral response curves reveal that the visual sensitivity of this insect ranges from ultraviolet (350 nm) to red (650 nm), peaking at 400–530 nm (blue-green to yellow) (Agee, 1985). Tree visual stimuli consist of green leaf color, silhouette of tree against background, and tree size and shape (Moericke et al., 1975). Although leaf shape is not important, leaf size and especially leaf color are important stimuli used by *R. pomonella* during intratree foraging (Prokopy and Owens, 1978, 1983; Owens and Prokopy, 1984; Owens, 1982). Individual fruit are thought to be detected at close range (up to 1 m) on the basis of fruit visual properties (shape, size, and color contrast against background) (Prokopy, 1968, 1977; Roitberg, 1985; Owens and Prokopy, 1986).

These combined findings on *R. pomonella* responses to host chemical and visual stimuli have led to the development of highly effective traps. After pioneering work by Oatman (1964), Prokopy (1968, 1973, 1975) developed a red sphere possessing supernormal fruit-mimicking characteristics. Reissig et al. (1982) attached a vial filled with the aforementioned blend of six esters to a red sphere. Such odor-baited red spheres captured significantly more apple maggot flies in the field compared to unbaited spheres (Reissig et al., 1982, 1985). Unbaited or odor-baited red spheres are currently used by commercial apple growers in many parts of North America for monitoring apple maggot fly abundance (Agnello et al., 1990) or for directly controlling flies (Prokopy et al., 1990; Prokopy, 1991).

Here, we present results of studies aimed at improving our understanding of the interplay of host-fruit visual and chemical stimuli during intratree fruit searching behavior of *R. pomonella* flies under seminatural conditions in field cages. Our objective was to study fly response to an array of host visual and chemical stimuli presented singly or in combination (e.g., red fruit model with and without host odor). Using a method developed by Aluja et al. (1989), we aimed to record three-dimensional search paths followed by flies while foraging for fruit or fruit models hung at varying densities in a host tree. We hoped that this approach would allow us to unravel behavioral mechanisms operative during *R. pomonella* intratree host searching behavior during periods of response to unbaited vs. baited sphere traps.

METHODS AND MATERIALS

Olfactory Stimuli. We used the same synthetic blend of six esters employed by Fein et al. (1982). The blend consisted of the following components: hexyl acetate (36%), butyl 2-methylbutyrate (7%), propyl hexanoate (12%), hexyl

propionate (5%), butyl hexanoate (29%), and hexyl butyrate (11%) [obtained in pure form (>99.5%) from Penta International, West Caldwell, New Jersey]. Two odor release rates were tested: 0.7 μg/hr (equivalent to one apple; Anne Averill, personal communication) and ca. 500 μg/hr [equivalent to ca. 700 apples; rate released by a commercial source of lure (Reissig et al., 1984; Prokopy et al., 1990)].

To release synthetic apple volatiles at the 0.7 μg/hr rate, we used Teflon fibers of 0.012 mm ID (TRE Tubing, Cat. No. R-6417-11, Cole-Parmer Instrument Co., Chicago, Illinois). Rate of release from a capillary is given by: rate $= K/L$, where K is a constant and L is the length of the vapor–air column above the liquid. Because the desired release rate of the ester blend was known, the required L was calculated as follows: $L = K/\text{rate}$. $K = -McD\ r^2 \ln (1 - P_{vap}/p) = McD\ r^2\ P_{vap}/p$, where M is molecular weight (nanograms), c is molar density expressed in moles per cubic centimeter (the same for all compounds), D is diffusion coefficient (centimeters square per second), r is the radius of the capillary (cm), P_{vap} is vapor pressure (millimeters of mercury), and p is atmospheric pressure (760 mm Hg). (Weatherston et al., 1985a,b; Iain Weatherston, personal communication).

Fibers were sealed on one end and loaded with the ester blend under a microscope using a 10-μl syringe, leaving varying distances from the opening of the fiber to the meniscus of the liquid column. This was done to permit equal release rates under varying temperatures. Each day, a set of four of each of the following fibers was taken to the field: liquid column 3.8, 5.6, 7.8, or 9.8 cm from opening of fiber. These were appropriate for and employed, respectively, at temperatures of 20.0–23.5, 24.0–27.0, 27.5–30.5, and 31.0–34.0°C (calculations based on information from Iain Weatherston, personal communication). When not in use, fibers were kept in a cooler with ice to prevent release of odor. Immediately prior to use, the distance from the opening of the fiber to the end of liquid column was verified again.

To release apple volatiles at ca. 500 μg/hr, we loaded 0.75 ml of the volatile mixture in polyethylene vials of the type used by Reissig et al. (1982), purchased from Andler Israel & Son (Everett, Massachusetts; 2 dram Wheaton Natural Cylinder #20298 and White Cap #15044). We waited three weeks between loading vials and measuring release rate (to allow volatiles to penetrate the polyethylene vial). Release rate was determined by weight loss using a high-precision electronic balance. One group was kept continuously under an exhaust hood at ca. 67% relative humidity and 25°C; another group was kept in a freezer overnight and then placed in the hood during the day. Release rate under both conditions was similar: for group 1 (constant T), average = 514 μg/hr (range: 397–644 μg/hr) (confirmed by A. Averill, personal communication); for group 2, average = 548 μg/hr (range: 404–656 μg/hr). Fibers were attached to the fruit models with clear tape; vials were fastened to the model-supporting wire.

Visual Stimuli. Visual stimuli consisted principally of real apples and apple mimics whose spectral reflectance curves are given in Figure 1. We used 1-year-old, ripe, red, ca. 7.5-cm-diam. Red Delicious apples (stored under controlled atmosphere conditions from harvest until use) and mimics, consisting of 7.5-cm-diam. wooden spheres covered with Tarter Red Dark enamel paint (Sherwin Williams, Cleveland, Ohio). We also used freshly picked, unripe, green, ca. 3.5-cm-diam. Red Delicious apples and mimics, consisting of 3.5-cm-diam. rubber spheres covered with the following mixture of Winsor and Newton artist pigments: Mars Black 248 SL Series 2 (0.69%); Windsor Green 170 SL Series 2 (1.62%); Titanium White (Permanent White) 244 SL Series 2 (33.03%), and Cadmium Yellow 222 SL Series 4 (64.67%). Before coating the rubber spheres with this mixture, we made two applications of Liquitex Acrylic Gesso #5332 (Binney & Smith Inc. Easton, Pennsylvania). We also used 7.5-cm-diam. trans-

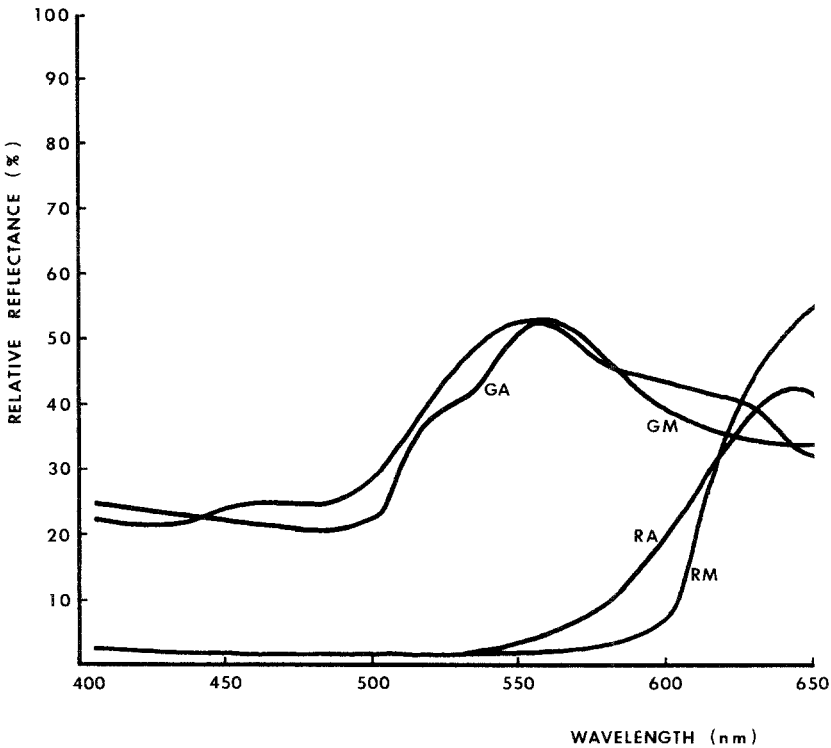

FIG. 1. Spectral reflectance curves of unripe (green) Red Delicious apples (GA), green apple models (GM), ripe (red) Red Delicious apples (RA), and red apple models (RM).

parent glass spheres. All fruit and models were wired for hanging on tree branches.

Research Arena. Experiments were conducted in two ca. 6-year-old semi-dwarf apple trees whose canopies were ca. 2.8 m diam. × 2.8 m high. Each tree was enclosed in a 3.5-m-diam. × 3.5-m-tall cylindrical, clear Saran screen cage. The roof of the cage was covered with a brown bed sheet to protect from direct sunlight. Cages were ca. 60 m apart. We manipulated the architecture of each tree until an approximately even distribution of foliage throughout the tree was obtained. We also clipped away 40% of all leaves to allow the researcher to view clearly almost any point within the tree from any location in the cage. Finally, we divided the research arena (=tree canopy) into imaginary cubes of space and then marked every tree part falling within a particular cube with a distinctive number, corresponding to x, y, and z coordinates. The arena had a volume of 54,880 cm^3 (14 × 14 × 14 20-cm cubes; total of 2744 cubes). For further details see Aluja et al. (1989).

Experimental Procedure. In each experiment, *R. pomonella* responses to color, odor, and density combinations were evaluated (Table 1). Two persons gathered data. Every morning each person was assigned at random to a cage and assigned at random the nature and order of treatments to be tested. The experimental procedure consisted of recording (using a portable audio-tape

TABLE 1. DESCRIPTION OF HOST FRUIT OR MODEL VISUAL, CHEMICAL, DENSITY COMBINATIONS TESTED DURING STUDY OF *R. pomonella* HOST FRUIT-SEARCHING BEHAVIOR

	Experiment number and fruit (model) density[a]/odor release rate[b]					
Color/odor combination	I 1/500	II 4/500	III 16/500	IV 16/500	V 1/500	VI 1/0.7
Clear, no odor	X	X	X	X	X	X
Clear, odor	X	X	X	X	X	X
Green, no odor				X	X	X
Green, odor				X	X	X
Green apple				X		
Red, no odor	X	X	X		X	X
Red, odor	X	X	X		X	X
Red apple	X	X	X			
Empty tree	X	X	X	X		

[a] Number of real fruit or models hung in field-caged apple tree.
[b] Release rate in micrograms per hour of synthetic apple volatiles from polyethylene vials or teflon fibers.

recorder) the behavior of an apple maggot female released individually into a cage. Only mated, sexually mature, wild *R. pomonella* females were used (14- to 16-day-old flies). Details on fly collection and maintenance are given in Aluja et al. (1993). On the afternoon previous to testing, 40 mature females showing no wing damage were allowed to oviposit twice each in a ripe hawthorn fruit (*Crataegus mollis* var. *toba*) and were then maintained overnight at 25°C and 65% relative humidity in Plexiglas cages with food (hydrolyzed protein and sugar) and water. The next morning, each test female was offered a hawthorn. Once a female commenced ovipositing, the fruit was carried to the tree. There, after ovipositor dragging, the fly was transferred gently to a predesignated leaf near the bottom center of the canopy (the same leaf was used throughout the experiment) and allowed to forage for a maximum of 20 min or until it flew to the cage wall or roof. By using the methodology described under "Research Arena" and in Aluja et al. (1989), we were able to record the three-dimensional path of a foraging fly and quantify its numerous behaviors. At the end of the trial (fly reaching 20 min limit or flying to cage wall), the fly was again offered a fruit. Only those that oviposited at this time were included in data analysis. Completion of oviposition just prior to and just after testing ensured as much as possible that assay flies were in a fruit-foraging mode during testing.

Data Collection and Analysis. Data from tape recorders were transcribed onto data sheets, entered into computer files, and subjected to data analysis and summarizing procedures that generated new files containing detailed information on each fly (for particulars see Aluja et al., 1989). The following dependent variables were examined: percentage of flies that visited a fruit or model; mean number of landings, mean time (minutes) spent on tree, and mean relative distance (meters) flown *before visiting a fruit or model*; mean directness of flight (beeline from release leaf to a fruit or model divided by total distance flown before landing on a fruit or model); total number of landings, mean rate of movement (alightments per minute), mean total relative distance (meters) flown, mean total number of cubes visited, and mean total time *while on tree*; mean distance traveled between alightment sites (meters); mean time spent at each alightment site (seconds); and mean number of flies attempting to oviposit.

We tested for homogeneity of variances using Levene's test (BMDP7D Procedure; BMDP, 1987). The proportion of flies that visited a fruit or model was compared using Fisher's exact test (Sokal and Rohlf, 1981). All other variables were subjected to a three-way analysis of variance (BMDP2V Procedure, BMDP, 1987) in which main effects (treatment, cage, researcher) and treatment × cage, treatment × researcher, cage × researcher, and treatment × cage × researcher interactions were tested. In those cases where treatment effects were statistically significant ($P < 0.05$), marginal means were compared by applying Bonferroni's mean separation procedure. Color × odor interaction effects were tested for 16-model density levels using a two-way analysis of

variance (BMDP2V Procedure, BMDP, 1987). Finally, we tested for differences in flight profiles (proportion of time spent at each alightment site) using a repeated-measures analysis (BMDP2V Procedure, BMDP, 1987).

RESULTS

General Description of Fly Behavior. We observed the following *R. pomonella* behaviors: foraging (moving by flight or walking), resting (sitting completely motionless), grooming, feeding, ovipositing, and defecating. Typically, a fly spent some time (10 sec to 2 min) grooming immediately after it had been transferred onto a tree. Flies then usually initiated a series of leaf-to-leaf flights, interrupted by brief grooming or resting periods. Figure 2 summarizes a subset of data on flight characteristics of flies exposed to 16 clear or 16 red models, with or without odor. Most (ca. 50%) intratree flights were short leaf-to-leaf hops of 5–20 cm. Of all flights, ca. 70% or more were 5–50 cm. Flies typically moved upward from the lower part of the canopy while performing these rather short hops. Occasionally (ca. 6% of all flights recorded), we observed sustained flight (spiraling loops) of 1 m or more. Typically, these were from the top of the canopy to lower branches. Flies foraging in a tree devoid of fruit or models averaged ca. 30 stops (alightments on a leaf or branch) before emigrating from the tree (flying to cage wall). After a fly alighted on a leaf or branch, it usually either soon moved to another leaf or branch (or fruit) or engaged in grooming, walking, or sitting. Among flies exposed to 16 clear or 16 red models with or without odor, ca. 80% or more of alightments were 1–50 sec in duration (Figure 2B).

Duration of fly residence on a tree varied from 1.2 to 20 min. Some flies remained motionless for the entire 20 min and were not considered for data analysis. Interestingly, mean total residence time in a tree did not appear to be affected by treatment (Tables 2–7; Figure 3C).

Fly Response to Host Fruit Visual and Chemical Stimuli. Results of each experiment (I–VI) are described separately in Tables 2–7 below. Each table is divided into two parts: one providing data on behaviors before the first fruit or fruit model was found; the other providing data on behaviors throughout the total time on a tree. For each treatment, if the number of flies visiting a fruit or model was less than eight, we did not analyze data relating to behaviors before the first fruit or model was visited.

Experiment I. Table 2 summarizes *R. pomonella* response to one red apple or one clear or red model in a tree. There were no significant differences in the proportion of flies (55, 50, 39%) that visited a red model with odor (released at ca. 500 μg/hr), a red model without odor, or a red apple. No fly visited a clear model without odor and only one fly (4%) did so when the clear model

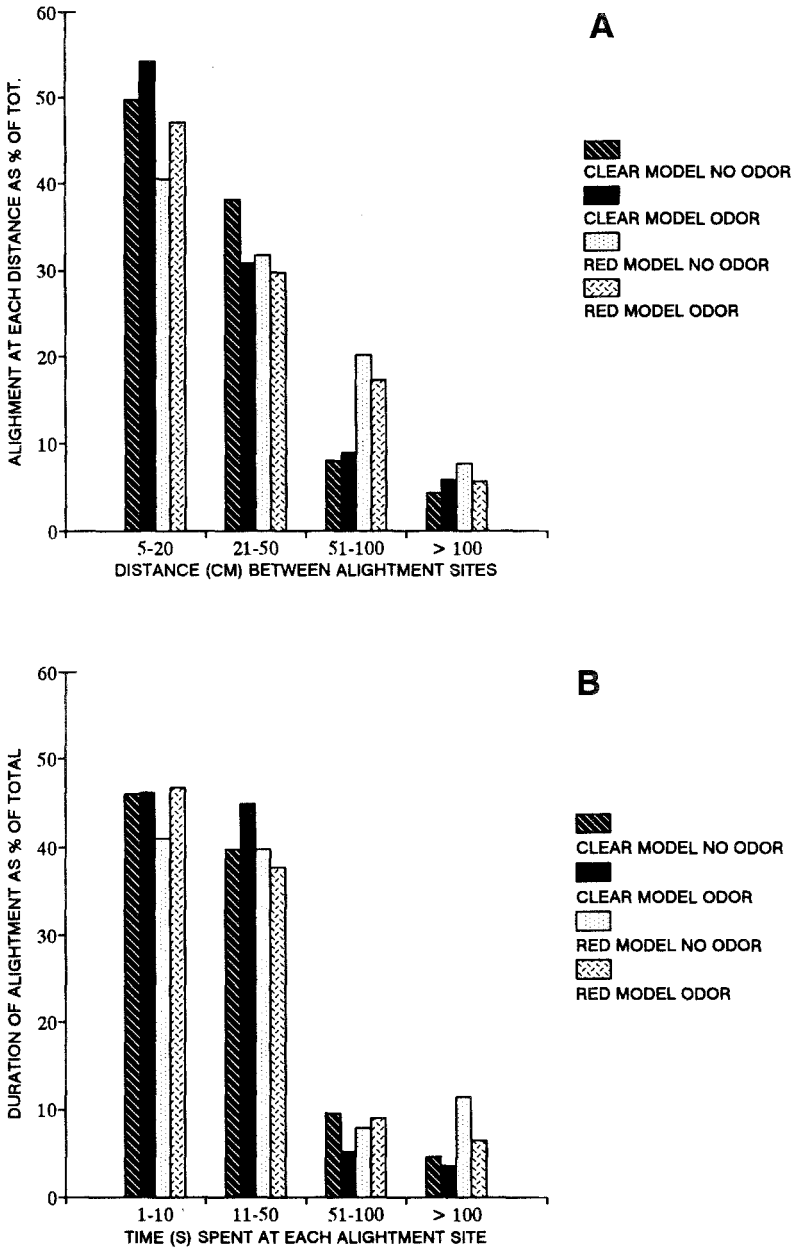

FIG. 2. Intratree flight characteristics of individually tracked *R. pomonella* flies foraging in a field-caged apple tree harboring 16 fruit models. (A) Distance (cm) between alightment sites; (B) time spent at each alightment site.

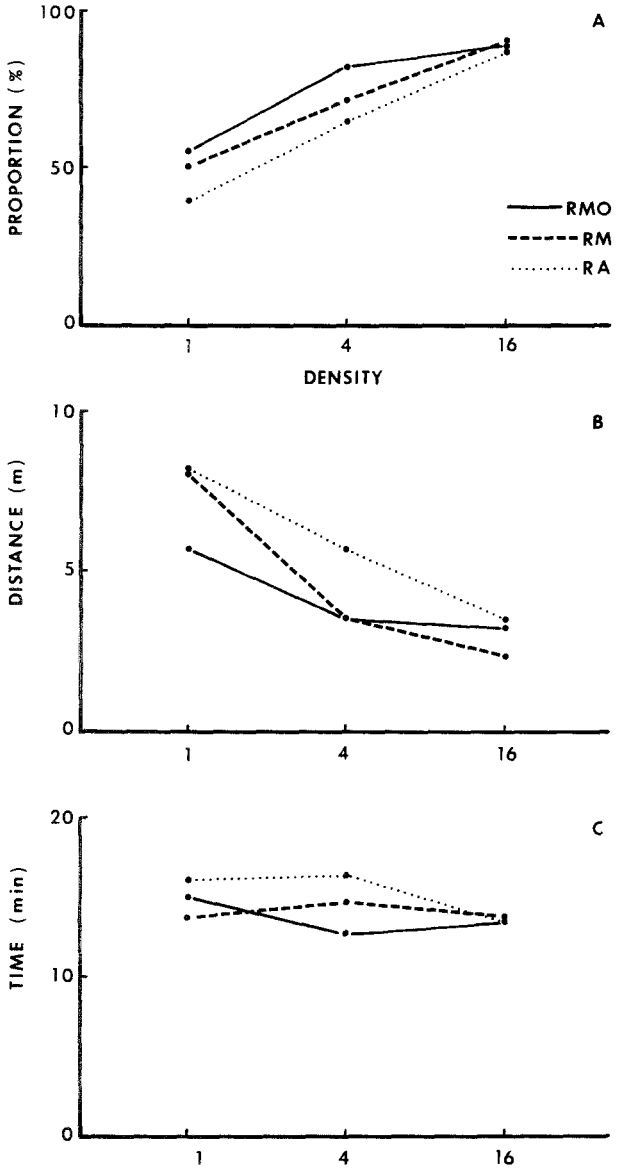

FIG. 3. Effect of density of real red fruit or red models (1, 4, or 16 per tree) on: (A) proportion of *R. pomonella* flies finding a model; (B) distance traveled before finding a model; (C) time spent on a tree before emigrating (flying to cage wall). RMO = red model with odor (released at ca. 500 μg/hr), RM = red model without odor, RA = red apple (data selected from Tables 2-4).

TABLE 2. RESPONSE OF INDIVIDUALLY TRACKED *R. pomonella* FEMALES TO 1 REAL RED FRUIT OR 1 RED OR CLEAR ODOR-BAITED OR UNBAITED MODEL HUNG IN FIELD-CAGED APPLE TREE (Experiment I of Table 1)[a]

	Treatment[b]					
	CM	CMO	RM	RMO	RA	ET
Flies tested (*N*)	27	25	30	29	23	26
Flies (%) that visited a "fruit"	0a	4a	50b	55b	39b	
Mean[c] no. landings before visiting a "fruit"			26.20a ±5.18	16.63b ±7.35	25.44a ±5.73	
Mean time (min) spent on tree before visiting a "fruit"			8.72a ±1.71	6.42a ±1.30	9.49a ±1.86	
Mean relative distance flown (m) before visiting a "fruit"			8.04a ±1.96	5.72a ±0.60	8.08a ±1.67	
Mean directness of flight to a "fruit"			7.79a ±1.85	5.83a ±0.61	7.98a ±2.03	
Mean total no. of landings while on tree	24.89a ±2.95	33.36a ±4.09	31.30a ±3.21	32.62a ±2.79	35.01a ±3.74	31.23a ±4.68
Mean rate of movement while on tree (landings/min)	2.02a ±0.16	2.69a ±0.26	2.44a ±0.23	2.21a ±0.17	2.31a ±0.22	2.42a ±0.28
Mean relative distance (m) flown while on tree	8.95a ±1.10	10.23a ±1.21	9.99a ±1.15	10.51a ±0.95	11.46a ±1.32	10.92a ±2.04
Mean total time on tree	13.01a ±1.19	13.04a ±0.95	13.71a ±1.09	15.11a ±0.91	16.13a ±1.13	13.41a ±1.23

[a] Means within a row followed by the same letter are not different at a 0.05 level of significance. Proportions were tested using Fisher's exact test; means were tested using Bonferroni's procedure ($P > 0.05$).
[b] CM, clear fruit model with no odor; CMO, clear fruit model with odor (odor released at ca. 500 μg/hr); RM, red fruit model with no odor; RMO, red fruit model with odor (odor released at ca. 500 μg/hr); RA, red delicious apple; ET, empty tree (no models).
[c] All mean values are followed by ±SE.

had odor. Mean time and mean relative distance flown before visiting a fruit or model and mean directness of flight to a fruit or model were not significantly different among treatments ($P = 0.23$, $P = 0.19$, and $P = 0.16$, respectively). However, the numbers of landings before visiting a fruit or model were significantly fewer ($P = 0.03$) under conditions of a red model with odor than a red model without odor or a red apple. There was also a significant treatment × cage interaction effect.

Mean total number of landings while on a tree, mean rate of movement while on a tree, mean relative distance flown while on a tree, and mean total time on a tree were not significantly different among treatments, including the

treatment of an empty tree ($P = 0.42$, $P = 0.17$, $P = 0.59$, and $P = 0.96$, respectively).

Experiment II. Table 3 summarizes *R. pomonella* responses to four red apples or four clear or red models. Proportions of flies that found red models without odor, with odor (released at ca. 500 μg/hr), and red apples (71, 81, and 65%, respectively) were not significantly different ($P > 0.05$). One fly (4%) found a clear model without odor, and two flies (8%) found a clear model with odor (difference not significant; $P > 0.05$). Differences in the proportion finding a clear model without or with odor versus the proportion finding a red model without or with odor or a red apple were significant ($P = 0.0001$). Mean number of landings before visiting a fruit or model and mean relative distance flown before visiting a fruit or model were not significantly different among treatments

TABLE 3. RESPONSE OF INDIVIDUALLY TRACKED *R. pomonella* FEMALES TO 4 REAL RED FRUIT OR 4 RED OF CLEAR ODOR-BAITED OR UNBAITED MODELS HUNG IN FIELD-CAGED APPLE TREE (Experiment II of Table 1)[a]

	Treatment[b]					
	CM	CMO	RM	RMO	RA	ET
Flies tested (*N*)	25	24	24	27	26	26
Flies (%) that visited a fruit	4a	8a	71b	81b	65b	
Mean[c] no. landings before visiting a "fruit"			10.41a ±2.33	9.39a ±1.25	14.66a ±1.85	
Mean time (min) spent on tree before visiting a "fruit"			3.78a ±0.82	4.59a ±0.82	8.51b ±1.27	
Mean relative distance flown (m) before visiting a "fruit"			3.44a ±0.55	3.44a ±0.47	5.72a ±0.76	
Mean total no. of landings while on tree	27.68a ±3.62	25.83a ±2.66	23.92a ±2.95	20.26a ±2.66	25.85a ±2.34	26.85a ±3.76
Mean rate of movement while on tree (landings/min)	2.46a ±0.28	1.81a ±0.20	1.79a ±0.21	1.71a ±0.20	1.70a ±0.18	2.14a ±0.24
Mean relative distance (m) flown while on tree	8.04a ±1.14	8.17a ±0.94	8.18a ±0.82	6.68a ±0.79	8.45a ±0.79	8.82a ±1.20
Mean total time on tree	12.65a ±1.21	15.78a ±0.97	14.69a ±1.17	12.78a ±1.23	16.29a ±1.09	13.50a ±1.12

[a] Means within a row followed by the same letter are not different as a 0.05 level of significance. Proportions were tested using Fisher's exact test; means were tested using Bonferroni's procedure ($P > 0.05$).
[b] CM, clear fruit model with no odor; CMO, clear fruit model with odor (odor released at ca. 500 μg/hr); RM, red fruit model with no odor; RMO, red fruit model with odor (odor released at ca. 500 μg/hr); RA, red delicious apple; ET, empty tree (no models).
[c] All mean values are followed by ±SE.

($P = 0.15$, 0.07 respectively). Mean time spent on a tree before visiting a fruit or model was significantly different among treatments ($P = 0.03$). No significant cage by treatment interactions were detected.

Mean total number of landings while on a tree, mean rate of movement while on a tree, mean relative distance flown, and mean total time on a tree were not significantly different among treatments, including the treatment of an empty tree ($P = 0.35$, $P = 0.14$, $P = 0.73$, and $P = 0.30$, respectively). No significant treatment × cage interactions were detected.

Experiment III. Table 4 summarizes *R. pomonella* responses to 16 red apples or 16 clear or red models in a tree. Differences in the proportion of flies visiting a red model without or with odor (released at ca. 500 μg/hr) or a red apple (90, 88 and 87%, respectively) were not significant ($P > 0.5$). The

TABLE 4. RESPONSE OF INDIVIDUALLY TRACKED *R. pomonella* FEMALES TO 16 REAL RED FRUIT OR 16 RED OR CLEAR ODOR-BAITED OR UNBAITED MODELS HUNG IN FIELD-CAGED APPLE TREE (Experiment III of Table 1)[a]

	Treatment[b]					
	CM	CMO	RM	RMO	RA	ET
Flies tested (*N*)	26	26	29	26	24	25
Flies (%) that visited a fruit	12a	35b	90c	88c	87c	
Mean[c] no. landings before visiting a "fruit"		20.78a ±3.09	5.35b ±0.73	8.09b ±1.67	11.19b ±1.69	
Mean time (min) spent on tree before visiting a "fruit"		9.48a ±1.88	2.81b ±0.50	3.49b ±0.72	5.10b ±1.07	
Mean relative distance flown (m) before visiting a "fruit"		8.46a ±1.48	2.28b ±0.31	3.11b ±0.55	3.55b ±0.63	
Mean total no. of landings while on tree	30.81a ±4.26	28.73a ±3.29	20.00a ±2.33	22.39a ±3.14	25.63a ±3.74	32.72a ±3.78
Mean rate of movement while on tree (landings/min)	2.58a ±0.25	2.51a +0.26	1.60b ±0.16	1.80b ±0.19	2.25a +0.27	2.34a ±0.22
Mean relative distance (m) flown while on tree	9.98a ±1.29	9.08a ±1.09	7.59a ±0.93	8.78a ±1.14	8.73a ±1.40	10.66a ±1.29
Mean total time on tree	12.85a ±1.34	12.96a ±1.20	13.69a ±1.14	13.32a ±1.16	13.28a ±1.41	14.80a ±1.27

[a] Means within a row followed by the same letter are not different at a 0.05 level of significance. Proportions were tested using Fisher's exact test; means were tested using Bonferroni's procedure ($P > 0.05$).

[b] CM, clear fruit model with no odor; CMO, clear fruit model with odor (odor released at ca. 500 μg/hr); RM, red fruit model with no odor; RMO, red fruit model with odor (odor released at ca. 500 μg/hr); RA, red delicious apple; ET, empty tree (no models).

[c] All mean values are followed by ±SE.

difference in proportion of flies visiting clear models with versus without odor (35 vs. 12%) was marginally significant ($P = 0.04$). A significantly higher proportion of flies visited red models without or with odor and red apples than visited clear models without or with odor. Significant differences among treatments for mean number of landings before visiting a fruit or model, mean time spent on a tree before visiting a fruit or model, and mean relative distance flown before visiting a fruit or model were detected ($P = 0.001$, $P = 0.01$, $P = 0.0002$, respectively). In this analysis, we incorporated mean values for the treatment of clear models with odor because more than eight flies found such a model. For all three parameters, values for flies on a tree having clear models with odor were significantly greater than values for flies on a tree having red models without or with odor or red apples. No significant treatment × cage interactions were detected.

Mean total number of landings while on a tree, mean relative distance flown while on a tree, and mean total time on a tree were not significantly different among treatments, including the treatment of an empty tree. However, rate of movement while on a tree was significantly different ($P = 0.005$). Means for treatments of red models without or with odor were significantly less than means for treatments of clear models without or with odor and an empty tree. No significant treatment × cage interactions were detected.

Experiment IV. Table 5 summarizes *R. pomonella* responses to 16 green apples or 16 clear or green models. Differences in the proportion of flies that found a green model without or with odor (released at ca. 500 μg/hr) or a green apple (33, 39 and 35%, respectively) were not significant ($P > 0.05$); nor was the difference in proportion of flies that found a clear sphere without or with odor (7 and 16%, respectively) significant ($P > 0.05$). Importantly, the proportion of flies finding a clear model with odor was not significantly different ($P = 0.05$) from the proportion finding a green model or a green apple. No significant differences among treatments were found for mean number of landings before visiting a fruit or model ($P = 0.37$), mean time spent on a tree before visiting a fruit or model ($P = 0.24$), and mean relative distance flown before visiting a fruit or model ($P = 0.31$). No significant treatment × cage interactions were detected.

Mean total number of landings while on a tree, mean rate of movement while on a tree, and mean relative distance flown while on a tree were not significantly different among treatments, including the treatment of an empty tree ($P = 0.62$, $P = 0.52$, and $P = 0.78$, respectively). However, total time on a tree was significantly different ($P = 0.02$). Flies spent less time on a tree harboring green models without or with odor than on a tree having clear models without or with odor, green apples, or an empty tree. No significant treatment × cage interactions were detected.

Experiment V. Table 6 summarizes *R. pomonella* responses to a single

TABLE 5. RESPONSE OF INDIVIDUALLY TRACKED *R. pomonella* FEMALES TO 16 REAL GREEN FRUIT OR 16 GREEN OR CLEAR ODOR-BAITED OR UNBAITED MODELS HUNG IN FIELD-CAGED APPLE TREE (Experiment IV of Table 1)[a]

	Treatment[b]					
	CM	CMO	GM	GMO	GA	ET
Flies tested (*N*)	27	25	24	28	26	26
Flies (%) that visited a fruit	7a	16ab	33b	39b	35b	
Mean[c] no. landings before visiting a "fruit"			29.25a ±8.34	14.55a ±2.70	12.67a ±2.27	
Mean time spent (min) on tree before visiting a "fruit"			11.46a ±2.51	6.54a ±1.09	8.08a ±2.12	
Mean relative distance flown (m) before visiting a "fruit"			9.27a ±2.43	4.78a ±1.16	3.82a ±0.69	
Mean total no. of landings while on tree	22.78a ±2.32	24.64a ±2.73	27.79a ±4.25	21.85a ±2.44	27.04a ±2.37	22.96a ±2.21
Mean rate of movement while on tree (landings/min)	1.61a ±0.15	1.81a ±0.31	2.20a ±0.25	1.95a ±0.18	1.59a ±0.15	1.72a ±0.18
Mean relative distance (m) flown while on tree	6.84a ±0.80	7.82a ±0.94	8.39a ±1.22	6.85a ±0.91	8.21a ±0.84	6.85a ±0.91
Mean total time on tree	15.31a ±1.12	15.98a ±1.08	13.04b ±1.27	12.35b ±1.04	17.83a ±0.86	15.35a ±1.04

[a] Means within a row followed by the same letter are not different at a 0.05 level of significance. Proportions were tested using Fisher's exact test; means were tested using Bonferroni's procedure ($P > 0.05$).
[b] CM, clear fruit model with no odor; CMO, clear fruit model with odor (odor released at ca. 500 μg/hr); GM, green fruit model with no odor; GMO, green fruit model with odor (odor released at ca. 500 μg/hr); GA, unripe green delicious apple; ET, empty tree (no models).
[c] All mean values are followed by +SE.

clear, green, or red model per tree. No flies landed on a clear model without or with odor (released at ca. 500 μg/hr). The difference in the proportion of flies that found a green model with or without odor (4 and 14%, respectively) or a red model with or without odor (50 and 49%, respectively) was not significant ($P = 0.05$). However, significantly more flies found a red model of either type than a green or clear model of either type. Mean number of landings before visiting a model, mean time spent on a tree before visiting a model, mean relative distance flown before visiting a model, or mean directness of flight to a model were not significantly different among treatments ($P = 0.89$, $P = 0.78$, $P = 0.61$, and $P = 0.70$ respectively). No significant treatment × cage interactions were detected.

Mean rate of movement and mean total time while on a tree were not

TABLE 6. RESPONSE OF INDIVIDUALLY TRACKED *R. pomonella* FEMALES TO 1 GREEN, RED, OR CLEAR ODOR-BAITED OR UNBAITED MODEL HUNG IN FIELD-CAGED APPLE TREE (Experiment V of Table 1)[a]

	Treatment[b]					
	CM	CMO	GM	GMO	RM	RMO
Flies tested (*N*)	24	26	29	28	41	30
Flies (%) that visited a fruit	0a	0a	14a	4a	49b	50b
Mean[c] no. landings before visiting a "fruit"					16.35a ±2.55	16.33a ±2.99
Mean time (m) spent on tree before visiting a "fruit"					7.88a ±1.27	6.89a ±1.37
Mean relative distance flown (m) before visiting a "fruit"					4.99a ±0.84	4.63a ±0.91
Mean directness of flight to a "fruit"					4.13a ±0.60	4.13a ±0.85
Mean total no. of landings while on tree	18.54a ±1.82	27.85a ±3.13	24.97a ±2.53	35.07b ±4.33	26.39a ±2.41	29.77a ±3.74
Mean rate of movement while on tree (landings/min)	1.43a ±0.21	1.88a ±0.18	2.06a ±0.18	2.45a ±0.27	2.23a ±0.22	2.25a ±0.22
Mean relative distance (m) flown while on tree	5.46a ±0.67	7.58a ±0.86	6.78a ±0.60	10.82b ±1.75	8.07a ±0.82	8.49a ±1.12
Mean total time on tree	16.06a ±1.13	15.60a ±0.97	13.33a ±1.19	14.85a ±1.13	14.20a ±1.03	13.74a ±1.10

[a] Means within a row followed by the same letter are not different at a 0.05 level of significance. Proportions were tested using Fisher's exact test; means were tested using Bonferroni's procedure ($P > 0.05$).
[b] CM, clear fruit model with no odor; CMO, clear fruit model with odor (odor released at ca. 500 μg/hr); GM, green fruit model with no odor; GMO green fruit model with odor (odor released at ca. 500 μg/hr); RM, red fruit model; RMO red fruit model with odor (odor released at ca. 500, μg/hr).
[c] All mean values are followed by ±SE.

significantly different among treatments ($P = 0.07$ and $P = 0.14$). However, mean total number of landings and mean relative distance flown while on a tree were significantly different ($P = 0.016$ and $P = 0.019$). In each case, the value was significantly greater for the treatment of green models with odor than for any of the other treatments. No significant treatment × cage interactions were detected.

Experiment VI. Table 7 summarizes *R. pomonella* responses to a single clear, green, or red model per tree. The same proportion (52%) of flies found a red model with as without odor (released at 0.7 μg/hr). Significantly fewer (4, 0, 8, and 0%, respectively) found a clear model with or without odor or a green model with or without odor. Mean number of landings, mean time spent

TABLE 7. RESPONSE OF INDIVIDUALLY TRACKED *R. pomonella* FEMALES TO 1 GREEN, RED, OR CLEAR ODOR-BAITED OR UNBAITED MODEL HUNG IN FIELD-CAGED APPLE TREE (Experiment VI of Table 1)[a]

	Treatment[b]					
	CM	CMO	GM	GMO	RM	RMO
Flies tested (*N*)	24	23	24	25	27	25
Flies (%) that visited a fruit	0a	4a	0a	8a	52b	52b
Mean[c] no. landings before visiting a "fruit"					14.50a ±1.62	12.62a ±2.77
Mean time (m) spent on tree before visiting a "fruit"					7.94a ±1.14	6.41a ±1.26
Mean relative distance flown (m) before visiting a "fruit"					5.37a ±0.80	4.49a ±1.41
Mean directness of flight to a "fruit"					4.70a ±0.63	3.97a ±1.10
Mean total no. of landings while on tree	28.00a ±3.27	24.74a ±2.46	27.50a ±3.19	26.76a ±3.83	27.67a ±2.69	25.12a ±3.56
Mean rate of movement while on tree (landings/min)	2.31a ±0.26	1.92a ±0.21	1.84a ±0.19	1.95a ±0.27	2.42a ±0.29	1.85a ±0.20
Mean relative distance (m) flown while on tree	9.54a ±1.17	7.70a ±0.89	8.63a ±1.09	8.23a ±0.87	10.27a ±1.19	7.95a ±1.04
Mean total time on tree	13.80a ±1.26	14.37a ±1.18	15.73a ±1.13	15.14a ±1.12	15.76a ±0.93	14.27a ±1.22

[a] Means within a row followed by the same letter are not different at a 0.05 level of significance. Proportions were tested using Fisher's exact test; means were tested using Bonferroni's procedure ($P > 0.05$).
[b] CM, clear fruit model with no odor; CMO, clear fruit model with odor (odor released at ca. 0.7 μg/hr); GM, green fruit model with no odor; GMO, green fruit model with odor (odor released at ca. 0.7, μg/hr); RM, red fruit model; RMO red fruit model with odor (odor released at ca. 0.7 μg/hr).
[c] All mean values are followed by ±SE.

on a tree, mean relative distance flown before visiting a model, and mean directness of flight to a model were not significantly different among treatments ($P = 0.30$, $P = 0.22$, $P = 0.13$, and $P = 0.24$, respectively). No significant treatment × cage interactions were detected.

Mean number of landings while on a tree, mean rate of movement while on a tree, mean relative distance flown while on a tree, and mean total time on a tree were not significantly different among treatments ($P = 0.99$, $P = 0.61$, $P = 0.92$, and $P = 0.53$, respectively). No significant treatment by cage interactions were detected.

Behavior after Alightment on Fruit or Model. After landing on a clear or green model with or without odor or a green apple, no fly attempted oviposition.

However, after landing on a red fruit or red model, some flies did attempt to oviposit. For example, at a density of one red model or red fruit in a tree, 20% of all alighting flies attempted to oviposit if the model lacked odor, 15% with odor, and 9% on fruit. At a density of four red models or red fruit, the values were 15, 33, and 15%, respectively. Where odor was present in association with models, flies searched near a point on the model just below the odor-emitting vial or fiber. Some even crawled upward on the vial-holding wire to reach a vial. Others walked to the opening of an odor-emitting fiber, circled rapidly around the opening, and attempted to oviposit. Interestingly, flies landing on models with odor spent more time attempting to oviposit than flies landing on models without odor. To illustrate, oviposition attempts lasted a mean of 1.6 min ($N = 6$) where a red model (1/tree) had no odor but 3.7 min ($N = 4$) where a red model (1/tree) had odor. When four red models were hung in a tree, oviposition attempts lasted a mean 3.0 min ($N = 8$) if models had no odor but 9.9 min ($N = 4$) if models had odor.

DISCUSSION AND CONCLUSIONS

Consistent with previous reports on *R. pomonella* responses to fruit and fruit mimics (Oatman, 1964; Prokopy, 1968, 1977), we found that fruit-seeking apple maggot females were highly attracted to 7.5-cm red fruit or fruit models and that vision played a major role in this process. Flies consistently discovered red fruit or red models in significantly higher proportions than green fruit or green or clear models in our field caged trees (Tables 2–7).

The probability of discovering a red fruit model increased as density of models increased (Figure 3A). Fifty, 71, and 90% of flies visited a red model without odor at densities of 1, 4, and 16 models/tree, respectively. Similarly, 55, 81, and 88% of flies visited a red model with odor at densities of 1, 4, and 16 models/tree, respectively (relationships drawn from a compilation of data from Tables 2–4). Moreover, flies traveled less distance before discovering the first red model (with or without odor) as model density increased (Figure 3B).

Flies located odorless red models quite rapidly. The minimum time until a fly landed on a red model without odor was 10 sec in trees with 16 models, 12 sec in trees with four models, and 32 sec in trees with one model. This contrasts sharply with the much longer minimum times until a fly landed on a clear model without odor: 216 and 274 sec in trees with 16 and four models, respectively. No fly landed on a clear model in trees with one model.

The role of olfaction during intratree fruit-finding was less obvious than that of vision. Judging by the proportion of flies that visited a fruit model, we conclude that presence of synthetic host plant odor did not measurably enhance the ability of flies to find green or red models. In the case of clear models, there

was some indication that at a high density (16 models) presence of odor did enhance the probability of flies alighting on a model (Figure 4A). The difference between the proportion finding a clear model with odor (35%) and one without odor (12%) was marginally significant (Table 4; $P = 0.04$). Under conditions of high fruit model density, clear models with odor appeared to be as apparent to flies as green models with or without odor (Figure 4A, Table 5). At lower model densities, the probability of a fly discovering a clear model with or without odor was very low (Tables 2–4, 6, and 7).

The strongest indication that olfaction may be playing some role in within-tree host fruit finding stems from our observation of *R. pomonella* behavior in trees harboring only one red fruit or model (Table 2). Flies landed significantly fewer times before finding a red model and followed a more direct path to a red model when the model had odor than when it did not. As already pointed out, odor effects also become apparent when the visual stimulus of a "fruit" is weak. Figure 4B shows that the distance traveled before finding the first green model in a tree having 16 models was reduced if the model had odor. Even though a formal analysis to determine color × odor interaction effects is not strictly appropriate due to the design of experiments (responses to green models were studied one month (Table 5) after responses to red models (Table 4) had been determined), we nevertheless felt justified in performing such an analysis to gain at least partial insight into a possible color × odor interaction. From this analysis it became apparent that odor effects varied according to the nature of the visual stimulus. Figure 4B shows that when models were red (very apparent), odor effects were insignificant (also see Table 4), but, when models were green, odor effects were significant. The color × odor interaction was highly significant ($P = 0.001$). Swift (1982), working with a series of trap types (including Pherocon AM traps with and without a red disk) and odors (*n*-butyl acetate, ammonium acetate, others), also found a significant trap type (color) × chemical lure (odor) interaction. For example, addition of a red disk to an otherwise yellow trap increased capture of *R. pomonella* flies by 4.5 times. Addition of ammonium acetate nullified this effect, but addition of *n*-butyl acetate significantly enhanced the effect.

Reissig et al. (1982, 1985) showed that sticky red spheres (essentially the same as the red models we used) baited with a blend of synthetic apple volatiles released at ca. 500 μg/hr (same volatile blend used by us in experiments I–IV) captured significantly more male and female apple maggot flies than unbaited spheres. Even though our results show that the presence of odor did not enhance the proportion of flies finding a red model, we believe that these two sets of findings are not incompatible. Aluja and Prokopy (1992) have shown that *R. pomonella* females are able to locate an odor-bearing tree within a patch of trees by performing a series of upwind tree-to-tree displacements that eventually lead them to the tree harboring the source of odor. In Reissig et al. (1982, 1985),

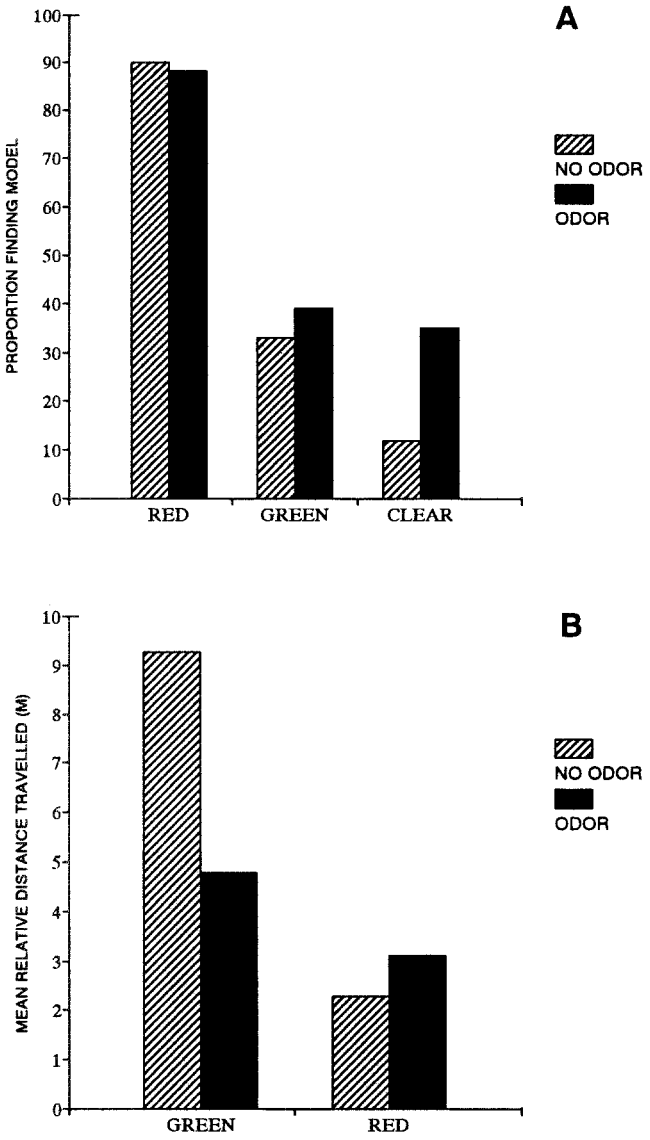

FIG. 4. Effect of host fruit odor (released at ca. 500 μg/hr) on finding of fruit models (16 model/tree) by *R. pomonella* females: (A) proportion of flies finding a red model, green model, and clear model; (B) relative distance traveled before finding a fruit model (due to the fact that too few flies found clear models without odor, no comparison between clear models without and with odor was made). Significant color effect, insignificant odor effect, and significant color $\times$ odor interaction (three-way ANOVA; $P = 0.0001$, $P = 0.67$ and $P = 0.001$, respectively).

the point sources of odor were trees in which a single baited sphere had been placed. Flies foraging in surrounding vegetation may have been drawn into the orchard and preferentially landed on trees emanating such odor. In addition, if the tree harboring a baited sphere were very large, a fly arriving on a side of the tree opposite the side with the sphere would likely have been unable to see the sphere. Under this scenario, it is conceivable that the presence of odor might have alerted the fly to the possible presence of a host fruit (sphere) in the same way as odor affected fly behavior in our experiments, especially if the volatile blend produced by apples in the tree were not as stimulating as synthetic volatiles (Averill et al., 1988) (Table 3).

During studies of *R. pomonella* intertree foraging behavior, we discovered that the same synthetic apple volatile blend used here (released at 500 μg/hr) acted to increase the rate of movement (landings per minute) of flies. This phenomenon was observed both in a wind tunnel and in the field under conditions in which flies were released in host trees devoid of any fruit or fruit models (Aluja and Prokopy, 1992; Aluja et al., 1993). We were therefore surprised to observe no clear effect of host fruit odor on *R. pomonella* rate of movement during intratree foraging under conditions where trees harbored clear models with versus without odor. One possible explanation may involve intermittent fly exposure to odor. As shown by Aluja and Prokopy (1992), flies foraging in a patch of 25 host trees were exposed to continuously shifting wind currents. This in turn caused odor plumes to exhibit a high degree of irregularity. A fly foraging under these conditions would be exposed in intermittent fashion to a point source of odor outside the patch. When attempting to determine effects of odor on rate of insect movement, one must pay careful attention to assessing precisely when the individual perceives odor-free versus odor-bearing air. In our 25-tree patch studies, it was only when flies were actually in a plume of fruit odor that they exhibited a higher rate of movement. We believe that when flies were foraging here within a tree having one or four fruit models with odor, it is quite possible they were exposed to odor intermittently. This is less probable, but still conceivable, in trees having 16 models. Models were hung only at ca. 1 m and ca. 2 m above the lowermost tree foliage. If a fly were foraging in tree parts below or above this height, it is conceivable (although unlikely) that odor plumes did not reach it. When we placed a $TiCl_4$-soaked cotton wick in the center of one of our test trees, we observed two types of plumes: when light wind blew through the cage walls (<0.3 m/sec), the "smoke" took the shape of an amorphous plume that shifted slowly toward the roof. Qualitatively, plume meander and width was considerable. When wind blew at higher speeds (>1.5 m/sec), a more narrow, uniform plume was generated that moved downwind, touching a narrow band of leaves and branches. We find it difficult to believe that under either of these wind speed scenarios, none of the 16 plumes would have impinged on a foraging fly.

An alternative is that flies exposed to such a high concentration of odor as ca. 8000 μg/hr (16 × ca. 500 μg/hr) habituated quite rapidly to odor, resulting in a lack of behavioral response to odor. To determine if habituation was a factor, we calculated rate of movement of flies foraging in trees having 16 clear spheres without odor versus 16 clear spheres with odor. Rate of movement was calculated during the time intervals 0–2 min, 0–5 min, 5–10 min, and 10–15 min. We found that under both odor-absent and odor-present conditions, rate of movement tended to diminish over time. Mean rates (alightments per minute) for the odor-absent treatment were 2.75 (0–2 min), 2.14 (0–5 min), 1.78 (5–10 min), and 1.21 (10–15 min). For the odor-present treatment, mean rates were 2.58, 2.47, 1.35, and 1.90, respectively. These data do not suggest possible habituation of flies after exposure to high concentrations of odor.

Still another alternative is the possibility of arrestment by odor. For example, in wind-tunnel studies, we observed that *R. pomonella* flies spent a high proportion of time in the wind-tunnel section in which the odor source was placed when air was calm (Aluja et al., 1993). We also observed that when flies were exposed to moderate wind (1.6 m/sec), odor caused them to stay in the minitree where they were released rather than move downwind, as was the case when wind carried clean air. It is thus conceivable that flies in our study were arrested in areas of high concentration of odor. Other authors have likewise reported arrestment of insects in close proximity to an odor source. For example, Judd (1986), working with *D. antiqua*, reported that after flies were able to locate an odor source (presumably through odor-mediated positive anemotaxis), their upwind movement seemed arrested.

To unveil possible arrestment by odor, we considered two possible mechanisms: (1) reduction of track lengths (distance between alightment sites) and (2) increase in time spent at each alightment site. First, we examined data for arrestment in trees bearing 16 models by comparing fly responses to clear models without versus with odor. We were unable to find any consistent pattern indicating that flies reduced the distance between alightment sites (Figure 2A) or that flies spent a longer time at sites of alightment in the presence versus the absence of odor (mean of 29.8 vs. 31.9 sec alightment duration under 16 clear models with versus without odor, $N = 25$, $P = 0.86$). We thus conclude that at the resolution level of this study, an arrestment effect of odor on flies in close proximity to an odor source may be operative but cannot be demonstrated through data analysis.

In a further attempt to unveil possible arrestment by odor, we also performed a detailed analysis of foraging behavior of flies released in trees harboring only one clear model without or with odor. We assessed fly behavior in close proximity to a model by selecting a spatial window of 1280 cm^3 (64 20-cm cubes) around a model and examining certain flight parameters within it. Rather than being arrested, flies appeared to be activated by presence of odor.

For example, flies foraging within the window in the presence of a clear model with odor moved at a rate of 4.3 ± 0.8 SE alightments per minute while those foraging within the window of a clear model without odor moved at a rate of 2.7 ± 0.6 SE alightments per minute ($P = 0.08$). Flies foraging in close proximity to a clear model with versus without odor also visited more cubes (3.2 ± 0.9 vs. 1.4 ± 0.4; $P = 0.04$), increased the interalightment track length (0.16 ± 0.05 vs. 0.07 ± 0.03 m; $P = 0.001$), reduced the mean residence time per alightment site (15.33 ± 3.5 vs. 29.46 ± 13.9 sec; $P = 0.08$), and traveled more distance (1.27 ± 0.5 vs. 0.42 ± 0.2 m; $P = 0.08$). The amount of time spent in the 1280-cm^3 window was 2.06 ± 0.45 vs. 1.04 ± 0.16 min around clear models with versus without odor ($P = 0.09$). As noted earlier, close proximity of a fly to a model in a tree having a single model does not necessitate a fly being exposed to odor.

In conclusion, we believe that a picture has emerged from various studies on the dynamics of host finding indicating that insects searching for hosts indeed use information from a variety of stimuli. It appears that host-finding behavior of *R. pomonella* is quite similar to that of *Delia* flies. The probable mechanisms uncovered here and by Aluja and Prokopy (1992) suggest that when sexually mature *R. pomonella* females begin searching for host fruit, both chemical and visual cues play an important role during the orientation process. Our findings support the following scenario: when a female is exposed to host fruit odor intermittently (high or low molecule concentration), it increases its level of activity (landing frequency), searches more thoroughly, straightens out its moves, increases interalightment track length, and is able to locate a point source of odor possibly by exhibiting mechanoreceptive anemotaxis (Aluja and Prokopy, 1992). When exposed continuously to a high concentration of host fruit odor and in close proximity to the source, a female may be arrested (Aluja et al., 1993). Females are also likely to be arrested when the speed of wind conveying host fruit odor is too high to permit a fly to forage (Aluja and Prokopy, 1992; Aluja et al., 1993). Exposure to host fruit odor may also increase the level of visual scanning by *R. pomonella* flies. Once a female has landed on a host tree, host visual and chemical stimuli appear to interact in the following manner: If the visual stimulus of a fruit is strong (dark-colored, ca. 8 cm diam.) flies seem able to locate individual fruit solely on the basis of vision. If the visual stimulus is weak (light-colored, smaller-size fruit), odor seems to facilitate host finding by increasing the alertness of a fly to visual stimuli and by acting to allow flies to reduce the distance traveled before locating a fruit.

During our studies we tested only sexually mature *R. pomonella* individuals. To gain a more complete picture of *R. pomonella* responses to host visual and chemical stimuli, there is still a need to study the effects of sex, age, nutritional status, mating status, and prior experience with fruit. As Judd (1986) has elegantly shown, several parameters are important sources of variability that

need to be considered when interpreting insect responses to host visual and chemical stimuli.

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PUKALIDE, A WIDELY DISTRIBUTED OCTOCORAL DITERPENOID, INDUCES VOMITING IN FISH

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Abstract—Tissue and extractable metabolites from the subtropical gorgonian *Leptogorgia virgulata* (Coelenterata: Anthozoa: Octocorallia: Gorgonacea) induce vomiting in a variety of fish species. To elucidate the chemical bases of this phenomenon, experiments were undertaken with purified pukalide, a cembranoid diterpene that comprises as much as 0.1–0.5% of the wet tissue weight of *L. virgulata*. When incorporated into artificial foods at concentrations corresponding to the levels found in *L. virgulata*, pukalide induced emesis when delivered orally to killifish (*Fundulus heteroclitus*). The threshold dose for pukalide-induced emesis was 0.05 mg/g fish body weight. Control pellets devoid of pukalide did not induce vomiting. The emetic effect of pukalide was dose-dependent, as fish ingesting more than 0.100 mg pukalide/g body weight exhibited a significantly higher incidence of vomiting than fish ingesting lower doses of pukalide ($G = 5.5$, $df = 1$, $P < 0.025$). The elapsed time between ingestion of pukalide-containing pellets and emesis was significantly longer in fish that ingested marginally emetic doses of pukalide (Kruskal-Wallis $H = 4.00$, significant with $P < 0.05$). Although not markedly unpalatable to fish, pukalide may function in nature as a defensive toxin by inducing emesis and learned aversion in potential octocoral predators.

Key Words—Vomiting, emesis, terpenoid, cembranoid, octocoral, pukalide, fish.

INTRODUCTION

The octocorals (Phylum Coelenterata: Class Anthozoa: Subclass Octocorallia), a group of sessile marine invertebrates that includes organisms commonly known

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as sea fans, sea whips, and soft corals, contain a plethora of secondary metabolites. During the dramatic expansion of marine natural products chemistry over the past 30 years, hundreds of unique compounds—primarily terpenoids, steroids, and acetogenins—were isolated from octocorals and other marine organisms (Tursch et al., 1978; Fenical, 1981; Faulkner, 1984; Coll, 1992). The potent pharmacological activity of some marine natural products (e.g., lophotoxin; Fenical et al., 1981) sparked biomedical investigations of marine metabolites as new medicines or as novel probes of physiological processes and cell function (Cohen et al., 1990; Scheuer, 1990). Other studies have focused on the roles that natural products from octocorals and other marine organisms play in the ecological interactions of marine and aquatic species; among the ecological roles most commonly considered have been chemical defense against predation, allelopathic effects on competitors, and antifouling functions (Bakus et al., 1988; Coll, 1992; Paul, 1992).

The sea whip *Leptogorgia virgulata* (Order Gorgonacea) is locally abundant on western North Atlantic coasts at subtropical and temperate latitudes (Bayer, 1961; Patton, 1972). Tissues and extracts of *L. virgulata* are active in two bioassays with potential ecological significance: (1) the inhibition of settlement by marine fouling organisms (Standing et al., 1982; Rittschof et al., 1984), and (2) the induction of emesis (vomiting) and subsequent learned aversion in fish (Gerhart, 1991). Bioassay-directed purification of fouling inhibitors from *Leptogorgia virgulata* led to the isolation of pukalide (Gerhart et al., 1988) (Figure 1), a furanocembranolide diterpene originally discovered in the alcyonacean

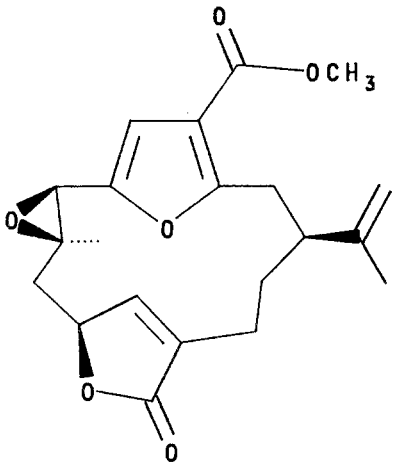

FIG. 1. Structure of the diterpenoid known as pukalide. This furanocembranolide and structurally similar compounds have been isolated from a variety of distantly related octocoral species.

octocoral *Sinularia abrupta* by Missakian et al. (1975). Pukalide, its derivatives, and structurally similar compounds are produced by a variety of octocorals in the orders Gorgonacea and Alcyonacea (Ksebati et al., 1984; Coll et al., 1989). Among the octocorals, the highest concentrations of pukalide occur in egg-bearing colonies and the recently released eggs of various *Sinularia* species (Coll et al., 1989). Despite the broad phylogenetic distribution of pukalide and the known emetic properties of *Leptogorgia virgulata*, the potential effects of orally administered pukalide on fish have not been experimentally examined and the ecological *raison d'etre* of pukalide has remained problematic (Coll et al., 1989; Coll, 1992). This paper reports the results of experiments designed to ascertain whether orally delivered purified pukalide at ecologically relevant doses would induce vomiting in the fish *Fundulus heteroclitus*, an abundant euryhaline teleost that occurs sympatrically with *Leptogorgia virgulata*.

METHODS AND MATERIALS

Colonies of *Leptogorgia virgulata*, killifish (*Fundulus heteroclitus*), and seawater were collected from coastal areas near Beaufort, North Carolina, U.S.A. Seawater was coarsely filtered, passed through a 100-kDa cutoff filter, and then aged a minimum of seven days at room temperature. Killifish were acclimated to marine aquaria (30–32 parts per thousand salinity) for at least one week prior to experiments.

Purified pukalide was prepared from freeze-dried colonies of Australian *Sinularia* sp. using methods described by Coll et al. (1989). Crude dichloromethane extracts of coral tissue were initially separated into fractions by chromatography on silica gel. Fractions containing pukalide were further purified by HPLC employing two silica gel columns in series and a mobile phase of ethyl acetate–light petroleum (35:65). Elution of compounds from the HPLC column was monitored using a refractive index detector. Structure verification of purified pukalide was accomplished by comparison of ^{1}H and ^{13}C NMR spectra with reported values.

Gelatin pellets for fish emesis experiments were produced by modification of the method of Gerhart (1984). Unflavored, food-grade gelatin (Knox) was dissolved with stirring in hot (80–90°C) distilled water to yield a 6% solution (w/w, solid–liquid). Commercial flaked fish food (Tetramin) was ground to a coarse powder with a mortar and pestle, then added to the dissolved gelatin at a concentration of 2.5% (w/w). The resulting mixture was cooled to 30–45°C. Pukalide solution was produced by dissolving a measured amount of the pure terpenoid in HPLC-grade dichloromethane to give a quantified concentration near 100 mg/ml. Ten microliters of the pukalide solution were added to 0.5 ml flavored gelatin and dispersed by stirring the mixture vigorously with a glass

rod. The resulting concentration of pukalide in gelatin pellets ranged from 0.2% to 0.5% of the wet weight. This level approximated the concentration of pukalide in the coenenchyme of some *L. virgulata* colonies as measured through bioassays of barnacle settlement inhibition (Rittschof and Hooper, unpublished data). Control gelatin contained 10 μl dichloromethane without pukalide. The gelatin mixture was transferred in 50-μl aliquots to the wells of a plastic microtiter plate and solidified into pellets by cooling to a temperature of 5–10°C.

Fish were placed singly in 1-liter, all-glass aquaria and offered pukalide-containing pellets. Fish that ingested pellets containing pukalide were monitored for 20 min to determine if vomiting occurred. Fish that did not feed were excluded from the study. Two to three hours later, the fish that consumed pukalide pellets were offered pellets containing dichloromethane but no pukalide. After these control observations were complete, the fish were weighed to the nearest 0.1 g (live weight) to allow calculation of the weight-specific dose of ingested pukalide. Each fish was used in only one trial. Trials were performed until 15 fish had received oral doses of pukalide ranging from 0.04 to 0.25 mg/g fish body weight. Additional trials were precluded by the limited availability of purified pukalide.

RESULTS

The ingestion of food pellets containing pukalide induced vomiting in 53% (8 of 15) of the killifish. Vomiting was defined as the emetic expulsion of swallowed pellets from the stomach through the mouth; the mere mouthing and spitting out of a pellet was not scored as vomiting. None of the killifish (0 of 15) vomited after ingestion of control pellets comprised of gelatin without pukalide. The proportion of fish vomiting after ingestion of pukalide-containing pellets was significantly greater than the proportion vomiting after ingestion of control pellets (G test for independence, Williams' corrected $G = 8.07$, $df = 1$, $P < 0.005$).

The incidence of pukalide-induced vomiting was dose-dependent (Figure 2), with a significantly higher proportion of fish exhibiting emesis after ingesting doses of pukalide greater than 0.100 mg/g body weight (G test for independence, Williams' corrected $G = 5.54$, $df = 1$, significant with $P < 0.025$). The threshold concentration, i.e., the lowest weight-specific dose of pukalide that induced vomiting, was 0.050 mg/g body weight.

The time that elapsed between ingestion of pukalide and vomiting was significantly longer in fish that ingested marginally emetic doses of pukalide (Figure 3). The two fish that received pukalide at doses near the vomiting threshold (0.05–0.06 mg/g) took significantly longer to vomit than fish that ingested higher doses of pukalide (0.07–0.24 mg/g) (Kruskal-Wallis test, $H =$

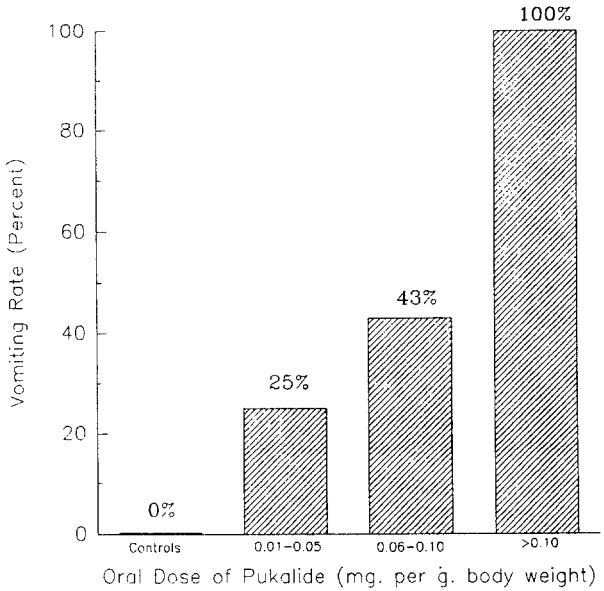

FIG. 2. Dose–response relationship for pukalide-induced vomiting. The vertical axis represents the percentage of tested fish that vomited at least once during the 20 min following pukalide ingestion. Of fish receiving the lowest dosages of pukalide, 25% (1 of 4) fish vomited, while 43% (3 of 7) fish vomited after ingesting doses of pukalide ranging from 0.05 and 0.10 mg/g body weight. Vomiting occurred in all fish (4 of 4) that received pukalide doses greater than 0.10 mg/g body weight.

4.00, significant with $P < 0.05$); prior to vomiting, these fish showed signs of apparent distress including rapid, erratic swimming and the repetitive gulping movements with the mouth. Fish that ingested pukalide but did not vomit also frequently exhibited signs of apparent distress. The shortest elapsed time between pellet ingestion and vomiting was 2.5 min; this fish ingested a weight-specific dose of pukalide equal to 0.13 mg/g. The longest elapsed time to vomiting was 16.4 min; this fish ingested a dose of pukalide equal to 0.064 mg/g.

DISCUSSION

Pukalide, when incorporated into artificial foods at concentrations approximating those found naturally in *Leptogorgia virgulata*, induced vomiting in *Fundulus heteroclitus* when delivered orally at doses greater than 0.05 mg/g body weight. The emetic activity of pukalide suggests that this terpenoid is partially or completely responsible for the emetic properties of *Leptogorgia virgulata* coenenchyme. Observations suggest that nonemetic doses of pukalide

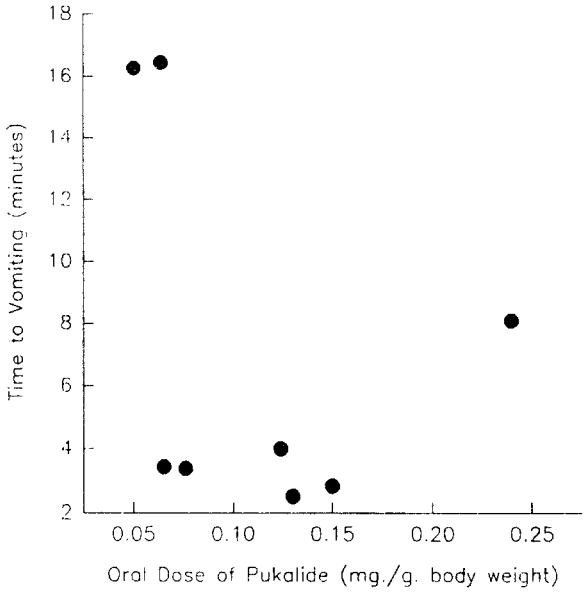

FIG. 3. Relationship between time to vomiting and the oral dose of pukalide. The two fish that ingested a marginally emetic dose of pukalide (0.05 mg/g body weight) exhibited a significantly longer time to vomiting than fish that consumed higher doses of pukalide.

may produce distress in fish; if so, then oral doses of pukalide below the emetic threshold may cause sufficient nausea to induce learned aversion in fish.

The results of the present study hold implications for the effectiveness of pukalide as a defense against marine predators. Present evidence indicates that the vomiting response is widespread among fish and that vomiting induces a learned aversion to the emetic food (Gerhart, 1984, 1991). Furthermore, materials that induce vomiting in a given fish species tend also to induce emetic responses in other species, even those that are distantly related (Gerhart, 1991). For example, prostaglandin A_2 induces vomiting in both killifish (family Cyprinodontidae) and the yellow-head wrasse, *Halichoeres garnoti* (family Labridae) (Gerhart, 1984, 1991). Thus, the present result with killifish may have ecologically relevant implications for other predator–prey interactions, such as the roles played by pukalide in the eggs of *Sinularia* sp. soft corals. Although eggs from many octocorals including *Sinularia* spp. are eaten by reef fish during episodes of mass spawning (Coll et al., 1989), this does not preclude the possibility that the ingestion of *Sinularia* spp. eggs induces nausea and vomiting in most fish species. Photographs of predation during nocturnal mass-spawning events suggest that the fish that consume octocoral eggs are not the most commonly encountered nocturnal, planktivorous species, but instead are fish such as ser-

geant-majors (genus *Abudefduf*) that are tolerant of octocoral toxins (Tursch, 1982). While the majority of fish may be dissuaded by the emetic properties of pukalide, the few fish species that consume octocoral eggs may possess adaptations that confer a degree of immunity to toxins and permit feeding upon noxious foods that most other fish species avoid. Biochemical adaptations of this type, in the form of biotransformation enzymes, have been found recently in fish that specialize on octocorals (Vrolijk, 1992). Thus, emetic chemicals such as pukalide, while not effective against all fish species, may inhibit feeding by fish species that lack resistance to toxins.

Leptogorgia virgulata tissue and its extractable metabolites, while emetic, do not initially inhibit the feeding of pinfish, *Lagodon rhomboides*, or of largemouth bass, *Micropterus salmoides* (Gerhart, 1991). Thus, even at concentrations between 0.1% and 0.5%, pukalide appears to lack ecologically significant palatability-reducing properties. This lack of repellency implies that if pukalide indeed functions as a defensive toxin, then predators must sample and subsequently learn to avoid foods containing the compound. Most other studies regarding the defensive properties of marine natural products have focused on properties such as their palatability reduction or "ichthyodeterrency" (Bakus et al., 1988; Coll, 1992). Emetic toxicity, however, is not necessarily equivalent to unpalatability; the converse is also true. In the terminology of Brower (1984), pukalide appears to function as a class I, not a class II, defensive chemical.

In addition to its emetic properties, pukalide is also a highly effective (but nontoxic) inhibitor of settlement by some marine invertebrate larvae (Gerhart et al., 1988). One possible explanation for this phenomenon is that pukalide may induce emesis through a mechanism of action that is similar or identical at the biochemical level to a mechanism involved in the inhibition of larval settlement. If so, then some octocoral metabolites may play dual roles, providing effective defenses both from predation and from biofouling.

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VOLATILE GLANDULAR SECRETIONS OF THREE SPECIES OF NEW WORLD ARMY ANTS, *Eciton burchelli*, *Labidus coecus*, AND *Labidus praedator*

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Abstract—The Dufour glands of workers of *Eciton burchelli* contain a mixture of small quantities of oxygenated compounds, some of which are derived from terpenes, and C_{17}–C_{25} hydrocarbons. The secretion of the Dufour glands of soldiers was either similar to that of workers, with geranylacetone a significant component, or they contained geranyllinalool in large amounts. The glands of workers and soldiers of *Labidus praedator* and *Labidus coecus* contained (*E*)-β-ocimene, a new substance for the Dufour glands of ants. 4-Methyl-3-heptanone was the dominant compound in the mandibular glands of *E. burchelli* and *L. coecus*. Skatole and indole were found in the gasters of *L. praedator*, and skatole was present in the venom glands of some soldiers of *E. burchelli*.

Key Words—Hymenoptera, Formicidae, Ecitoninae, army ants, *Eciton*, *Labidus*, (*E*)-β-ocimene, Dufour gland, mandibular gland.

INTRODUCTION

The behavior of foraging along well-established trails, one of the characteristics of ant societies, has developed to exceptional proportions in the army ants, among which a migratory existence and group predation are the two essential

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and diagnostic characteristics (Wilson, 1958). The most spectacular and extreme form of this group-foraging and raiding is displayed by the Dorylinae and Ecitoninae, the army ants of the Old and New World, respectively. Most of the available knowledge on army ants is on the Ecitoninae, and in particular the genus *Eciton*, including reports on its taxonomy (Watkins, 1976), anatomy and glandular ultrastructure (Whelden, 1963; Billen, 1985), social behavior and foraging strategy (Topoff, 1972; Rettenmeyer et al., 1983; Franks, 1985), thermoregulation (Franks, 1989), and review papers on their general biology (Schneirla, 1971; Gotwald, 1982). The few reports available on their use of pheromones deal with the existence of trail substances (Blum and Portocarrero, 1964; Watkins et al., 1967; Torgerson and Akre, 1970) and the existence of sternal and tergal glands (Hölldobler and Engel, 1978; Hölldobler and Wilson, 1990, p. 235), and there is a contribution by Franks and Hölldobler (1987) on sexual competition and possible queen pheromones. Until now, only behavioral studies have been carried out; this report is the first chemical identification of any of their exocrine secretions.

Three species of the Ecitoninae have been studied here. *Labidus coecus* and *Labidus praedator* are "column raiders," where the small and medium workers run along the trails while the soldiers remain stationary on either side. *Eciton burchelli* is known as a "swarm raider," where workers spread out into a broad fan-shaped raiding front.

We have examined the chemical contents of the mandibular gland, venom gland, Dufour gland, postpharyngeal gland, cuticle, and sixth and seventh abdominal sternites of workers and soldiers of *Eciton burchelli*.

We report the composition of mandibular gland and Dufour gland secretions of workers of *Labidus praedator* as well as the volatile products from their whole abdomens, and the contents of the Dufour glands of soldiers. We also report on the mandibular and Dufour gland contents of minor and medium workers and soldiers of *Labidus coecus*. These data form part of a comparative survey of representative species of the major ant subfamilies. We have recently reported the first such chemical study on the secretions of an Old World army ant (subfamily Dorylinae) *Dorylus* (*Anomma*) *molestus* (Bagnères et al., 1991).

METHODS AND MATERIALS

Collection

Eciton burchelli (Westwood 1842) was collected at Reserva Ducke in Manaus, AM Brazil in March 1991. *Labidus coecus* (Latreille 1802) was collected at Bowmer Ranch near Waco, Texas, U.S.A. in June 1985 and *L. praedator* (Fr. Smith 1858) near Rio Claro, SP Brazil in November 1989. Raiding workers and soldiers were taken live to the laboratory and dissected. Whole

abdomens, heads, venom glands, Dufour glands with sting attached, and isolated Dufour glands (free of other tissues) were sealed singly or in groups in soft glass capillaries (Morgan, 1990), which were then posted to Keele, where they were kept in a refrigerator until ready for analysis.

Analysis

The sealed capillaries were placed in the injector area of the gas chromatograph, and after 2 min heating, were crushed in the device described by Morgan and Wadhams (1972) to introduce the volatile material onto the column without the intervention of solvents. Gas chromatography linked to mass spectrometry was performed as described by Bagnères et al. (1991). A fused silica capillary column (12 m × 0.2 mm) coated with OV-1 of 0.33-μm film thickness was used with helium carrier gas at 1 ml/min. The oven temperature was programmed from 30°C at 8°C/min to 250°C.

Some samples were analyzed by gas chromatography on a Carlo Erba Fractovap Series 4160 using a column of the same type and dimensions as for GC-MS. The carrier gas was helium at 1 ml/min, and the oven was programmed from 100°C at 6°C/min to 270°C. The detector was connected to a Shimadzu CR-3A recording integrator for quantification.

The double bond position in tricosene of the cuticular hydrocarbons was determined by GC-MS of the dimethylthioether derivative, prepared with dimethyl disulfide as described by Billen et al. (1986).

Preparation of Reference Compounds

2,3-Dihydrofarnesol. 2,3-Dihydrofarnesol was prepared essentially as described by Dawson et al. (1988). Farnesol (5 g, 25 mmol, Aldrich) was stirred at room temperature in an atmosphere of hydrogen in the presence of platinum oxide (0.1 g) until 500 ml (25 mmol) of hydrogen had been absorbed. The catalyst was filtered off and the solvent removed to give a mixture of dihydrofarnesol isomers, IR, liquid film, 3500–3300 cm^{-1} (OH), 1720 cm^{-1} (C=C), NMR (60 MHz) δ 0.89 (d, CH_3) δ 1.2–1.3 (m, 5H, CH_2 and CH) δ 1.65 (m, 9H CH_3—C=), δ 2.0 (m, 6H, CH_2), δ 3.2 (t, CH_2—OH), δ 5.04 (broadened t, 2H, CH=). GC—MS resolved the crude product into a number of peaks with R_t between 20 and 22 min identified as isomeric dihydrofarnesols and tetrahydrofarnesol. The main peak at R_t 20.79 min, representing 60% of the total, was (*E*)-2,3-dihydrofarnesol, identified by its mass spectrum (M^+ 224 (0.1%), *m/z* 209 (0.1), 181 (3.3), 164 (0.3), 162 (2.1), 149 (0.6), 139 (0.8), 123 (10.2), 121 (2.5), 119 (0.2), 109 (6.4), 107 (26), 95 (16), 91 (24), 82 (10.4), 81 (33), 71 (3.4), 69 (100).

2,3-Dihydrofarnesyl Acetate. Crude dihydrofarnesol (2.24 g) in dry pyridine (3 ml) and acetyl chloride (0.86 g), held at −10°C for 10 min and then

at room temperature for 2 hr, gave, after the usual work-up, crude dihydrofarnesyl acetate, IR, 1740 cm^{-1} (C=O), 1230 cm^{-1} (C—O), NMR 60 MHz, δ 0.7 (d, CH_3), δ 1.1–1.3 (m, 5H, CH_2 and CH), δ 1.4 (br, 9H, CH_3), δ 1.9 (s CH_3CO), δ 2.0 (m, 6H, CH_2) δ 4.1–4.3 (t, CH_2—O), δ 5.0 (m, 2H, CH=). GC—MS gave only two peaks, R_t 21.46 and 21.85 min, identified as the *E* and *Z* isomers, respectively, of 2,3-dihydrofarnesyl acetate. Mass spectrum of (*E*)-2,3-dihydrofarnesyl acetate gave M^+ 224 (0.1%), *m/z* 223 (2), 191 (0.2), 189 (0.5), 163 (2.7), 149 (0.8), 135 (1.9), 123 (11), 109 (7), 95 (16), 93 (14), 81 (48), 79 (7), 69 (100), 67 (73), 57 (3), 55 (19), 53 (14), 43 (91), 41 (87).

7-Methyl-2-undecanone. The Grignard reagent prepared from 2-bromohexane (8.2 g, 50 mmol) in tetrahydrofuran, was added to a cooled (0°C), stirred solution of 1,4-dibromobutane (10.7 g, 50 mmol) and dilithium tetrachlorocuprate (10 mmol) in tetrahydrofuran (Friedman and Shani, 1974). After 1 hr, isolation and distillation gave 1-bromo-5-methylnonane. The Grignard reagent prepared in turn from 1-bromo-5-methylnonane was treated with acetyl chloride at −70°C. Work-up in the usual way gave 7-methyl-2-undecanone (bp 70°/0.05 mm in bulb-tube distillation), NMR (270 MHz) δ 0.9 (m, 6H, CH_3), δ 1.1 (m, 12H, CH_2), δ 1.4 (m, 1H, CH), δ 2.2 (s, CH_3CO), δ 2.4 (t, CH_2CO); mass spectrum M^+ 184 (0.1%), *m/z* 166 (1), 151 (1), 124 (10), 109 (8), 95 (12), 71 (25), 58 (75), 43 (100), 41 (30).

7-Methyl-2-undecanol. Reduction of 7-methyl-2-undecanone (200 μl) with sodium borohydride (40 mg) in methanol (2 ml) gave 7-methyl-2-undecanol, NMR (270 MHz) δ 0.9 (m, 6H, CH_3) δ 1.1 (m, 12H, CH_2), δ 1.4 (m, CH and CH_3—C—OH, 4H), δ 1.7 (m, 2H, CH_2—C—OH), δ 3.2 (s, OH), δ 3.8 (m, CH—OH); mass spectrum, M^+ 186 (not seen), *m/z* 153 (2%), 126 (3), 111 (15), 84 (20), 69 (40), 55 (38), 45 (100), 43 (80), 41 (48).

Other compounds were identified by comparison of their retention times and mass spectra with authentic specimens, except for the cyclic acetal **4**, dimethyldisulfide and trisulfide, and the alkenes, which were identified by their mass spectral fragmentation patterns alone.

RESULTS

The presentation of the analyses of Dufour glands of soldiers and workers of *Eciton burchelli* provided difficulties because the composition of the secretion varied considerably among the 23 individuals examined (13 workers and 10 soldiers). There was a pattern of linear hydrocarbons present that was more or less constant in proportions throughout the sample, but when these were taken together with the very variable amounts of oxygenated compounds also present, the percentage composition was rather variable, as indicated by the standard deviations found in Table 1. Of these hydrocarbons, tricosene was the major

TABLE 1. MAJOR COMPOUNDS IN DUFOUR GLAND SECRETION OF WORKER AND SOLDIER CASTES OF *Eciton burchelli*, PRESENTED, WHERE POSSIBLE, AS MEAN PERCENTAGE OF TOTAL SECRETION IN GLAND WITH STANDARD DEVIATION[a]

Compound	Workers ($N = 13$)[b] ($\bar{X} \pm$ SD)	Soldiers I ($N = 5$)[c] ($\bar{X} \pm$ SD)	Soldiers II ($N = 5$)[c] ($\bar{X} \pm$ SD)
2-Methylcyclopentanone	0.2 ± 0.2	t[d]	t
2-Methylcyclopentanol	0.3 ± 0.2	t	t
Cyclic acetal **4**	5.7 ± 1.9	—	c
6-Methyl-5-hepten-2-one	0.4 ± 0.3	t	c
6-Methyl-3-octanone	t		
(*Z*)-β-Ocimene	—	t	t
(*E*)-β-Ocimene	1.1 ± 2.0	[e]	t
7-Methyl-2-undecanone	1.1 ± 0.8		
7-Methyl-2-undecanol	0.8 ± 0.8		
Geranylacetone	4.9 ± 3.0	0.4 ± 0.6	4.4 ± 5.3
Pentadecane	0.6 ± 1.1		
Farnesol	(12.7)[f]		
Heptadecane	1.7 ± 0.4		
Nonadecene	2.1 ± 1.4		
Nonadecane	1.0 ± 0.4		
Geranyllinalool	—	44.2 ± 14.4	
Eicosene	2.0 ± 0.2		
Eicosane	0.2 ± 0.2		
Heneicosadiene	—	—	2.3 ± 1.5
Heneicosene	20.7 ± 5.2	8.6 ± 5.1	15.6 ± 9.6
Heneicosane	4.5 ± 1.4	— —	5.1 ± 1.0
Docosene	2.4 ± 1.3	2.6 ± 1.3	3.1 ± 0.2
Tricosene	42.2 ± 3.0	19.9 ± 8.9	37.0 ± 3.0
Tricosane	3.0 ± 1.1	1.6 ± 0.8	2.3 ± 1.0
Mean total amount (μg)	1.4 ± 0.6	4.7 ± 2.4	2.7 ± 1.7

[a] Soldiers have been divided into two groups, those containing (I) or not containing (II) geranyllinalool.
[b] Not all minor components listed, so total does not equal 100%, 36 substances were quantified, 20 of them hydrocarbons.
[c] Gaps in the columns means either the substance was not present or that amounts were small and quantitation too difficult.
[d] t = trace constituent, <0.5%, — = not detected.
[e] Present but very variable.
[f] Identified in only one individual.

one, followed by heneicosane, and thereafter a large number of C_{17}–C_{25} alkanes and alkenes in smaller proportion. The analysis was further complicated by finding geranyllinalool **(1)** (Figure 1) in large quantities in five of the soldiers' Dufour glands, together with variable amounts of (*E*)-β-ocimene **(2a)**, while

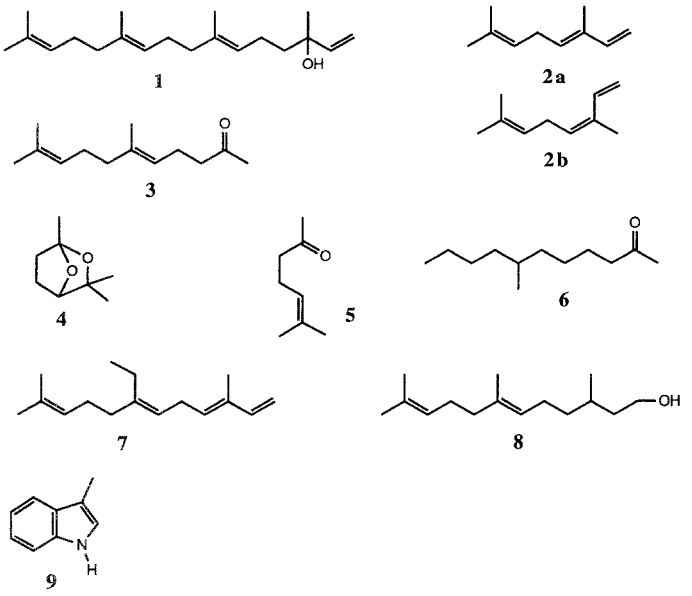

FIG. 1. Chemical structures: **1,** geranyllinalool; **2a,** (*E*)-β-ocimene; **2b,** (*Z*)-β-ocimine; **3,** geranylacetone; **4,** 1,3,3-trimethyl-2,7-dioxabicyclo[2,2,1]heptane; **5,** 6-methyl-5-hepten-2-one; **6,** 7-methyl-2-undecanone; **7,** (*E,E*)-α-homofarnesene; **8,** 2,3-dihydrofarnesol; **9,** skatole.

the other five soldiers did not contain geranyllinalool but had more geranylacetone **(3)** (Figure 2). Chemically, the latter group of soldiers more closely resembled the workers, which all contained geranylacetone but had no geranyllinalool. The workers' Dufour glands also contained the cyclic acetal 1,3,3-trimethyl-2,7-dioxabicyclo(2,2,1)heptane **(4)** and 6-methyl-5-hepten-2-one **(5),** which can be regarded as a biosynthetic precursor of **4.** Three groups of results are therefore presented in Table 1, and many minor components have had to be omitted for lack of useful quantitation. The species was notable for containing chemicals of very wide range of volatility from the very volatile cyclic acetal **4** to pentacosane. This is unusual in Dufour glands. It was also notable for having present very small quantities (individually less than 1% of total) of many other compounds, most of them identified by their mass spectra, e.g., methyl-branched hydrocarbons related to the major hydrocarbons, but also some other unidentified compounds. The quantities of secretion in glands of both workers and soldiers were large for ants of their size, with more than 1 μg secretion in workers and up to 8 μg in soldiers that contained geranyllinalool.

Geranyllinalool was identified by comparison of mass spectrum and retention time with an authentic sample contained in jasmine oil (Demole and Led-

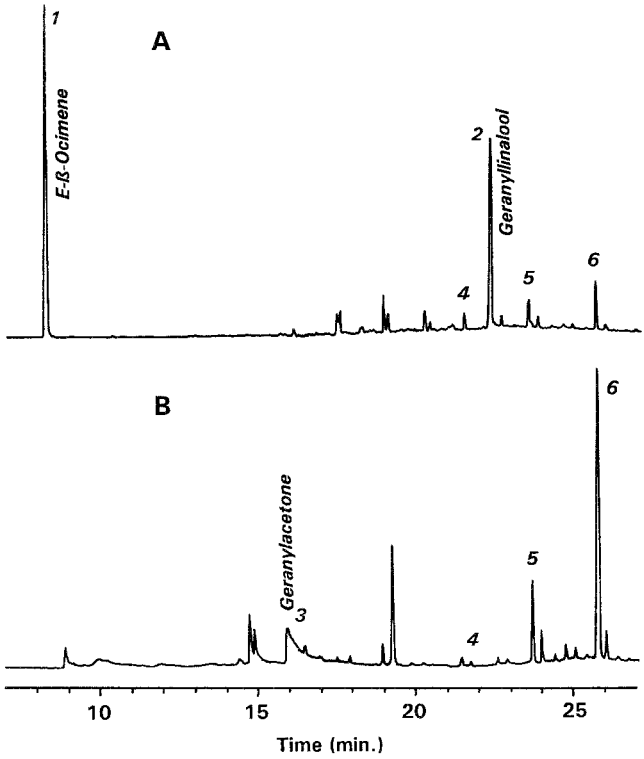

FIG. 2. Total ion chromatograms of the two different patterns seen in the secretions of Dufour glands of soldiers of *Eciton burchelli*: (A) one dominated by (*E*)-β-ocimene (peak 1) and geranyllinalool (peak 2), and (B) one where these are replaced by geranylacetone (peak 3); other peaks are nonadecane (4), heneicosene (5), and tricosene (6).

erer, 1958). 7-Methyl-2-undecanone **(6)** and 7-methyl-2-undecanol are not previously recorded in the chemical literature. The ketone was recognized as a methyl-branched 2-ketone by its mass spectrum and retention time. Possible structures were deduced using biosynthetic considerations and the correct structure was proved by synthesis. When the ketone was reduced to the corresponding alcohol, that was found to be identical with another minor product in the gland.

The Dufour glands of workers of *L. praedator* contained principally (*E*)-β-ocimene (**2a,** 83% of the total secretion, Table 2). The linear hydrocarbon nonadecene was the second most important compound. Minor amounts of (*Z*)-β-ocimene **(2b),** (*E,E*)-β-homofarnesene **(7),** (*E*)-2,3-dihydrofarnesol **(8)** and its acetate and hexadecyl acetate were also present. (*Z*)-β-Ocimene was identified by comparison with authentic material in a mixture of (*E*)- and (*Z*)-β-ocimene. (*E,E*)-β-homofarnesene was identical with that described by Attygalle and Mor-

TABLE 2. PERCENTAGE COMPOSITION OF DUFOUR GLAND SECRETIONS OF WORKERS AND SOLDIERS OF *Labidus praedator*[a]

Compound	Clean worker glands (% ± SD)	All worker glands (% ± SD)	Worker abdomens (%)	Soldier abdomens (%)
(*Z*)-β-Ocimene	1.2 ± 0.3	0.5 ± 0.1	1.4	0.5
(*E*)-β-Ocimene	83.1 ± 0.8	74.7 ± 11.2	42.1	74.7
Monoterpene isomer **1**	—	1.0 ± 0.9		1.0
Homoocimene	—	1.8 ± 0.7	0.3	0.3
Monoterpene isomer **2**	—	1.9 ± 0.6		0.4
2-Decanone	—	—	0.2	
Geraniol	—	—	0.4	
Indole	—	—	1.2	—
2-Undecanone	—	—	—	0.2
Methyl 6-methylsalicylate	—	0.4 ± 0.1		
Skatole	—	—	36.5	
(*E*,*E*)-β-Homofarnesene	—	0.1 ± 0.1	0.3	0.4
(*E*)-2,3-Dihydrofarnesol	1.7 ± 0.6	0.6 ± 0.3	0.8	1.6
Heptadecene	2.0 ± 0.3	1.2 ± 0.5	0.8	2.5
Heptadecane	1.2 ± 0.5	0.1 ± 0.1		0.8
(*E*,*E*)-Farnesol	—	0.3 ± 0.1	0.8	t
Farnesal	—			t
Dihydrofarnesyl acetate	0.6 ± 0.2	0.4 ± 0.2		0.5
Nonadecadiene	0.5 ± 0.1	1.6 ± 2.0	0.2	0.8
Nonadecene	9.6 ± 0.3	12.0 ± 4.6	5.7	7.7
Nonadecane	0.4 ± 0.2	0.6 ± 0.3	0.3	0.6
Hexadecyl acetate	0.6 ± 0.2	1.4 ± 1.8		
Geranyl-linalool	—	—	0.5	
Heneicosene	—	—	0.3	
Heneicosane	—	—	2.2	
Tricosadiene	—	—	0.6	
Tricosene	—	—	4.0	
Tricosane	—	—	1.2	
Mean total amount (μg)	86	90	34	490

[a]Worker samples were either carefully dissected free of other tissues ($N = 2$), or had some tissue attached ($N = 8$). Mean values for the two and ten samples are given. The mean values for whole abdomens of worker ($N = 20$) and soldiers ($N = 2$) are also given. — indicates substance not detected.

gan (1982). 2,3-Dihydrofarnesol and its acetate, prepared as described above, were identical in mass spectra and retention times with the ant compounds. Small differences in composition were found between cleanly dissected workers' Dufour glands and those to which the sting (but not the venom gland) and some tissue were still attached (Table 2). Some minor amounts of farnesol and some

unidentified monoterpenes were present in samples of the sting apparatus but were not found in the cleanly dissected Dufour glands, and it is presumed they come from some other structure. Whole abdomens were found to contain the same mixture of substances as the Dufour glands with the addition of skatole (3-methylindole, **9**), in quantity similar to that of the (*E*)-β-ocimene (Figure 3), and some higher hydrocarbons (C_{21} and C_{23}) probably from the cuticle (Table 2). The glands of soldiers were very similar to those of workers. The glands of *L. praedator* workers contained much less secretion than those of *E. burchelli* but soldiers' glands of *L. praedator* were correspondingly larger than those of workers of the same species.

The Dufour glands of *Labidus coecus* contained a much simpler mixture of substances, essentially (*E*)-β-ocimene **(2a)** in minor and medium workers and soldiers, with over 90% of this compound in all but one sample of a medium worker gland (Table 3). The amount of glandular secretion was intermediate in amount between that of *E. burchelli* and *L. praedator*.

The heads of both workers and soldiers of *E. burchelli* contained essentially 4-methyl-3-heptanone with much smaller amounts of 4-methyl-3-heptanol, two substances already identified in the mandibular glands of a number of ant species (Table 4).

The heads of *L. praedator* contained very small amounts of 2-nonanone, 2-decanone, and 2-undecanone, evidently from the mandibular gland since such volatile ketones are often found in mandibular glands (Attygalle and Morgan, 1984). There were rather large amounts of tricosane, tricosene, tricosadiene, heneicosane, and heneicosene, characteristic substances of the postpharyngeal

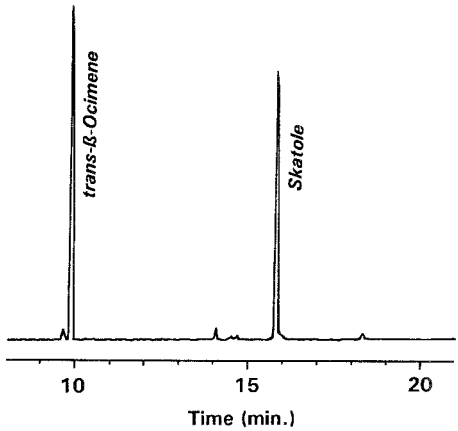

FIG. 3. Total ion chromatogram of the volatile compounds in a whole gaster of a worker of *L. praedator*. A Dufour gland chromatographed under the same conditions showed only one peak for β-ocimene.

TABLE 3. PERCENTAGE COMPOSITION OF DUFOUR GLAND SECRETIONS OF MINOR WORKERS, MEDIUM WORKERS, AND SOLDIERS OF *Labidus coecus* AND MEAN TOTAL AMOUNT OF SECRETION PER GLAND

Compound	Minor workers ($N = 6$)	Medium workers ($N = 3$)	Soldiers ($N = 4$)
(*Z*)-β-Ocimene	1.6	—	1.4
(*E*)-β-Ocimene	97.8	80.0	98
2-Nonanone	—	2.4	—
Nonanal	—	8.0	—
Decanal	—	10.4	—
Nonadecene	0.1	—	—
Tricosene	0.4	—	—
Mean total amount (μg)	0.62	0.25	0.94

TABLE 4. MEAN VALUES OF PERCENTAGE COMPOSITION OF VOLATILE COMPOUNDS IN HEADS OF WORKERS ($N = 5$) AND SOLDIERS ($N = 2$) OF *E. Burchelli*, WORKERS OF *L. praedator* (10 Heads in One Sample), AND MEDIUM WORKERS OF *L. coecus* ($N = 4$).

Compound	*E. burchelli*		*L. praedator* workers	*L. coecus* workers
	Workers	Soldiers		
4-Methyl-3-heptanone	92.6	91.2	—	89.6
4-Methyl-3-heptanol	7.4	8.9	—	8.3
2-Carene	—	—	9.8	—
1-Hexanol	—	—	11.2	—
Hexanal	—	—	4.5	—
2-Nonanone	—	—	14.2	—
Nonanal	—	—	17.1	—
2-Decanone	—	—	19.4	—
1-Decanol	—	—	—	2.1
2-Undecanone	—	—	17.0	—
Tridecane	—	—	6.7	—
Mean total amount (ng)	2,200	3,900	4.8	760

gland (Bagnères and Morgan, 1991), also present in this head sample, which are not included in Table 4.

The heads of *L. coecus* workers contained chiefly 4-methyl-3-heptanone. In this, and in the amount of secretion, they resembled *E. burchelli* much more than *L. praedator* (Table 4).

Venom glands were also dissected from three workers and two soldiers of

E. burchelli. The worker glands contained a very variable mixture of alkylpyrazines among other things (Table 5). The soldier glands were without volatile substances. Samples of sixth and seventh abdominal sternites from *E. burchelli* were also available. No volatile substances were detected in the samples of worker sternites. Of six soldiers' sternites prepared, two contained relatively large amounts of (~30 ng) of skatole, two contained small amounts (1–2 ng) of skatole, and two contained no detectable amount of skatole (<0.1 ng).

Samples of antennae, legs, abdominal cuticle, and the postpharyngeal glands of soldiers and workers of *E. burchelli* were also examined. The composition of hydrocarbons on the legs and abdominal cuticle and in the postpharyngeal glands was very similar; 9-tricosene, tricosane, heneicosane, pentacosane, and heptacosane (in decreasing order of importance) were all present. The antennae also contained these hydrocarbons but also up to 40% of cholesterol, not previously identified in cuticular wax.

DISCUSSION

Although the New World army ants of the subfamily Ecitoninae have been extensively studied in the field, the difficulty of confining them in a laboratory and keeping them alive with normal behavior has until now prevented laboratory-based studies of their exocrine secretions or pheromones. By the use of our microchemical studies on single isolated glands sealed in glass capillaries (Morgan, 1990), it has been possible to prepare glands from freshly collected ants to transport the samples and to carry out the analyses later. The great interest in this subfamily and the absence of chemical data on them made it interesting

TABLE 5. PERCENTAGE COMPOSITION OF VENOM GLANDS OF THREE SAMPLES OF WORKERS OF *Eciton burchelli*[a]

	Worker		
Compound	1	2	3
Dimethyldisulfide	24.8	—	—
Dimethyltrisulfide	20.7	—	—
2,5-Dimethylpyrazine	7.3	—	20.2
Trimethylpyrazine	—	35.5	—
3-Ethyl-2,5-dimethylpyrazine	4.4	64.5	27.5
Indole	32.8	—	52.2
2-Decanone	9.6	—	—
Total amount (ng)	642	4.5	2185

[a] Venom glands from two soldiers gave no volatile compounds.

to record these preliminary and fragmentary studies of three species of Ecitoninae. Of the five genera in the subfamily, we have examined two species of *Labidus* and one of *Eciton*. The Dufour glands of the two *Labidus* species were notable for having the relatively volatile monoterpene substance (*E*)-β-ocimene **(2a)** as the major substance while it was a minor substance in *E. burchelli*. Ocimene ($C_{10}H_{16}$) is a highly volatile substance with a pleasant odor for humans. It was first isolated from the leaves of the herb sweet basil (*Ocimum basilicum*). It is found in the leaves and flowers of many plants (cf. Sutton et al., 1992), along with other monoterpenes, and is a constituent of many perfumes. Although Dufour gland substances are usually less volatile than ocimene, monoterpenes have been found in other ants. *Myrmicaria natalensis* venom glands contain a mixture of pinene, sabinene, phellandrene, and camphene (Brand et al., 1974). β-Pinene was reported in the mandibular glands of *Atta sexdens* (Schildknecht, 1976) and perillene in the mandibular glands of *Lasius fuliginosus* (Bernardi et al., 1967). β-Ocimene is found frequently in the Isoptera, for example, in the frontal glands of soldiers of several species of termites (Baker et al., 1981). This is, however, the first time a monoterpene has been found as the major substance of the Dufour gland of an ant.

Ocimene was usually absent from the Dufour glands of *E. burchelli* workers or present only as minor substances, but it appeared as a major substance in some of the soldier glands. The workers of *E. burchelli* were more like that of many other ant species, having some oxygenated and terpenoid compounds in a mixture of higher alkanes, alkenes, and methyl-branched alkanes.

Another minor component of the Dufour glands was found to have a mass spectrum similar to that of ocimene **(2a, 2b)** but contains one more carbon atom (Figure 4). We have tentatively identified it as a homoocimene with the structure 3,7-dimethyl-1,3,6-nonatriene. Its mass spectrum does not correspond to the known 4,8-dimethyl-1,3,7-nonatriene. The presence of homofarnesene accompanying farnesene is a common occurrence in ants (Attygalle and Morgan, 1982). There are a number of other homologous sesquiterpenes, such as the juvenile hormones, but homomonoterpenes are very rare. Complete identification of the homoocimene will depend upon its unambiguous synthesis.

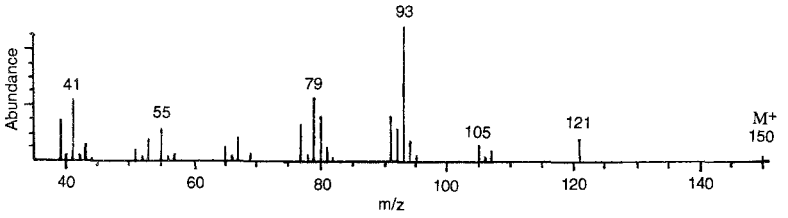

FIG. 4. Mass spectrum of the minor compound in the Dufour glands of *Labidus praedator*, identified as homoocimene.

E. burchelli is distinguishable from the two *Labidus* species by its very variable Dufour gland secretion. That of *E. burchelli* is much more like that of many formicine ants with a mixture of odorous terpene and oxygenated chemicals in a hydrocarbon mixture. The two cleanly dissected Dufour glands of *L. praedator* workers gave surprisingly consistent results (Table 2). The *Labidus* species, with their predominance of ocimene, are quite unusual. *L. praedator* and *L. coecus* can nevertheless be distinguished by the absence of linear hydrocarbons in *L. coecus*, but also they can be distinguished very easily by their mandibular gland secretions (Table 4).

Billen (1992) found that an extremely powerful and persistent trail pheromone was located in the seventh abdominal sternite of *E. burchelli*, and he was able to dissect and prepare samples of sixth and seventh sternite cuticle of several workers and soldiers of this species. We were unable to detect any volatile substances in the worker sternites but we identified skatole **(9)** in some soldier sternites. Since no skatole was detected in the Dufour glands of *E. burchelli*, it is possible the skatole is a product of the well-developed glandular epithelium on the seventh sternite described by Hölldobler and Engel (1978) for *E. hamatum* (see Hölldobler and Wilson, 1990, p. 235), and the possibility of skatole being the trail pheromone must be considered. The presence of relatively large quantities of skatole (and some of its homolog, indole) in the abdomens (but not the Dufour glands) of *L. praeditor* is also very interesting. Skatole has been found in the poison gland of major workers of *Pheidole fallax*, but no pheromone function has been assigned to it (Law et al., 1965). Indole has recently been shown to be one of the components of the trail pheromone from the poison gland of the ant *Tetramorium meridionale* (Jackson et al., 1990). We must await the opportunity to test the behavioral effect of skatole.

Eciton burchelli will require a more detailed examination to understand the variation in our results on the Dufour and venom glands. Is it possible that the two chemically distinct groups of soldiers reflect some morphologically or behaviorally distinct castes in this species or do they represent individuals at different stages of development in the nomadic–stationary cycle of colony life? The presence of cholesterol on the worker antennae also needs further study. We have discovered some interesting substances, unusual in ant secretions, some of them readily available, which we hope will stimulate interest in behavioral studies of the Ecitoninae.

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SPECIES DISCRIMINATION IN FIVE SPECIES OF WINTER-FLYING GEOMETRIDS (LEPIDOPTERA) BASED ON CHIRALITY OF SEMIOCHEMICALS AND FLIGHT SEASON

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Abstract—Enantiomer separation of (6*Z*,9*Z*)-*cis*-3,4-epoxynonadecadiene and (3*Z*,9*Z*)-*cis*-6,7-epoxynonadecadiene could be achieved using chiral high-resolution gas chromatography and a cyclodextrin-bond column. (3*Z*,9*Z*)-(6*R*,7*S*)-Epoxynonadecadiene was identified from ovipositor extracts of *Colotois pennaria*, while in *Erannis defoliaria* the 6*S*,7*R*-enantiomer was found. In field trapping tests pure synthetic enantiomers caught only conspecific males of these species. (3*Z*,6*Z*,9*Z*)-Nonadecatriene was found in both species, while the presence of (3*Z*,6*Z*,9*Z*)-heneicosatriene was indicated in *C. pennaria* only. A 10:10:3 blend of (3*Z*,9*Z*)-(6*R*,7*S*)-epoxynonadecadiene, (3*Z*,6*Z*,9*Z*)-heneicosatriene, and (3*Z*,6*Z*,9*Z*)-nonadecatriene was found to be optimal for catching *C. pennaria*, while *E. defoliaria* males were optimally caught by a 1:1 mixture of (3*Z*,9*Z*)-(6*S*,7*R*)-epoxynonadecadiene and (3*Z*,6*Z*,9*Z*)-nonadecatriene. (6*Z*,9*Z*)-(3*S*,4*R*)-Epoxynonadecadiene was identified from ovipositor extracts of *Agriopis (Erannis) aurantiaria*. In field tests the pure enantiomer proved to be a highly specific sex attractant for both the late autumn/early winter flying *A. aurantiaria* and the late winter/early spring flying *A. leucophearia*. Males of *Agriopis marginaria*, which fly in late win-

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ter/early spring, were attracted to (3*Z*,9*Z*)-(6*S*,7*R*)-epoxynonadecadiene. The addition of (3*Z*,6*Z*,9*Z*)-nonadecatriene to the *S*,*R*-enantiomer increased captures. Optimal catches were recorded with a 10:3 epoxide–hydrocarbon blend. Enantiomer specificity in all species was confirmed in EAG measurements.

Key Words—Chirality, enantiomers, sex pheromones, (3*Z*,9*Z*)-(6*R*,7*S*)-epoxynonadecadiene, (3*Z*,9*Z*)-(6*S*,7*R*)-epoxynonadecadiene, (6*Z*,9*Z*)-(3*S*,4*R*)-epoxynonadecadiene, *Colotois pennaria*, *Erannis defoliaria*, *Agriopis aurantiaria*, *A. marginaria*, *A. leucophearia*, Lepidoptera, Geometridae, electroantennogram, field trapping.

INTRODUCTION

The group of winter-flying geometrids (Lepidoptera) includes a few genera with a limited number of species. Adults are adapted to low ambient temperature: some of the species fly in late autumn/early winter, others in late winter/early spring. Females cannot fly: they have no or only rudimentary wings. In mate finding, males search for chemically signaling females. In order to reveal the chemical basis of species specificity in the communication channels, we studied the pheromone systems of the most frequently occurring Middle-European species of the *Erannis*/*Agriopis* genus, as well as of *Colotois pennaria* L. Among these, *Erannis defoliaria* Cl., *Agriopis aurantiaria* Hbn., and *C. pennaria* fly in late autumn/early winter, while the flight season for *A. marginaria* F. and *A. leucophearia* Den. et Schiff. is late winter/early spring (Vojnits, 1980). Since these species are regarded as occasional pests of deciduous forests and orchards in large areas in Europe (Escherich, 1931), the establishment of specific sex attractants for practical monitoring by means of pheromone traps was another objective of our study.

Earlier, (3Z,9Z)-*cis*-6,7-epoxynonadecadiene (3Z,9Z-*cis*-6,7-epo-19Hy) and (3Z,6Z,9Z)-nonadecatriene (3Z,6Z,9Z-19Hy) were identified as pheromone components of *E. defoliaria* (Hansson et al., 1990). The presence of the epoxide in *C. pennaria* was also indicated (Hansson et al., 1990). A mixture of the synthetic racemic epoxide and the triene was found to attract males of *E. defoliaria*, *A. marginaria*, and *C. pennaria* (Hansson et al., 1990). At that time the absolute configuration of the natural compounds remained unknown. Here we report on the identification of the absolute configuration of chiral epoxydienes found as female pheromone components of *E. defoliaria*, *C. pennaria*, and *A. aurantiaria*, and on the biological activity of enantiomerically pure synthetic samples in electroantennogram measurements and field trapping tests. In addition, the EAG response of *A. marginaria*, and field attraction of *A. marginaria* and *A. leucophearia* males towards enantiomerically pure epoxydienes were also tested.

METHODS AND MATERIALS

Insects. Cultures of *C. pennaria* and *E. defoliaria* were initiated from adults collected in forests near Budapest, Hungary. Adult males and females were kept outdoors in glass jars with a sheet of filter paper for egg-laying. Pieces of filter paper with some overwintered eggs were fastened to twigs of pear (*C. pennaria*) or cherry (*E. defoliaria*) trees before bursting of the buds in the experimental orchard of the Plant Protection Institute at Julianna-major (near Budapest). Subsequently, the whole twig was covered by a linen bag. When the larvae reached the last instar, the twigs were cut and placed in plastic containers (diameter: 42 cm; height: 16 cm). For pupation, a small amount of sterilized soil was added. Pupae were taken out of the soil in September, sexed, and placed in separate Petri dishes on sheets of slightly moistened filter paper. Emerging adults were collected daily and placed in glass jars. All developmental stages were kept outdoors under natural ambient temperature and photoperiod.

A culture of *A. aurantiaria* was initiated from last (or penultimate) instar larvae collected from oak trees at Síkfökút, Bükk Mountains, Hungary. This species was reared on oak trees at Síkfökút, in a similar way as described above.

Extracts. Ovipositors of unmated calling females were extracted in redistilled hexane or pentane as described earlier (Hansson et al., 1990).

Synthesis. Pure enantiomers **6** and **7** were prepared as shown in Figure 1. The approach is based on the acetylene methodology used by Mori and Ebata (1986) and Mori and Takeuchi (1989) and resembles that of Becker et al. (1990) and Millar et al. (1990a). Nonylbromide **1** was elongated three times with protected propargyl alcohol to yield the triynol derivative **4.** Hydrogenation over P-2-Ni (Brown and Ahuja, 1973) followed by Sharpless epoxidation of the resulting trienols (30 hr at −30°C) afforded the enantiomers of the epoxy alcohol **5.** Treatment of the corresponding tosylates with lithium dimethylcuprate yielded the target epoxydienes **6** and **7,** which were chromatographed on silica gel (230–400 mesh, elution with *n*-hexane–ethyl acetate 100 : 1). The final products showed a chemical purity of ca. 98% containing ca. 2% of epoxyenes produced by partial overhydrogenation of the epoxydienes. Chiral gas chromatography (see below) showed the enantiomeric purity of the epoxides to be ca. 95% ee (ee = enantiomeric excess; here: 97.5 : 2.5) $\{[\alpha]_D^{21}$ (6) = −3.7° (c = 0.96, CH_2Cl_2) and $[\alpha]_D^{21}$ (7) = +3.7° (c = 1.18, CH_2Cl_2)$\}$. As compared to results reported in the literature (Millar et al., 1990a), our products showed higher optical purities, which may be due to the lower temperatures used in our procedure. The synthesis of both enantiomers of 3*Z*,9*Z*-*cis*-6,7-epo-19Hy has been described in detail by Mori and Brevet (1991). The products showed a chemical purity of higher than 99.5% and an optical purity of at least 99% (chiral gas chromatography).

Chemical Analysis. Structure elucidation of the target compounds was car-

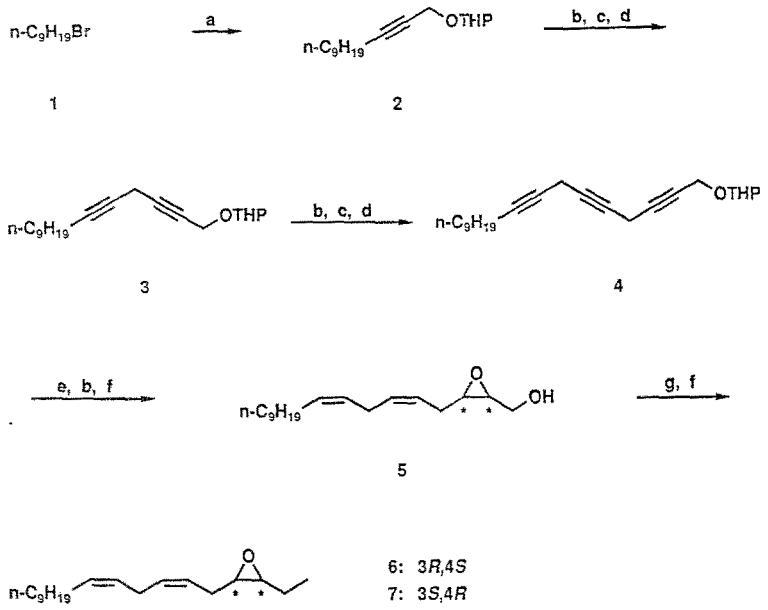

FIG. 1. Syntheses of pure enantiomers of (6Z,9Z)-*cis*-3,4-epoxydienes: (a) $THPOCH_2C{\equiv}CLi$/liq.NH_3/DMSO/$-33°C \rightarrow 20°C$; (b) *p*-TsOH/MeOH/20°C; (c) $PPh_3Br_2/CH_2Cl_2/-20°C$; (d) $THPOCH_2C{\equiv}CMgBr$/CuCl/THF/40°C; (e) H_2 P2-Ni/EtOH/20°C; (f) $Ti(OiPr)_4$/diethyl tartrate/*t*BuOOH/$CH_2Cl_2/-30°C$/30hr; (g) TsCl/KOH/Et_2O/0°C; (h) $Me_2CuLi/Et_2O/-60°C \rightarrow -30°C$.

ried out by combined gas chromatography–mass spectroscopy (GC-MS) under the same conditions as described earlier (Hansson et al., 1990). Mass spectra and gas chromatographic retention times (coinjection) of racemic samples served as references. Enantiomeric separation of chiral epoxides was achieved by gas chromatography using a 1 : 1 mixture of heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin and OV-1701 as the stationary phase and hydrogen (2 ml/min) as the carrier gas. Synthesis of the cyclodextrin as well as preparation of our tailor-made columns have been described in detail (König et al., 1992, Pietruszka et al., 1992). Separation conditions were carefully optimized. (3Z,9Z)-*cis*-6,7-Epoxynonadecadiene (3Z,9Z-*cis*-6,7-epo-19Hy) was analyzed on a 50-m, 0.25-mm-ID fused silica column held at 135°C for 1 hr, programmed to 155°C within 2 min, then held at 155°C. Under these conditions the enantiomers showed an α value, i.e., $R_t(6R,7S):R_t(6S,7R)$, of 1.02 (Figure 2). Assignment of absolute configuration of naturally occurring epoxydienes was performed by chiral gas chromatography using synthetic optically active reference compounds (Figure 2B and C). The (6Z,9Z)-*cis*-3,4-epoxynonadecadienes were analyzed

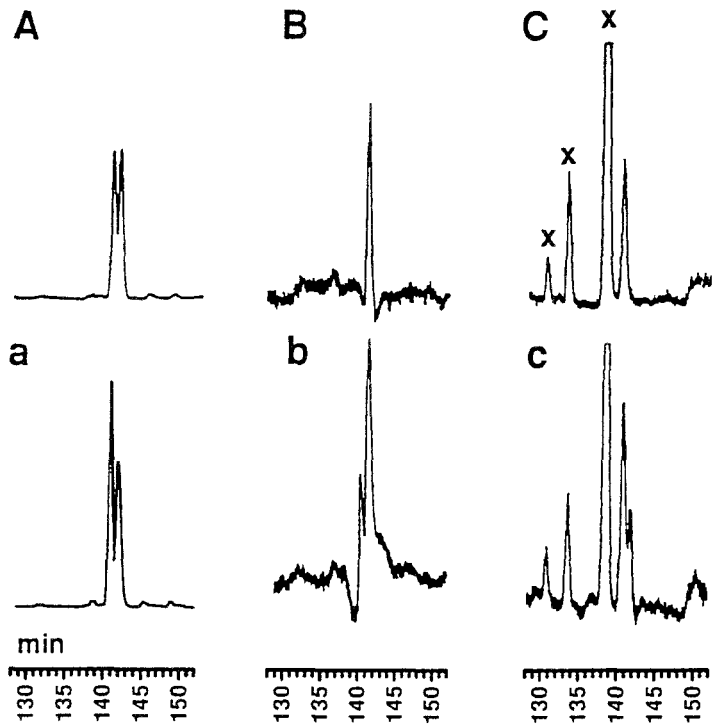

FIG. 2. Enantiomeric separation of 3*Z*,9*Z*-*cis*-6,7-epo-19Hy on a 1:1 mixture of heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin and OV-1701 (FID detector); for conditions see text. (A) racemate; (a) racemate + 6*R*,7*S* enantiomer; (B) *E. defoliaria* extract; (b) racemate + *E. defoliaria* extract; (C) *C. pennaria* extract; (c) racemate + *C. pennaria* extract. X: hydrocarbons.

on a 25-m, 0.25-mm-ID fused silica column programmed from 80°C to 145°C within 4 min, then held at 145°C.

Under these conditions, the enantiomers showed an α value, i.e., $R_t(3\,S,4R):R_t(3R,4S)$, of 1.02. An α value of 1.02 at a retention time of 140 min (as in the present case) represents a difference in retention times of almost 3 min, which enables one to clearly distinguish between the enantiomers.

Due to slight tailing of the peaks, the detectable amounts of enantiomeric impurities contained in the test compounds depend on whether they elute before or after the corresponding main stereoisomer. The first-eluting enantiomers were easily detectable in less than 0.1% contained in the synthetic samples and in ca. 0.5–1% in the gland extracts, while the minimum detectable amounts of later-eluting enantiomers was 0.5–1% in the synthetic samples and 2–3% in the gland extracts. As compared to synthetic compounds, determination of the enan-

tiomeric composition of natural products is less exact due to less material being available (higher signal-to-noise ratio) and the presence of other contaminants.

Electroantennograms. Electroantennograms (EAG) were recorded from excised male antennae, with platinum electrodes set at the tip and the basal part of the antenna. Connection between the electrodes and the insect tissues was maintained by an electrically conducting gel (Valleylab, Boulder, Colorado). Responses were amplified by a high-impedance amplifier (Rumbo, 1981), and displayed on an OH 850 chart recorder (Radelkis, Budapest, Hungary). Test compounds in required amounts were applied on a 10 × 10-mm piece of filter paper positioned inside a Pasteur pipet. Stimuli were provided by injecting 1 ml of air through the Pasteur pipet into an airstream (0.3 m/sec) flushing over the antenna. The interval between stimuli was at least 1 min. Responses were normalized against responses to a common standard, which was administered before and after the series of stimuli. In addition to males reared according to the above-mentioned procedure, feral males collected by a live-catching light trap were used in these experiments.

Field Trapping. Triangular traps with sticky bottoms (sticky material: Tanglefoot, Tanglefoot Co., Grand Rapids, Michigan) were used, similar in shape and size as described by Arn et al. (1979), but made of transparent plastic sheets. Test compounds were applied in hexane solution to 1 × 1-cm pieces of rubber tubing (Taurus, Budapest, Hungary, MSZ 9691/6). In the field, traps were suspended from branches at a height of 1–1.5 m above ground level in a complete block design (Tóth et al., 1992). At inspections, captured moths were recorded, sticky bottoms exchanged, and traps moved one position further within the block. Trappings were conducted in mixed oak forests, near Budapest, Hungary. In statistical analysis, catches recorded at each trap inspection were regarded as replicates. Trap capture data were statistically analyzed after transformation to log $(x + 1)$, tested for homogenity (Bartlett's test), and then submitted to a one-way ANOVA. If the F value was significant, differences between mean catches were then tested for significance by Duncan's new multiple-range test (Steel and Torrie, 1960).

RESULTS

Colotois pennaria (Feathered Thorn)

Chemical Analysis. Using ovipositor extracts, GC-MS analysis on a DB-5 column (Hansson et al., 1990) showed the presence of (3Z,6Z,9Z)-nonadecatriene (3Z,6Z,9Z-19Hy), 3Z,9Z-*cis*-6,7-epo-19Hy, and (3Z,6Z,9Z)-heneicosatriene (3Z,6Z,9Z-21Hy) in a ratio of about 1:2:0.1. Traces of (3Z,9Z)-*cis*-6,7-epoxyheneicosadiene (3Z,9Z-*cis*-6,7-epo-21Hy) and (3Z,6Z)-*cis*-9,10-epoxyheneicosadiene (3Z,6Z-*cis*-9,10-epo-21Hy) were also detected. Gas chromatog-

raphy on a modified cyclodextrin phase proved the natural epoxynonadecadiene to be the 6*R*,7*S*-enantiomer showing high optical purity (Figure 2C).

Electroantennograms. Higher responses were elicited from male antennae by (3*Z*,9*Z*)-(6*R*,7*S*)-epoxynonadecadiene (3*Z*,9*Z*-6*R*,7*S*-epo-19Hy) as compared to its enantiomer, in a dose range of 1–0.001 μg (Figure 3A). The dose–response relationship on a semilogarithmic scale showed a tight linearity with an increase at higher doses, characterized by a higher slope for the *R*,*S*-enantiomer.

Field Trapping. In a preliminary trial 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy attracted males in large numbers, while no males were caught by the *S*,*R*-enantiomer (Figure 4).

In optimizing the ratio of 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy, 3*Z*,6*Z*,9*Z*-21Hy, and 3*Z*,6*Z*,9*Z*-19Hy, the addition of any of the two hydrocarbons to the epoxide slightly enhanced captures over ranges of 30–1000%, and 30–100% for 3*Z*,6*Z*,9*Z*-21Hy and 3*Z*,6*Z*,9*Z*-19Hy, respectively (Figure 5). Combined addition of both hydrocarbons resulted in a superposed enhancement. The highest capture was recorded at the 10:10:3 ternary blend of 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy, 3*Z*,6*Z*,*Z*-21Hy, and 3*Z*,6*Z*,*Z*-19Hy. The addition of 1.5, 5, 15, and 50 μg of 3*Z*,9*Z*-*cis*-6,7-epo-21Hy as a fourth component to the above-mentioned ternary mixture at a dose of 50:50:15 μg exerted no effect on captures (Nagy-Hárshegy, Budapest, Hungary, November 12–18, 1991, 10 traps/bait combination). In preliminary tests the two hydrocarbons on their own attracted no males into traps (Kétbükkfa-nyereg, Pilis Mountains, Hungary, November 11, 1990–January 27, 1991, six replicates).

Erannis defoliaria (Mottled Umber)

Chemical Analysis. Using ovipositor extracts, GC-MS analysis on a DB-5 column confirmed the presence of 3*Z*,6*Z*,9*Z*-19Hy and 3*Z*,9*Z*-*cis*-6,7-epo-19Hy in a ratio of about 1:3 (Hansson et al., 1990). Gas chromatography on a modified cyclodextrin proved the natural product to be the 6*S*,7*R*-enantiomer, showing high optical purity (Figure 2B).

Electroantennograms. Higher responses were elicited from male antennae by 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy as compared to its enantiomer in a dose range of 1–0.01 μg (Figure 3B). The dose–response relationship on a semilogarithmic scale showed a tight linearity with an increase at higher doses, characterized by a higher slope for the *S*,*R*-enantiomer.

Field Trapping. In a test with pure synthetic enantiomers of 3*Z*,9*Z*-*cis*-6,7-epo-19Hy, only the *S*,*R*-enantiomer attracted *E. defoliaria* males to traps (Figure 4).

In a further test aimed at studying the effect of the addition of 3*Z*,6*Z*,9*Z*-19Hy to the activity of enantiomerically pure 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy, signifi-

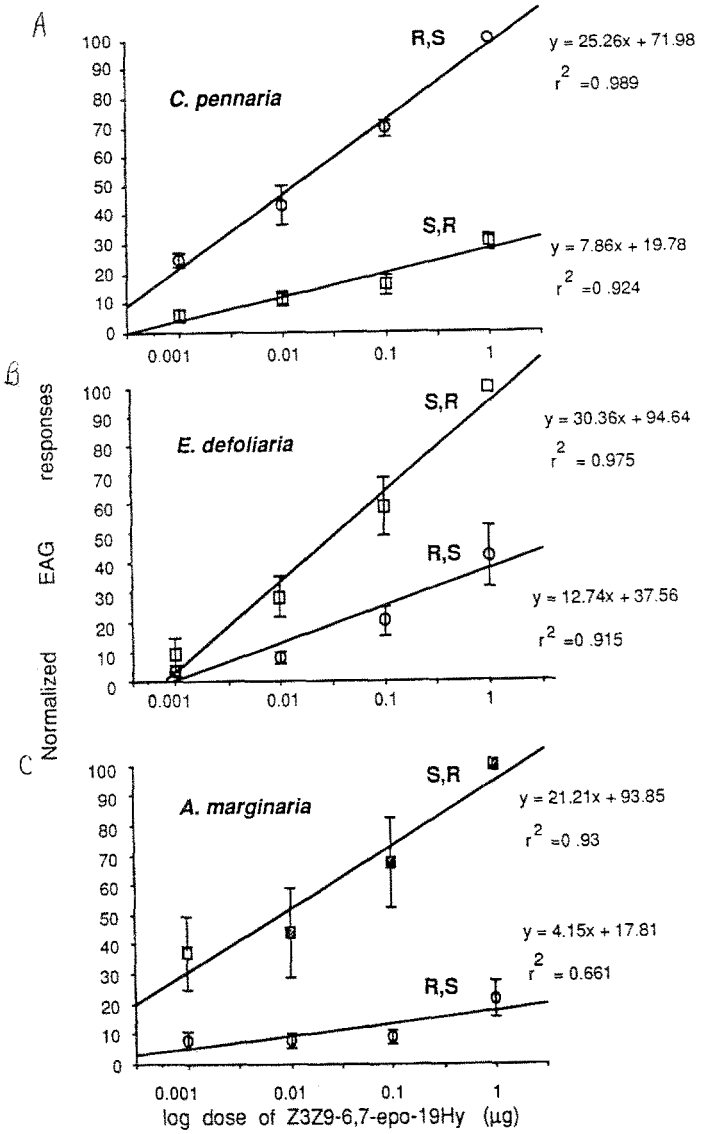

FIG. 3. Electroantennogram responses of *C. pennaria* (A), *E. defoliaria* (B), and *A. marginaria* (C) to *S,R* and *R,S* enantiomers of 3*Z*,9*Z*-*cis*-6,7-epo-19Hy. Responses were normalized against the response to 1 µg of 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy (*C. pennaria*), or to 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy (*E. defoliaria* and *A. marginaria*). Stimuli were tested consecutively on five male antennae. Bars represent SD.

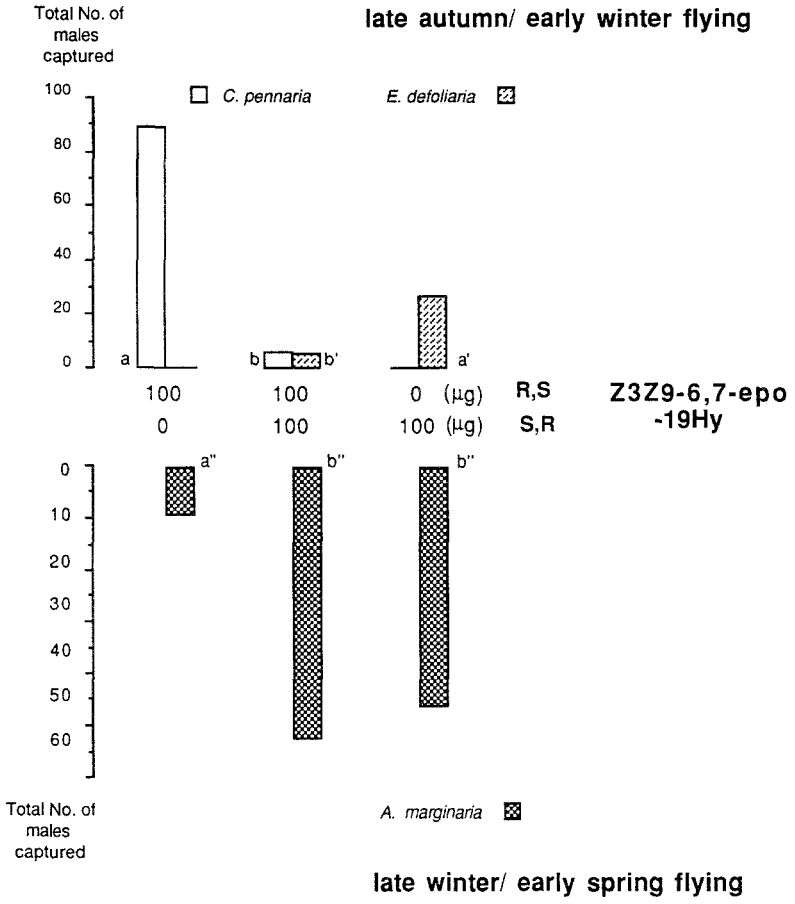

FIG. 4. Field captures of *C. pennaria*, *E. defoliaria*, and *A. marginaria* males in traps with *S,R* and *R,S* enantiomers of 3Z,9Z-*cis*-6,7-epo-19Hy. *C. pennaria* and *E. defoliaria*: Julianna-major, near Budapest, Hungary, November 2–29, 1991; 5 traps/bait combination. *A. marginaria*: Nagy-Hárshegy, Budapest, Hungary, February 27–March 26, 1992; 5 traps/bait combination. ANOVA—*C. pennaria*: $F = 36.93$, $P < 0.1\%$; *E. defoliaria*: $F = 9.95$, $P < 5\%$; *A. marginaria*: $F = 9.39$, $P < 0.1\%$. Captures followed by same letters within a species are not significantly different at the 5% probability level (Duncan's new multiple-range test). Catches significantly differ from zero catch within a species at the 5% probability level (Wilcoxon's signed-ranks two-tailed test).

cantly higher catch was recorded at the 1 : 1 mixture (Table 1) than the epoxide alone. In preliminary tests, 3Z,6Z,9Z-19Hy on its own attracted no males into traps.

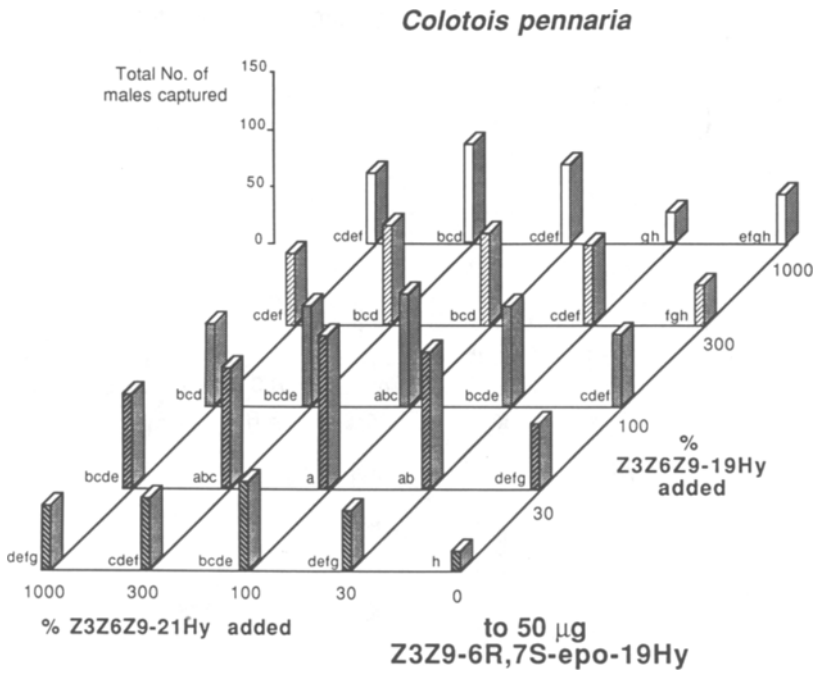

FIG. 5. Field captures of *C. pennaria* males by various ternary mixtures of 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy, 3*Z*,6*Z*,9*Z*-21Hy, and 3*Z*,6*Z*,9*Z*-19Hy. Nagy-Hárshegy, Budapest, Hungary, October 24–November 11, 1991; 8 traps/bait combination. ANOVA—$F = 5.28$, $P < 0.1\%$. Captures followed by same letters are not significantly different at 5% probability level (Duncan's new multiple-range test).

Agriopis aurantiaria (Scarce Umber)

Chemical Analysis. Using ovipositor extracts, GC-MS analysis on a DB-5 column confirmed the presence of (6*Z*,9*Z*)-*cis*-3,4-epoxynonadecadiene (6*Z*,9*Z*-*cis*-3,4-epo-19Hy). Gas chromatography on a modified cyclodextrin revealed the compound to be the 3*S*,4*R*-enantiomer.

Electroantennograms. Higher responses were elicited from male antennae by 6*Z*,9*Z*-3*S*,4*R*-epo-19Hy as compared to its enantiomer at 1- and 0.1-µg doses (100 vs. 48.8, and 34.6 vs. 5.4, respectively, five antennae). Responses were generally low at lower doses and did not differ significantly from each other.

Field Trapping. Large catches were recorded with 6*Z*,9*Z*-3*S*,4*R*-epo-19Hy (Figure 6). While the *R*,*S*-enantiomer did not catch, the racemic mixture, produced by mixing equal amounts of the *S*,*R* and *R*,*S*-enantiomer, caught many fewer males as compared to catches with the *S*,*R*-enantiomer. When the same test was run in early spring, the *S*,*R*-enantiomer attracted males of the spring

TABLE 1. FIELD CAPTURES OF *E. defoliaria* AND *A. marginaria* AT VARIOUS BLENDS OF Z3,Z9-6*S*,7*R*-epo-19Hy AND Z3,Z6,Z9-19Hy[a]

Bait composition (μg)		Total No. of males captured	
Z3,Z9-6*S*,7*R*-epo-19Hy	Z3,Z6,Z9-19Hy	*E. defoliaria*	*A. marginaria*
50		28 bc	62 c
50	0.5	13 c	60 c
50	1.5	45 abc	77 c
50	5	51 ab	103 bc
50	15	84 ab	181 a
50	50	93 a	176 a
50	150	not tested	171 ab

[a] *E. defoliaria:* Nagy-Hárshegy, Budapest, Hungary, November 25–December 6, 1991; 10 traps/treatment. *A. marginaria:* Julianna-major, near Budapest, Hungary, March 2–16, 1992; 10 traps/bait composition. ANOVA—*E. defoliaria:* $F = 9.40$, $P < 0.1\%$; *A. marginaria:* $F = 4.83$, $P < 0.1\%$. Captures followed by same letters within a species are not significantly different at the 5% probability level (Duncan's new multiple-range test).

umber, *A. leucophearia* to traps (Figure 6). Four specimens were also caught by the racemic mixture.

Agriopis marginaria (Dotted Border)

Electroantennograms. Higher responses were elicited from male antennae by 3Z,9Z-6*S*,7*R*-epo-19Hy as compared to the *R*,*S*-enantiomer, in a dose range of 1–0.001 μg (Figure 3C). The dose–response relationship on a semilogarithmic scale showed a linearity with an increase at higher doses, characterized by a higher slope for the *S*,*R*-enantiomer.

Field Trapping. In a test with pure enantiomers of the 3Z,9Z-*cis*-6,7-epo-19Hy, the *S*,*R*-enantiomer attracted males in significantly higher numbers than the *R*,*S*-enantiomer (Figure 4). A racemic mixture attracted males in similar numbers as compared to the pure *S*,*R*-enantiomer.

Mixtures of 3Z,9Z-6*S*,7*R*-epo-19Hy with 3Z,6Z,9Z-19Hy in ratios of 10:3 to 1:3 caught significantly more males than the epoxide alone (Table 1). In preliminary tests, 3Z,6Z,9Z-19Hy on its own attracted no males into traps.

DISCUSSION

Gas chromatographic separation of enantiomers of chiral epoxydienes produced as pheromones by female geometrid species has been achieved for the first time. Our analysis enabled unambiguous assignment of the absolute con-

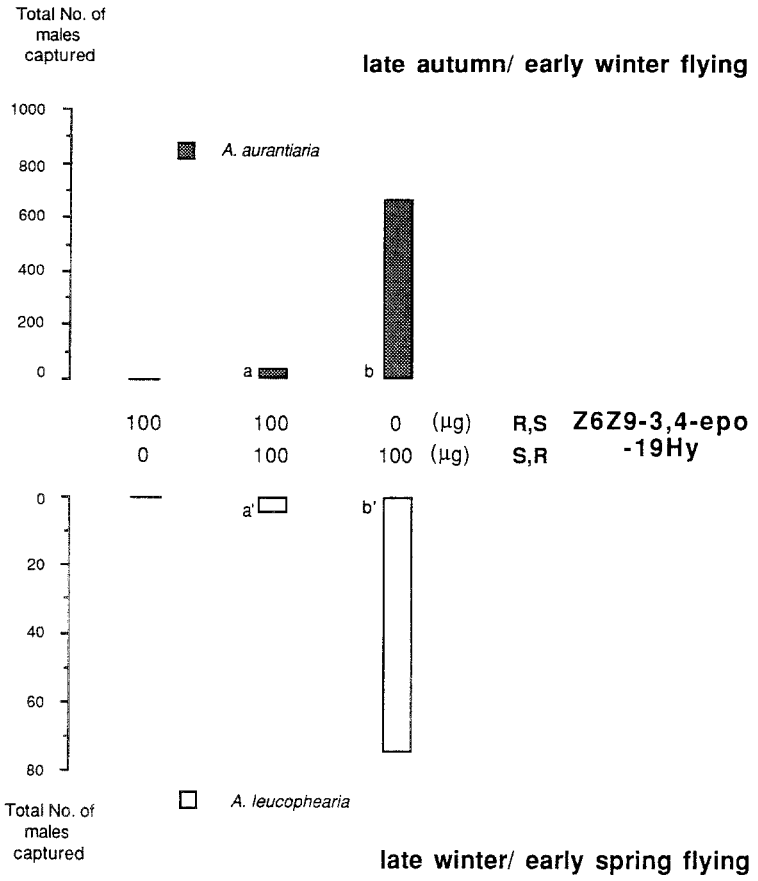

FIG. 6. Field captures of *A. aurantiaria* and *A. leucophearia* males at *S,R* and *R,S*-enantiomers of 6*Z*,9*Z*-*cis*-3,4-epo-19Hy. *A. aurantiaria*: Nagy-Hárshegy, Budapest, Hungary, November 11–15, 1991; 10 traps/bait combination *A. leucophearia*: Julianna-major, near Budapest, Hungary, March 2–13, 1992; 2 traps/bait combination. ANOVA—*A. aurantiaria*: $F = 457.7$, $P < 0.1\%$; *A. leucophearia*: $F = 7.34$, $P < 5\%$. For further details of statistical analysis, see Figure 4.

figuration of the natural products and revealed that the species under investigation produce these pheromone components in high enantiomeric purity. In this study, 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy was identified as a female-produced pheromone component of *C. pennaria*, and it was shown to attract only conspecific males in the field. Its enantiomer was identified from *E. defoliaria* and found to attract males of this species. No interspecific attraction was observed: each enantiomer attracted only males of the respective species. When both enantio-

mers were present in a ratio of 1 : 1 (racemate), captures of both species were significantly lower as compared to captures at the corresponding pure enantiomers. In EAG studies, significantly higher responses were evoked in the two species by the corresponding naturally occurring enantiomers than by the "nonnatural" enantiomer. It can not be ruled out that responses attributed to the "nonnatural" enantiomers were actually evoked by the natural enantiomers that occurred in extremely low percentages in the synthetic samples. 3*Z*,6*Z*,9*Z*-19Hy was also identified from both *C. pennaria* and *E. defoliaria*; however its bis homologe, 3*Z*,6*Z*,9*Z*-21Hy was present only in the former species. The hydrocarbons per se were not attractive, but when added to the corresponding epoxide, showed a catch-increasing effect in both species. The ecology of these two species broadly overlaps: they live in the same habitats, their flight period partially overlaps (Escherich, 1931), and the diurnal rhythm of calling and mating also coincides (Szöcs & Tóth, unpublished). It seems that the absolute configuration of the epoxide pheromone component forms the basis for maintaining species-specificity in the pheromonal communication between *C. pennaria* and *E. defoliaria*.

Chirality seems to play an important role also in the pheromonal communication of all other *Erannis*/*Agriopis* species dealt with in the present study. 6*Z*,9*Z*-3*S*,4*R*-epo-19Hy was identified as a pheromone component of *A. aurantiaria*, and males of both the late autumn/early winter flying *A. aurantiaria* and the late winter/early spring flying *A. leucophearia* were trapped by using this enantiomer. Its antipode did not catch males of these species, and when added to 6*Z*,9*Z*-3*S*,4*R*-epo-19Hy, catches drastically decreased, indicating an antagonistic effect. Males of *A. marginaria* were better attracted to 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy, showing a close relation to *E. defoliaria* with respect to males' attraction. Further investigation is needed, however, to reveal the chemical and enantiomeric composition of the pheromone produced by *A. marginaria* females. It is interesting to note that *S*,*R*-enantiomers of the 3,4- or 6,7-epoxynonadecadienes were found to be the pheromone components and/or attractants for males of all *Erannis*/*Agriopis* species investigated in this study, while an *R*,*S*-enantiomer was found the essential pheromone component in *C. pennaria*, a species only distantly related to *Erannis*/*Agriopis* spp.

This study casts some light on parallelisms in chirality-based separation of pheromonal channels between late autumn/early winter and late winter/early spring flying species. 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy is an essential sex pheromone component for the late autumn/early winter flying *E. defoliaria* and a sex attractant for the late winter/early spring flying *A. marginaria*. However, the addition of the 6*R*,7*S*-enantiomer decreased captures of *E. defoliaria*, but did not affect captures of *A. marginaria*. The different influence of the enantiomer may reflect species specificity in the communication channel: during the flight period of *E. defoliaria*, the *R*,*S*-enantiomer is a key pheromonal component of *C. pennaria*,

which flies at the same time. In contrast, no species was found that used this compound during the flight period of *A. marginaria*. As for 6*Z*,9*Z*-3*S*,4*R*-epo-19Hy, it is the sex pheromone of the late autumn/early winter flying *A. aurantiaria* and a sex attractant for the late winter/early spring flying *A. leucophearia*. There again, pheromonal communication channels are based on the same compounds, which are used by different species in late autumn/early winter or in late winter/early spring, respectively.

Based on the present study, the following attractant combinations are recommended for practical monitoring (numbers represent dosages in micrograms on rubber septa): for *C. pennaria*, a 50:50:15 blend of 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy, 3*Z*,6*Z*,9*Z*-21Hy, and 3*Z*,6*Z*,9*Z*-19Hy; for *E. defoliaria*, a 50:50 blend of 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy and 3*Z*,6*Z*,9*Z*-19Hy; for *A. aurantiaria* and for *A. leucophearia*, 100 μg of 6*Z*,9*Z*-3*S*,4*R*-epo-19Hy; for *A. marginaria*, a 50:15 blend of 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy and 3*Z*,6*Z*,9*Z*-19Hy.

Among known chiral sex attractant or pheromone components of Lepidoptera, monoepoxide derivatives of homoconjugated polyenes represent the most frequent type, occurring exclusively in Geometridae, Arctiidae, and in some subfamilies of Noctuidae (Arn et al., 1992). Of the epoxide components identified in the present study, 3*Z*,9*Z*-6*S*,7*R*-epo-19Hy was reported by Millar et al. (1990b) as a pheromone component of another geometrid, *Eufidonia convergaria*. After assignment of the gross structure, field tests with optically active samples strongly indicated the natural product to show 6*R*,7*S* configuration. The same compound was also described as a sex attractant component for geometrids, *Anavitrinella pampinaria* (Millar et al., 1990a), *Caripeta angustiorata* (Millar et al., 1990b), *Hypagyrtis piniata* (Underhill, cited in Arn et al., 1992), as well as for the noctuid, *Rivula propinqualis* (Millar et al., 1990b). 3*Z*,9*Z*-6*R*,7*S*-epo-19Hy was reported as a sex attractant component for the geometrid, *Probole amicaria* (Millar et al., 1990a). 6*Z*,9*Z*-*cis*-3,4-epo-19Hy was identified as a sex pheromone component of *Ascotis selenaria* (Geometridae) (Becker et al., 1990, Cossé et al., 1992). The 3*S*,4*R* configuration showed high biological activity in both EAG studies and field tests, while the enantiomer proved to be much less active. It remains, however, unknown whether the natural pheromone is optically pure or represents a mixture of enantiomers. 6*Z*,9*Z*-(3*S*,4*R*)-epo-19Hy was also reported as a sex attractant for the two geometrids, *Probole americana* and *Sicya macularia* (Millar et al., 1990a). Our results represent the first unambiguous structure assignments of chiral epoxydiene pheromones by direct chemical method.

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ROLE OF ALLELOPATHY IN HAY-SCENTED FERN INTERFERENCE WITH BLACK CHERRY REGENERATION

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Abstract—Black cherry (*Prunus serotina* Ehrh.) seedlings survive and grow poorly under dense hay-scented fern (*Dennstaedtia punctilobula* Michx.) ground cover in the understory of partially cut Allegheny hardwood stands. Previous field studies showed that there were about 80% fewer black cherry seedlings where fern was present than where it was absent. Allelopathic interference with black cherry seed germination, seedling survival, and growth by hay-scented fern foliage leachates, root washings, and soil transformation products was evaluated in a series of field, greenhouse, and laboratory experiments. Black cherry seeds germinated as well in the presence of hay-scented fern or its leachates as when they were absent in both the laboratory and the field. Fern foliage leachates and root washings did not affect black cherry growth in sand or natural soil cores in the greenhouse. There also was no evidence that hay-scented fern natural products or their soil transformation products built up in the soil. A two-year manipulative field experiment to separate effects of hay-scented fern foliage shade from foliar leaching showed that foliage shade significantly reduced black cherry seedling survival and growth; foliage leachates had no effect. Results of the studies led to the conclusion that allelopathy does not play a direct role in hay-scented fern interference with black cherry seedling establishment in partially cut Allegheny hardwood stands.

Key Words—Allelopathy, interference, *Prunus serotina*, *Dennstaedtia punctilobula*, Allegheny hardwood type, regeneration failures.

INTRODUCTION

Significant difficulty has been encountered in regenerating the cherry–maple (*Prunus–Acer*) Allegheny hardwood forests on the Allegheny Plateau in north-

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western Pennsylvania, U.S.A. Herbivory by white-tailed deer [*Odocoileus virginianus virginianus* (Boddaert)] is considered the most important reason for regeneration failure (Marquis and Brenneman, 1981; Tilghman, 1989). Selective browsing by deer has either eliminated or greatly reduced the abundance of palatable species in the forest understory. The abundance of unpalatable and browse-tolerant species has increased greatly (Horsley, 1992). Black cherry (*Prunus serotina*) is low in palatability and frequently is the only important timber species present in the understory (Grisez and Peace, 1973). Hay-scented fern (*Dennstaedtia punctilobula*), an aggressive weed of agricultural fields, roadsides, and forests in the northeastern United States and eastern Canada, is unpalatable to deer and has expanded greatly in many partially cut (thinned or shelterwood seed cut) Allegheny hardwood stands (Horsley, 1981). Where dense fern cover is present, there are about 80% fewer black cherry seedlings, and surviving seedlings invariably fail to become established and grow even when they are protected from deer for as long as five years (Horsley, 1977b; Horsley and Marquis, 1983). Thus, hay-scented fern interferes strongly with regeneration of black cherry.

Plant–plant interference may occur through a variety of biotic and abiotic mechanisms including allelopathy, resource competition, or other biotic interactions such as herbivory (Harper, 1977; Horsley, 1991). In 1985, a comprehensive series of experiments was undertaken to evaluate the mechanisms of interference between hay-scented fern and black cherry. A series of alternate hypotheses was made to evaluate the roles of allelopathy and resource competition for soil water, soil nitrogen, phosphorus, and light. Results of experiments to evaluate resource competition are presented elsewhere (Horsley, 1993). Direct allelopathic interference of a donor plant with a receiver may occur through effects of natural products released by the donor in foliage leachings (Nilsson and Zackrisson, 1992), root washings (Rovira, 1969), rhizosphere transformation products (Dalton et al., 1983), or through volatilization (Muller, 1969) on seed germination (Nilsson and Zackrisson, 1992) or seedling growth (Horsley, 1977a) of the receiver. Allelopathy also may occur indirectly through effects of donor natural products or rhizosphere transformation products on mycorrhizal fungi, which help plants acquire phosphorus (Rose et al., 1983), or on soil organisms which control fixation, mineralization, and nitrification of nitrogen (Rice and Pancholy, 1972). This report contains the results of field, greenhouse, and laboratory experiments to evaluate the role of direct allelopathic interference between hay-scented fern and black cherry.

METHODS AND MATERIALS

Design of Experiments. The present work builds on previously reported field and greenhouse studies of allelopathic interference between hay-scented fern and black cherry (Horsley, 1977b; Horsley and Marquis, 1983). In this

work, field tallies of black cherry abundance in the presence of hay-scented fern were made on an annual basis, so it was not clear whether reduced numbers of black cherry seedlings were the result of failure of seeds to germinate or of poor seedling growth and survival, or both. A greenhouse dose–response experiment in which black cherry seedlings were grown in sand cultures watered with hay-scented fern foliage soak leachates suggested that fern reduced the growth of black cherry seedlings, although reductions were small and not statistically different from the control (Horsley, 1977b).

The present work attempted to avoid the pitfalls of earlier experiments by using methods that accounted for field conditions at the time of symptom development. Experiments conducted indoors or in semisterile media (sand, peat) suggesting the presence of allelochemical interference were verified with field experiments in forest soil. All experiments compared seeds or seedlings growing with fern plants or a dose–response series of fern leachates against a no-fern or no-leachate control. Previous work showed that black cherry seed germination and seedling growth after exhaustion of cotyledonary reserves were sensitive to allelochemical interference (Horsley, 1977a). Both of these growth responses were evaluated. Fern effects on black cherry seed germination are presented first, followed by experiments to evaluate the effects of fern foliage leachings, root and rhizome washings, and microbial soil transformation products on seedling growth.

Field Sites. Field studies were established in Allegheny hardwood stands at two sites within a few kilometers of each other on the Allegheny National Forest. Both sites were underlain by the Cookport soil series (Aquic Fragiudults), which comprises a major portion of the Allegheny Plateau on the Allegheny National Forest. These soils are medium textured, moderately well-drained, and have a fragipan 20–90 cm below the soil surface. Both stands had received a partial overstory removal (shelterwood seed cut), leaving 60% relative density in the overstory; cuttings were made at site 1 in April 1986, and at site 2 in 1980 (Stout et al., 1987). These conditions were typical of those where black cherry regenerates if fern ground cover is not present but fails to regenerate if fern ground cover is dense (Horsley and Marquis, 1983). Site 1 was used for the field seed germination experiment and site 2 for the fern foliage shading/leaching field experiment described in the section that follows. Soil cores for several greenhouse and growth-chamber experiments also were collected at site 2.

Phenology of Black Cherry and Hay-Scented Fern. The protocol used to make preparations containing potentially allelopathic natural products should mimic the conditions prevailing in nature during symptom development because the phenology of the donor and receiver species may be important factors in detection of allelopathy (Horsley, 1991). In northwestern Pennsylvania, overstory tree leaves generally emerge in mid-May; black cherry flowers appear in

late May to early June (Grisez, 1974). Seeds are shed from late August to early September (Grisez, 1974). A minimum of four months of moist, low temperature in the forest floor is required to relieve dormancy. Some seeds germinate the following growing season, while others remain dormant in the forest floor seed bank for as long as three to five years (Marquis, 1975). Seed germination usually begins in early to mid-April.

Hay-scented fern fronds emerge from preformed, partially expanded fiddleheads on a perennial rhizome early in May (Horsley, 1984). Frond expansion is complete by mid-to late June. Mature fronds form a canopy 60–75 cm above the forest floor. This canopy remains intact (green) until early September when fronds begin to senesce (yellow). By early to mid-October, senescence of all fronds is complete, and they stand as brown skeletons on the forest floor. With the first snows in November, the withered fronds are bent to the ground.

Preparation of Foliage Leachates. Foliage leachate preparation was based on the average amount of fern foliage on 15-cm-radius (0.09 m^2) plots at site 2 and the 25-year average amount and frequency of rainfall for Warren, Pennsylvania, during the months of June through August (NOAA, 1985). The mean amount of fern foliage on 20 randomly located plots in a portion of the stand with 100% fern ground cover was 13 $\pm$ 0.6 fronds/0.09 m^2. At the end of the growing season in early September 1985, 13 fronds weighed about 80 g fresh wt (13.5 g dry wt). The 25-year average rainfall for Warren, Pennsylvania, is 23,168 ml during June through August in 24 rainfall events (2/week). Leonard (1961) reported that about 10% of the rainfall in northern hardwood stands was intercepted by trees; thus, about 20,851 ml or 869 ml/rainfall event/0.09 m^2 should be available for leaching fern fronds.

Fern foliage leachates were prepared in a concentration series by leaching 0, 0.5, 1, or 2 times ($\times$) the amount of foliage found on 0.09-m^2 field plots with 869 ml of distilled water adjusted to the pH of local rainwater (4.1, H_2SO_4). The 0 $\times$ leachate control was pH-adjusted distilled water. Since the intensity of rainfall influences the amount and quality of natural products leached, fern foliage was leached in two ways: misting and soaking (Tukey, 1970). Foliage was collected weekly (foliage leachate watering experiment) or as needed (laboratory seed germination experiment) at site 2. Leachates were prepared at the 2$\times$ concentration; other concentrations were made from the 2$\times$ leachate by dilution with pH-adjusted water. Mist leachate was made by suspending foliage in plastic screen baskets and spraying with a fine mist of pH-adjusted water. Foliage drip was collected in glass containers. Soak leachate was made by covering foliage with pH-adjusted water in glass containers for 16 hr at 4°C. Leachate to be used in germination experiments was cold sterilized by filtration (0.22 μm) under vacuum; leachate to be used in the watering experiment was not sterilized. Osmotic potential (Wescor HR-33T and C-52 sample chamber) was determined on three samples and pH on one sample of each freshly prepared

leachate. Leachates not used immediately were stored in glass containers at −20°C for a maximum of four days. Leachates applied to sand medium in the foliage leachate watering experiment were amended immediately before use with one-quarter strength, complete, balanced (for cations and anions) mineral nutrient stock solutions containing nitrate as the nitrogen source (Machlis and Torrey, 1956). All other leachates were unamended.

Potting Media, Seedling Selection, Greenhouse Conditions, and Seedling Measurements. Two potting media were used in the experiments, natural soil cores, which preserved the structural, organic matter, and microbial characteristics of the forest soil, and semisterile sand. Soil cores were removed in 15-cm-long × 10-cm-diameter pieces of polyvinyl chloride (PVC) pipe from an area of site 2 with no fern cover. After extraction, the pipes were fitted with perforated pipe caps to make them suitable as pots. Similar pipes were filled with sand. Differences in results among treatments between sand and soil media were attributed to the chemical, physical, and biological properties of the media. Soil was considered the standard against which allelopathic interference must be shown to attribute regeneration failures to the participation of allelopathy.

Seedlings used in the foliage extract watering and the root and rhizome washing experiments were prepared by planting pregerminated, half-sib black cherry seeds from a single seed lot in the PVC pipe containers of sand or soil cores and growing them in the greenhouse for three weeks until cotyledonary reserves were exhausted (Horsley, 1977a). Replicate groups of seedlings with similar leaf development and height were selected for treatment application.

Greenhouse temperatures varied depending on the time of year. Evaporative coolers maintained maximum temperatures at 30°C. Natural daylength was extended to 16 hr with cool white fluorescent lamps.

At harvest, seedling height, number of leaves ≥20 mm long (Horsley and Gottschalk, 1993), and dry weight (70°C) of leaves, stems, and roots were measured. In the foliage extract watering and the root and rhizome washing experiments, foliar nutrient concentrations also were determined. After weighing, the middle 50% of the leaves from plants in each treatment were composited, petioles were removed, and the leaves were ground to pass a 20-mesh sieve. Analysis of foliar nutrient concentrations was performed by the University of Minnesota Research Analytical Laboratory (Munter, 1982). Total nitrogen was determined by the Kjeldahl method. P, K, Ca, Mg, Mn, Al, Fe, Na, Zn, Cu, and B were determined simultaneously by inductively coupled plasma (ICP) atomic-emission spectrometry.

Laboratory Seed Germination Experiment. The effect of fern foliage leachates on black cherry seed during the overwinter dormancy-breaking period was tested in the laboratory by stratifying black cherry seeds in peat moistened with frond mist or soak leachates obtained in autumn when foliage was alive and green (September 13), senescing and yellow (October 11), or dead and brown

(October 29). Treatments included two methods of leachate preparation, three times of foliage collection, four leachate concentrations (0×, 0.5×, 1×, and 2×), and five replicates. Each treatment combination was applied to 100-seed replicates obtained from a single half-sib seed lot in a zip-lock polyethylene bag containing 30 g of horticultural peat moistened with 270 ml of leachate. Bags were stored at 4°C for 166 days, then moved to plant growth chambers with 25°C, 16-hr days and 15°C, 8-hr nights. Seed germination counts began 125 days after bags were placed in storage and continued at 14-day intervals until germination was complete, 194 days after storage. Seeds were considered germinated when the radical reached 5 mm in length. Germination was expressed as a percentage of the starting number of seeds and as the germination value (*GV*), a measure of speed and completeness of germination (Czabator, 1962):

$$GV = PV \times MDG$$

where *PV*, the peak value, is the largest value of the quotient of the cumulative germination percent per day since the beginning of the germination test and *MDG* is the mean daily germination for the test.

Field Seed Germination Experiment. Black cherry seeds also were stratified naturally in the forest floor at site 1 in areas with (90%) and without (0%) hay-scented fern ground cover. The two areas were about 10 m apart and differed only in the presence or absence of hay-scented fern. In each fern cover treatment, 15-cm-long × 10-cm-diameter pieces of PVC pipe were driven into the ground to within 1 cm of the soil surface on October 17. Ten half-sib seeds were sown on the soil surface inside each of 20 replicate pipes and covered with 1 cm of moist sand to ensure good contact with the soil. Tops of the pipes were covered with plastic screen to exclude extraneous seeds and rodent depredation. The pipes were covered naturally by falling leaves and fern fronds. Seeds remained in the forest floor for 137 days until the pipes were moved to a greenhouse on March 3. Seed germination counts were made at one to three day intervals, beginning 14 days after movement to the greenhouse, until germination was complete (44 days).

Foliage Leachate Watering Experiment. The effect of fern foliage leachates on growth of black cherry seedlings that had exhausted cotyledonary reserves was tested in a greenhouse experiment. Seedlings were grown in sand and soil cores. Five replicate groups of eight seedlings were selected from each potting medium for random assignment to treatment with mist or soak leachate in one of four leachate concentrations. Seedlings were grown for 60 days from July 17 to September 16. Each seedling received 100 ml of leachate 2×/week. Leachates applied to sand medium were amended with nutrients; those applied to soil were not amended.

Root and Rhizome Washing Experiment. The effect of washings from fern roots and rhizomes on the growth of black cherry seedlings was tested in another

greenhouse experiment. Hay-scented fern was grown in sand from rhizome segments for six months in ten 19-liter plastic pots fitted with thin polyethylene inner liners. After ferns became established they were watered 3×/week with 500 ml of a complete mineral nutrient solution using nitrate as the nitrogen source (Machlis and Torrey, 1956); on other days they were watered with a similar quantity of distilled water. In a preliminary experiment, it was found that the effluent from pots of fern had a lower nitrate concentration than pots of sand with no fern (Horsley, unpublished). The disparity in nitrate was large enough to cause significant differences in growth of black cherry seedlings receiving the effluents. To prevent confounding of nutritional differences in the pot effluent with differences due to the presence of potentially allelopathic fern root and rhizome natural products, effluent from fern pots was collected in bulk and half of the effluent was percolated through a glass column filled with Amberlite XAD-4 styrene divinyl benzene resin (Tang and Young, 1982) prepared according to the method of Junk et al. (1974) and Tateda and Fritz (1978); the other half of the effluent was unaltered. XAD-4 resin removes hydrophobic organic molecules but does not retain inorganic molecules. Thus, two watering solutions were made from the effluent of potted fern plants: one with and one without potentially allelopathic hydrophobic natural products. Fern root and rhizome washings were prepared in this manner 2×/week on days when distilled water was used to water fern pots. These two watering solutions were applied at the rate of 100 ml/pot to black cherry seedlings grown in sand and soil cores. There were 10 replicate seedlings in each treatment. Treatments were applied for 1.5 months from July 8 to August 22.

Soil Transformation Products Experiment. Some investigators have noticed that soil in which one plant is grown becomes toxic to other plants at certain times of the year, particularly after the annual accumulation of litter (Martin et al., 1972; Kimber, 1973; Dalton et al., 1983), or after a period of years when a threshold of toxicity is reached (Reitveld et al., 1983). This hypothesis was tested by growing black cherry seedlings in soil cores removed from two areas of site 2 that were only a few meters apart, one with dense ground cover of hay-scented fern (100%) and the other with no fern (0%). Twenty fern cores and 20 no fern cores were removed August 27, just prior to fern foliage senescence, and April 22, just prior to spring seed germination. Fern fronds were trimmed to the top of each core, and any fern regrowth was trimmed as it appeared to prevent shading of black cherry seedlings. Each core was planted with a germinated black cherry seed, and seedlings were grown in a plant growth chamber under standard conditions for two months: 335 μmol/m^2/sec photosynthetic photon flux density from cool white fluorescent and incandescent lamps; 25°C, 16-hr days and 18°C, 8-hr nights; humidity was not regulated. Each seedling was watered daily with distilled water.

Foliage Leaching/Shading Field Experiment. Two possible influences of

fern foliage on black cherry seedlings growing beneath them are shading and leaching of allelopathic natural products. The effect of presence or absence of fern foliage shade and presence or absence of fern foliage leachates on black cherry seedling survival, height, and diameter growth was measured in a two-year field experiment at site 2. Sixty points were selected in dense fern cover prior to fern emergence in the spring. At each point, a 15-cm-long × 10-cm-diameter core of soil was removed in PVC pipe and replaced with a similar core taken from an area of the stand without fern. Use of PVC pipe prevented interaction between below-ground organs of fern and seedling roots inside the pipe. Each pipe was planted with germinated black cherry seed on May 6. Fifteen replicates of four treatments were applied to these 60 pipes. The four treatments were: fern foliage and leachate present; fern foliage present, fern leachate absent; fern foliage absent, fern leachate present; and fern foliage and leachate absent.

The presence or absence of foliage shade was controlled by allowing fern foliage to close over the top of seedlings or by restraining fern foliage behind a fiberglass rod and string barrier as it grew. Leaching of potentially allelopathic fern foliage natural products was controlled by erecting 58 × 58-cm canopies of clear polyethylene film over the top of ferns and seedlings. A rainwater collector with a water delivery tube from the collector to the pot was placed outside each canopy either above or below fern cover to simulate the absence or presence of leachate from fern foliage. Seedling survival, height, and diameter 2.54 cm above the ground were made one and two growing seasons after treatment in October after all growth had stopped. The polyethylene canopies were removed from November until May to prevent overwinter damage.

Statistical Analysis. Least-squares analysis of variance was used to test for differences in seed germination or measures of seedling survival or growth due to the treatments. Replicates were treated as random effects and treatments as fixed effects in the analysis of variance. Percentage data were transformed with the arc-sin transformation prior to analysis. Means were separated using the Bonferroni procedure in SYSTAT (Wilkinson, 1988). Linear regression was used to investigate the relationship between leachate osmotic potential and pH on the germination value and seed germination percentage. The coefficient of determination (R^2) was used as a measure of the strength of the relationship; $\alpha = 0.05$ was the value of P accepted as significant.

RESULTS

Laboratory Seed Germination Experiment. Time of foliage collection (foliage condition) did not significantly effect either the germination value ($P = 0.091$) or the final germination percentage ($P = 0.241$) (Table 1). Method of

TABLE 1. GERMINATION VALUES AND FINAL GERMINATION PERCENTAGE ± SE FOR BLACK CHERRY SEEDS STRATIFIED WITH FOLIAGE LEACHATES OF HAY-SCENTED FERN PREPARED BY MISTING OR SOAKING GREEN, YELLOW, OR BROWN FOLIAGE IN LABORATORY SEED GERMINATION EXPERIMENT

	Foliage condition							
	Green		Yellow		Brown		All conditions	
	Mist	Soak	Mist	Soak	Mist	Soak	Mist	Soak
	Germination Value							
Leachate Preparation Method Mean[a]	0.096 ± 0.006 a	0.070 ± 0.010 b	0.112 ± 0.010 a	0.085 ± 0.006 b	0.096 ± 0.008 a	0.081 ± 0.004 a	0.101 ± 0.005 a	0.079 ± 0.003 b
Foliage Condition Mean[b]	0.083 ± 0.005 a		0.098 ± 0.006 a		0.088 ± 0.004 a		0.090 ± 0.003	
	Final Germination %							
Leachate Preparation Method Mean[a]	54 ± 2 a	47 ± 3 b	58 ± 3 a	50 ± 2 b	53 ± 3 a	50 ± 1 a	55 ± 1 a	49 ± 1 b
Foliage Condition Mean[b]	50 ± 2 a		54 ± 2 a		52 ± 1 a		52 ± 1	

[a] Leachate mist and soak means for the same foliage condition followed by the same letter were not significantly different.
[b] Foliage condition means followed by the same letter were not significantly different.

leachate preparation was associated with small, but significant differences in both the germination value ($P = 0.000$) and the final germination percentage ($P = 0.001$) (Table 1, All Conditions). Both measures were higher for mist than for soak leachate preparation. Neither the germination value ($P = 0.840$) nor final germination percentage ($P = 0.675$) were affected by including foliage leachates of up to 2× concentration (data not shown).

Mean osmotic potential was in the order: green (-0.024 ± 0.002 MPa) > yellow (-0.012 ± 0.001 MPa) > brown (-0.009 ± 0.001 MPa) foliage leachate. Leachate prepared by soaking (-0.019 ± 0.002 MPa) had higher osmotic potential than that prepared by misting (-0.011 ± 0.001 MPa). Osmotic potential of leachates increased with leachate concentration; however the highest osmotic potential measured in any leachate was only -0.039 MPa in 1× and 2× green soak leachate.

The mean pH of all leachates (5.83 ± 0.05) was higher than that of the leaching water (4.26) used. pH was in the order: brown (6.02 ± 0.09) > yellow (5.81 ± 0.06) > green (5.31 ± 0.12) foliage leachate. Green (6.13 ± 0.11) and brown (6.43 ± 0.09) mist leachates had higher pH than corresponding soak leachates (5.20 ± 0.01, 5.62 ± 0.00), respectively. For yellow foliage leachate, the reverse was true (mist = 5.51 ± 0.05, soak = 6.12 ± 0.01). Leachate pH generally increased with increase in leachate concentration.

Linear regressions of osmotic potential, pH, and osmotic potential + pH on the germination value accounted for little of the variance in the relationship. Coefficients of determination (R^2) were, respectively, 0.068 ($P = 0.009$), 0.011 ($P = 0.303$),and 0.069 ($P = 0.031$). Nor did these variables contribute substantially to the variance of the final germination percentage. Coefficients of determination were, respectively, 0.063 ($P = 0.012$), 0.007 ($P = 0.410$), and 0.063 ($P = 0.043$).

Field Seed Germination Experiment. There were no significant differences in the germination value or the final germination percentage for seeds stratified overwinter in an Allegheny hardwood stand with fern than for seeds stratified without fern. Germination values with and without fern were, respectively, 0.058 ± 0.005 and 0.061 ± 0.004 ($P = 0.582$); final germination percentage values with and without fern were, respectively, 74 ± 4 and 76 ± 3 ($P = 0.514$).

Foliage Leachate Watering Experiment. In general, black cherry plants grown in sand with complete mineral nutrient solution increased more in height, dry weight, and number of leaves than plants grown in soil cores (Table 2). Seedlings grown in soil cores were unaffected by leachates made with up to twice the amount of fern foliage found at site 2, regardless of whether the leachate was produced by misting or soaking foliage. Seedlings potted in sand generally grew more in treatments where leachate was present than in control treatments where it was absent; growth of leachate-treated seedlings was not

TABLE 2. HEIGHT, DRY WEIGHT, AND NUMBER OF LEAVES ± SE OF BLACK CHERRY SEEDLINGS POTTED IN SAND OR SOIL CORES RECEIVING HAY-SCENTED FERN LEACHATE PRODUCED BY MISTING OR SOAKING FOLIAGE IN FOLIAGE LEACHATE WATERING EXPERIMENT

Leachate concentration	Growth measures					
	Height (mm)	Leaf dry wt (g)	Root dry wt (g)	Shoot dry wt (g)	Total dry wt (g)	Leaves (*N*)
Mist foliage leachate–soil core medium[a]						
0×	143 ± 45 a	0.403 ± 0.176 a	0.463 ± 0.209 a	0.193 ± 0.096 a	1.059 ± 0.480 a	5.6 ± 1.4 a
0.5×	168 ± 26 a	0.586 ± 0.113 a	0.693 ± 0.140 a	0.271 ± 0.060 a	1.550 ± 0.304 a	7.6 ± 1.5 a
1×	184 ± 12 a	0.708 ± 0.058 a	0.691 ± 0.047 a	0.264 ± 0.026 a	1.664 ± 0.102 a	8.6 ± 0.2 a
2×	176 ± 63 a	0.923 ± 0.385 a	0.601 ± 0.322 a	0.312 ± 0.178 a	1.836 ± 0.780 a	9.6 ± 1.4 a
Mist foliage leachate–sand medium[a]						
0×	245 ± 69 a	1.238 ± 0.369 a	1.053 ± 0.248 a	0.527 ± 0.156 a	2.818 ± 0.686 a	11.0 ± 2.5 a
0.5×	338 ± 18 a	1.943 ± 0.083 b	1.413 ± 0.121 a	0.896 ± 0.176 a	4.253 ± 0.244 b	13.8 ± 0.7 a
1×	373 ± 42 b	2.177 ± 0.097 b	1.289 ± 0.075 a	0.961 ± 0.129 b	4.426 ± 0.199 b	14.6 ± 1.0 b
2×	412 ± 52 b	2.221 ± 0.073 b	1.394 ± 0.168 a	1.093 ± 0.155 b	4.708 ± 0.147 b	14.6 ± 0.8 b
Soak foliage leachate–soil core medium[a]						
0×	182 ± 33 a	0.767 ± 0.130 a	0.607 ± 0.159 a	0.274 ± 0.082 a	1.648 ± 0.295 a	8.2 ± 1.2 a
0.5×	143 ± 38 a	0.656 ± 0.201 a	0.653 ± 0.122 a	0.209 ± 0.069 a	1.518 ± 0.365 a	6.2 ± 1.4 a
1×	166 ± 33 a	0.682 ± 0.093 a	0.606 ± 0.067 a	0.241 ± 0.059 a	1.529 ± 0.176 a	7.8 ± 1.2 a
2×	169 ± 45 a	0.603 ± 0.206 a	0.595 ± 0.194 a	0.211 ± 0.067 a	1.409 ± 0.466 a	6.2 ± 1.7 a
Soak foliage leachate–sand medium[a]						
0×	304 ± 24 a	1.603 ± 0.108 a	1.156 ± 0.070 a	0.648 ± 0.092 a	3.407 ± 0.255 a	12.2 ± 0.4 a
0.5×	290 ± 28 a	1.774 ± 0.166 a	1.275 ± 0.097 a	0.675 ± 0.036 a	3.723 ± 0.228 a	12.8 ± 1.1 a
1×	355 ± 16 a	1.777 ± 0.068 a	1.346 ± 0.061 a	0.884 ± 0.034 b	4.008 ± 0.078 a	12.4 ± 0.8 a
2×	296 ± 10 a	1.565 ± 0.124 a	1.067 ± 0.070 a	0.625 ± 0.051 a	3.257 ± 0.232 a	13.0 ± 0.4 a

[a] Values of the same growth measure potted in the same medium and receiving the same foliage leachate followed by the same letter were not significantly different from the 0× leachate control.

less than control seedlings in any treatment. Seedlings in sand watered with mist leachate grew significantly more in height, leaf, shoot, and total dry weight and produced more leaves under treatment than control seedlings. There was no difference in dry weight of roots among seedlings receiving mist leachate. Soak leachates had no effect on growth of seedlings potted in sand medium. Foliar nutrient concentration data was not tested statistically because it was measured on composite samples from each treatment; however, there were no obvious trends in the data related to the treatments (data not shown).

Root and Rhizome Washing Experiment. Use of the XAD-4 resin technique to create two watering solutions that were nutritionally similar was successful. Foliar nutrient concentrations of all measured elements were similar for plants grown in the same potting medium. Foliar nitrogen concentrations are shown in Table 3; other elements are not shown. There was no evidence that constituents of hay-scented fern root and rhizome washings interfered with black cherry seedling growth. Regardless of whether seedlings were grown in soil cores or sand, there were no significant differences in any growth attribute measured (Table 3).

Soil Transformation Products Experiment. There was no evidence that hay-scented fern natural products or soil transformation products built up in the soil by the end of the growing season in August or by the beginning of the next growing season in April. Black cherry height, dry weight, and number of leaves were not significantly different for seedlings grown in soil cores removed from areas of site 2 with fern than those without fern cover in either August or April (Table 4).

Foliage Leaching/Shading Field Experiment. Small mammals clipped some seedlings from each treatment in the experiment (Table 5). Most animal-caused mortality occurred during the first growing season and was distributed nearly equally among treatments. These seedlings were removed from the experiment and evaluation of the results was based on the remaining seedlings. Survival of black cherry seedlings growing in fern foliage shade was half as great the first growing season and one tenth as great the second growing season as that of seedlings growing where fern foliage was restrained behind a barrier (Table 5). By the end of the second year, seedling survival averaged 4% for shaded treatments and 82% for unshaded treatments. Differences in seedling height and stem diameter among treatments were small at the end of the first growing season (Table 5). By the end of the second growing season, only one seedling remained in the shaded treatments. There was no difference in height or diameter of seedlings due to presence or absence of fern foliage leachate in the unshaded treatment.

DISCUSSION

None of the evidence in this study supports the hypothesis that interference between hay-scented fern and black cherry is mediated by direct allelopathic effects of fern on black cherry seed germination, seedling survival, or growth.

TABLE 3. HEIGHT, DRY WEIGHT, NUMBER OF LEAVES ± SE AND FOLIAR NITROGEN CONCENTRATION OF BLACK CHERRY SEEDLINGS POTTED IN SAND OR SOIL CORES RECEIVING HAY-SCENTED FERN ROOT AND RHIZOME WASHINGS WITH OR WITHOUT ORGANIC NATURAL PRODUCTS IN ROOT AND RHIZOME WASHING EXPERIMENT

Organic natural products	Growth measures						
	Height (mm)	Leaf dry wt (g)	Root dry wt (g)	Shoot dry wt (g)	Total dry wt (g)	Leaves (*N*)	Foliar N (%)[a]
Soil core medium[b]							
Present	514 ± 34 a	3.156 ± 0.345 a	0.814 ± 0.092 a	1.493 ± 0.195 a	5.464 ± 0.608 a	25.5 ± 1.1 a	3.12
Not Present	542 ± 51 a	3.378 ± 0.419 a	0.931 ± 0.077 a	1.694 ± 0.315 a	6.003 ± 0.762 a	26.6 ± 1.7 a	3.01
Sand medium[b]							
Present	490 ± 25 a	2.670 ± 0.168 a	1.021 ± 0.071 a	1.079 ± 0.099 a	4.769 ± 0.312 a	25.9 ± 0.4 a	2.10
Not Present	424 ± 31 a	2.485 ± 0.284 a	1.009 ± 0.098 a	0.951 ± 0.116 a	4.445 ± 0.481 a	24.1 ± 1.2 a	2.07

[a] Analysis of foliar nitrogen (N) was conducted on composite samples; no statistical analysis was made.
[b] Values of the same growth measure potted in the same medium followed by the same letter were not significantly different.

TABLE 4. HEIGHT, DRY WEIGHT, AND NUMBER OF LEAVES ±SE OF BLACK CHERRY SEEDLINGS GROWN IN SOIL CORES WITH OR WITHOUT HAY-SCENTED FERN, REMOVED FROM FOREST IN AUGUST OR APRIL IN THE SOIL TRANSFORMATION PRODUCT EXPERIMENT

	Growth measures					
Fern	Height (mm)	Leaf dry wt (g)	Root dry wt (g)	Shoot dry wt (g)	Total dry wt (g)	Leaves (*N*)
Soil cores removed from forest in August[a]						
Present	244 ± 26 a	0.362 ± 0.054 a	0.486 ± 0.064 a	0.321 ± 0.054 a	1.170 ± 0.163 a	12.9 ± 0.8 a
Absent	221 ± 12 a	0.319 ± 0.039 a	0.519 ± 0.053 a	0.245 ± 0.023 a	1.083 ± 0.105 a	12.2 ± 0.5 a
Soil cores removed from forest in April[a]						
Present	282 ± 23 a	0.337 ± 0.042 a	0.379 ± 0.060 a	0.330 ± 0.048 a	1.045 ± 0.141 a	13.4 ± 0.6 a
Absent	293 ± 18 a	0.372 ± 0.030 a	0.510 ± 0.054 a	0.360 ± 0.034 a	1.242 ± 0.109 a	13.7 ± 0.5 a

[a]Values of the same growth measure potted in soil cores removed from the forest in the same month followed by the same letter were not significantly different.

TABLE 5. MORTALITY, SURVIVAL, HEIGHT (±SE) AND STEM DIAMETER (±SE) OF BLACK CHERRY SEEDLINGS 1 AND 2 GROWING SEASONS AFTER GERMINATION IN PRESENCE OR ABSENCE OF FERN FOLIAGE SHADE OR FERN FOLIAGE LEACHATE IN LEACHING/SHADING FIELD EXPERIMENT

	Foliage shade/foliage leachate			
	Present/present	Present/absent	Absent/present	Absent/absent
One growing season after germination				
Mortality (number)				
Animal caused	2	2	3	3
Treatment caused	7	8	1	0
Survival				
number	6	5	11	12
%[a]	46	39	92	100
Mean seedling height (mm)[b]	24 ± 4 b	28 ± 6 b	31 ± 4 b	36 ± 3 a
Mean stem diameter (mm)[b]	0.029 ± 0.001 s	0.032 ± 0.002 s	0.032 ± 0.002 s	0.039 ± 0.002 r
Two growing seasons after germination				
Mortality (number)				
Animal caused	1	0	0	1
Treatment caused	5	4	1	2
Survival				
number	0	1	10	9
%[a]	0	8	83	82
Mean seedling height (mm)[b]		43b	138 ± 35 a	139 ± 35 a
Mean stem diameter (mm)[b]		0.040 s	0.070 ± 0.008 r	0.076 ± 0.008 r

[a] Seedlings lost to animals were removed from the experiment. Percentage survival values are for seedlings remaining after subtraction of animal losses.
[b] Values of seedling height or seedling diameter for the same year after germination followed by the same letter were not significantly different.

Neither the speed of germination (germination value) nor the final germination percentage was affected by the presence or absence of fern cover in the field seed germination experiment. The same conclusion was reached in the laboratory seed germination experiment. While soak leachates produced significantly smaller germination values and final germination percentages than mist leachates, no concentration of leachate differed from its respective control.

Fern foliage leachates also did not cause a reduction in any measure of seedling growth. Seedlings receiving fern foliage leachate grew as well or better than those which did not receive fern leachate, regardless of whether seedlings were grown in sand or soil cores. This result was confirmed in the foliage shading/leaching field experiment at site 2. The presence or absence of fern foliage leachate made no difference in black cherry seedling survival or growth over the two-year experiment. A suggestion from earlier work that black cherry seedling shoot growth was reduced by hay-scented fern foliage leachate was not supported by the present work (Horsley, 1977b). The earlier work was conducted in sand culture using leachate prepared by soaking an amount of foliage equivalent to about one fourth the $1\times$ amount used here. The major difference between the former and the present work was the temperature and length of leachate storage. In the earlier study, leachate was stored for up to seven days at 5°C, whereas in the present work leachate was stored for up to four days at -20°C. It is possible that changes in the leachate may have occurred during the longer, higher temperature leachate storage of the earlier work. In any event, growth differences between treated and control plants in the earlier work were small and not statistically significant. The present work shows that leachates that were many times more concentrated than those used in the earlier study had no adverse effect on black cherry seedlings.

Washings of below-ground fern roots and rhizomes did not affect growth or nitrogen availability to black cherry seedlings. The data give no evidence that hay-scented fern natural products build up to toxic levels in the soil or are transformed into toxic products by soil microorganisms. Regardless of the date of soil core collection, there was no difference in any growth measure for black cherry seedlings grown in soil cores from areas of site 2 with or without fern.

Seedling growth was affected by the presence or absence of shade from fern foliage. Where foliage shade was present, nearly all seedlings died; where foliage shade was removed by restraining it behind a barrier as it grew, survival was more than 80%.

Other experiments in this series evaluated the competitive role of hay-scented fern in altering the levels of soil water, soil phosphorus, soil nitrogen, and light, their availability to black cherry seedlings, and the response of black cherry seedlings to changes in the level of each factor (Horsley, 1993). These experiments led to the conclusion that fern interfered with the survival and

growth of black cherry seedlings by altering the availability of light close to the forest floor. Measurements of light above and below fern ground cover in the partially cut stand at site 2 showed that fern greatly modified both the quantity and the quality of light available to small black cherry seedlings growing beneath it. Light below fern was less than 0.5% of full sunlight and had a red/far-red (R/FR) ratio of only 0.04–0.07. Marquis (1973) also found that black cherry seedlings survive poorly in the low photon flux density (about 10% of full sunlight), high far-red (R/FR ratio = 0.15) light environment of uncut Allegheny hardwood stands. A similar conclusion was reached in another manipulative experiment at site 2, which tested the effects of presence or absence of above-ground and presence or absence of below-ground fern organs on survival and growth of black cherry seedlings (Horsley, 1993). The results showed that the presence of above-ground fern foliage greatly reduced survival of black cherry seedlings; the presence of below-ground fern organs had no effect. The critical role of light as the primary interference factor is supported by the fact that other experiments in this series show no significant differences in the availability of soil water (predawn and midday seedling water potential), soil phosphorus, or soil nitrogen (foliar concentrations) between seedlings growing with or without fern cover.

Allelopathic interference by one plant with its neighbor also may occur indirectly through interference of donor plant allelochemicals with mycorrhizae formation or function of the neighbor (Melin, 1953; Olsen et al., 1971; Brown and Mikola, 1974; Fisher, 1979; Rose et al., 1983), or with microorganisms that fix or transform nitrogen (Rice and Pancholy, 1972). Examination of roots of newly germinated black cherry seedlings growing with and without fern at monthly intervals for the first five months after germination showed that nearly all root tips became mycorrhizal within two months after germination regardless of the presence or absence of fern (Horsley, 1993). Fungal infiltration of the seedling root cortex was slower for the first three months after seed germination where fern was present, but by the fourth month after germination, the degree of mycorrhizal colonization was the same where fern was present as where it was not. Measurements of phosphorus availability (foliar concentration) suggested that there was no functional difference in the mycorrhizal relationship as a result of fern presence. Further, a two-year study of rates of net ammonium- and nitrate-nitrogen production in in situ buried bags at site 1 also showed that the presence or absence of hay-scented fern had no effect on production rates (Horsley, 1993). Thus, I conclude that neither direct nor indirect allelopathic interference by hay-scented fern is responsible for poor survival and growth of black cherry seedlings in partially cut Allegheny hardwood stands. All of the evidence supports the conclusion that alteration of light availability by fern is the responsible factor.

Acknowledgments—I thank Vonley Brown for technical assistance in this project.

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PHYTOTOXIC AND ANTIMICROBIAL ACTIVITY OF VOLATILE CONSTITUENTS OF *Artemisia princeps* VAR. *orientalis*

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Abstract—The volatile constituents of *Artemisia princeps* var. *orientalis* (wormwood) were investigated for phytotoxic and antimicrobial activities. The germination and radicle elongation of receptor plants were inhibited by volatile substances emitted from wormwood leaf and effects were concentration-dependent. Essential oil of the plant extracted by Karlsruker's apparatus suppressed seed germination and seedling elongation of the receptor plants at a threshold concentration of 4.8 μl/100 ml. *Escherichia coli* was not susceptible to the wormwood essential oil, but the growth of *Bacillus subtilis*, *Aspergillus nidulans*, *Fusarium solani*, and *Pleurotus ostreatus* was inhibited severely.

Key Words—*Artemisia princeps* var. *orientalis*, volatile constituent, essential oil, phytotoxic activity, antimicrobial activity.

INTRODUCTION

Artemisia princeps var. *orientalis* (wormwood) is known to exhibit allelopathic effects. In earlier papers we reported that water extracts and residues of the plant inhibited seed germination and seedling elongation of receptor plants (Kil and Yun, 1992; Yun and Kil, 1992).

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Allelopathic activity of volatile constituents released from many plants have been cited in the literature. For example, *Artemisia californica* (Halligan, 1975, 1976), *A. absinthium* (Funke, 1943), *Trichostema lanceolatum* (Heisey and Delwiche, 1984), *Thymus capitatus* (Vokou and Margaris, 1986), and Cruciferae species (Oleszek, 1987) produce volatile phytotoxins.

Kasenberg and Traquair (1988) investigated the effects of phenolics from lettuce on *Fusarium oxysporum* f.sp. *radicis-lycopersici* and concluded that growth inhibition of the pathogen by phenolic compounds may be part of an allelopathic mechanism. Antimicrobial activity of volatile constituents from aromatic plants has also been documented by several researchers (Vokou et al., 1984; Yashphe et al., 1987).

In these experiments, we evaluated the effects on seed germination, seedling growth, and antimicrobial activity of volatile substances emitted from *A. princeps* var. *orientalis*.

METHODS AND MATERIALS

Phytotoxic Activity. In a 1.8-liter glass chamber (Baker, 1966), 50 seeds of the receptor plants were placed on the filter paper, which was layered on absorbent cotton with sufficient moisture (Figure 1). Sliced leaves of the wormwood plant in different quantities (5, 10, 15, 20, 25, 30 g) were placed in a glass beaker within the chamber. An identical chamber without sliced leaves was used as the control. Results obtained from the experiments were calculated in grams per 1.8 liters to grams per 100 ml. Chambers were covered with vinyl wrap and placed at room temperature (ca. 25°C). After two to four days, germination percentage and radicle elongation were measured. Further experiments were conducted with essential oil extracted from the leaves. Water distillation

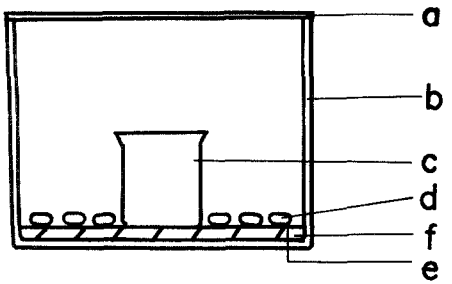

FIG. 1. Diagrammatic section through test chamber: a, vinyl wrap; b, glass chamber; c, glass beaker for wormwood leaf; d, seeds; e, filter paper; f, absorbent cotton moistened with water.

of crushed wormwood leaves was performed in Karlsruker's apparatus (Stahl, 1973). The water distillate was saturated with sodium sulfate and then extracted with ether. The ether extract was concentrated at 15°C under vacuum in an evaporator. The resulting essential oils were tested for activity on seed germination and seedling growth. Fifty seeds of test plants were placed in each of four 210-ml Petri dishes, on filter paper over absorbent cotton with 10 ml water. In addition, each Petri dish contained a 1-cm-diameter aluminum vessel. The quantities of essential oil put in the containers were 5, 10, 15, and 20 μl, respectively. Results obtained from the experiments were calculated in grams per 210 ml to grams per 100 ml. Variables for the estimation of the phytotoxic effects were germination ratio and seedling length (Vokou and Magaris, 1986). All the results of the experiments were means of the four replicates.

Antimicrobial Activity: Antibacterial Test. Escherichia coli was cultured in Luria-Bertani (LB) media and *Bacillus subtilis* in nutrient broth (NB) medium by the method of Costilow (1981). Essential oil extracted from the wormwood plant was added to the media to produce concentrations of 10, 50, and 100 ppm. Five milliliters of suspension of bacterial culture was added to the media. The culture flask was swirled in a shaking incubator at 120 rpm and 37°C. Twelve hours after inoculation, the optical density (OD) of each culture was measured at 600 nm using a colorimeter (Spectronic 20).

Antimicrobial Activity: Antifungal Test. Sterile polyethylene plates containing 25 ml of complement media (CM) and 100, 500 and 1000 ppm of the essential oils were inoculated under light condition (Harsani et al., 1976). *Fusarium solani*, *Aspergillus nidulans*, and *Pleuroteus ostreatus* were inoculated in the plates. The plates of *F. solani* and *A. nidulans* were incubated for three days and then colony diameter was measured. *P. ostereatus* was incubated for 10 days at 28°C and then colony diameter was measured (Costilow, 1981).

RESULTS

Germination and Radicle Elongation of Receptor Plants in Volatile Substances from Wormwood. In concentrations of 0.3, 0.6, 0.8, and 1.1 g/100 ml, germination of receptor plants was generally not inhibited, but *Achyranthes japonica* was inhibited by 0.3 g/100 ml while *Plantago asiatica* was inhibited by 0.8 g/100 ml (Figure 2). The higher concentrations of 1.4 g and 1.7 g/100 ml severely inhibited germination of most species. Germination of *Echinochloa crus-galli* and *Melandryum firmum* was slightly stimulated at below 1.1 g/100 ml treatments, while germination of *M. firmum* was inhibited at over 1.4 g/100 ml. Inhibition patterns for radicle length were similar to germination.

Radicle elongation of receptor plants in volatile substances of wormwood

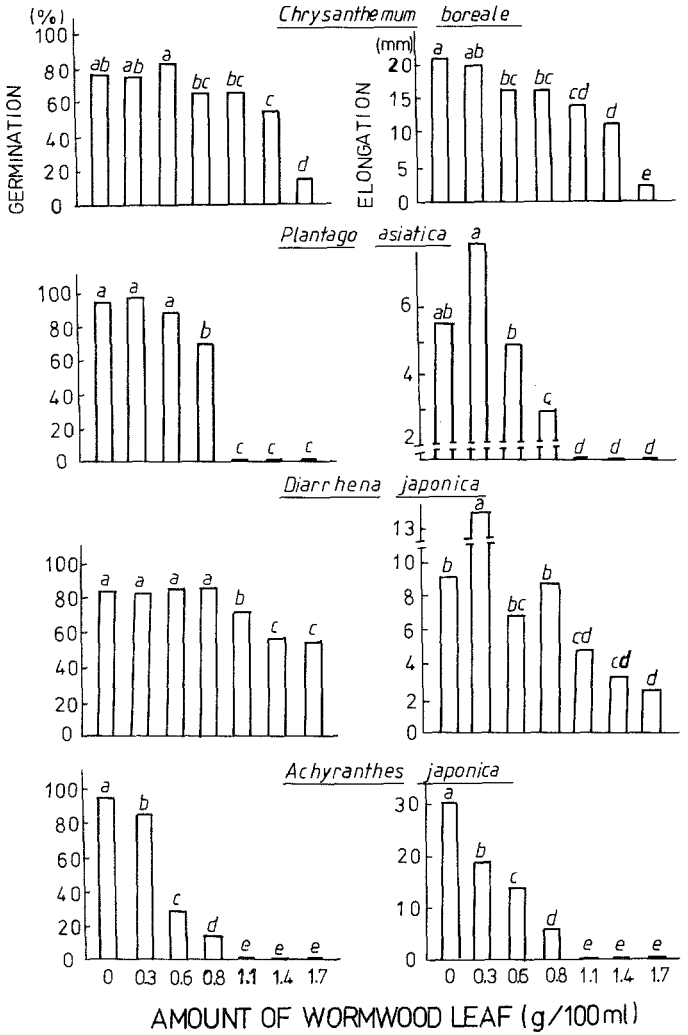

FIG. 2. Germination percentage (left) and mean radicle length (right) of receptor plants treated at different concentrations of wormwood leaf volatile substances. Bars topped by different lowercase letters are significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

leaf were generally inhibited, although the threshold concentration for inhibition varied among the species. The higher the concentration of volatile substances of wormwood leaf, the less the radicle elongation.

Water distillation of the leaf yielded 320 mg essential oils per 100 g of

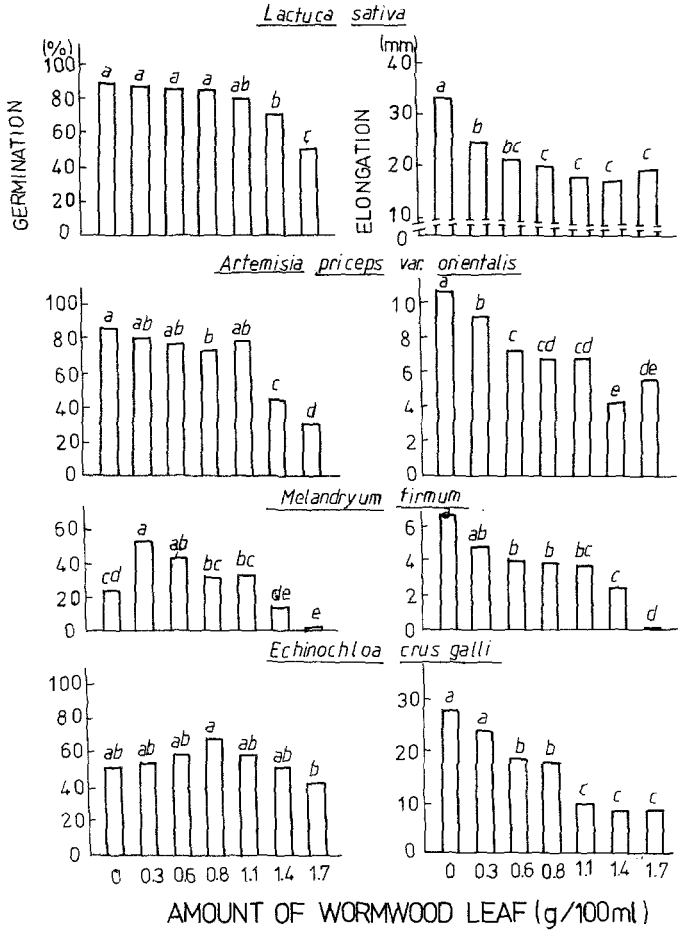

FIG. 2. Continued

tissue. Water-distilled essential oil of wormwood leaf strongly inhibited seed germination and radicle elongation of the receptor plants (Figure 3). The germination inhibition of *A. japonica* and *A. princeps* var. *oientalis* was dependent upon the essential oil concentration. Mean germination percentage of wormwood plant itself treated with 9.5 μl/100 ml of wormwood plant essential oil was below 0.1%. Elongation of all three receptor plants was heavily inhibited. *A. japonica* at concentrations of 7.1 and 9.5 μl/100 ml germinated but radicle elongated was less than one fourth that of controls (Figure 3).

Antimicrobial Potential: Antibacterial Activity. Concentration of up to 100 ppm of the wormwood essential oil had no effect on the growth of *E. coli*

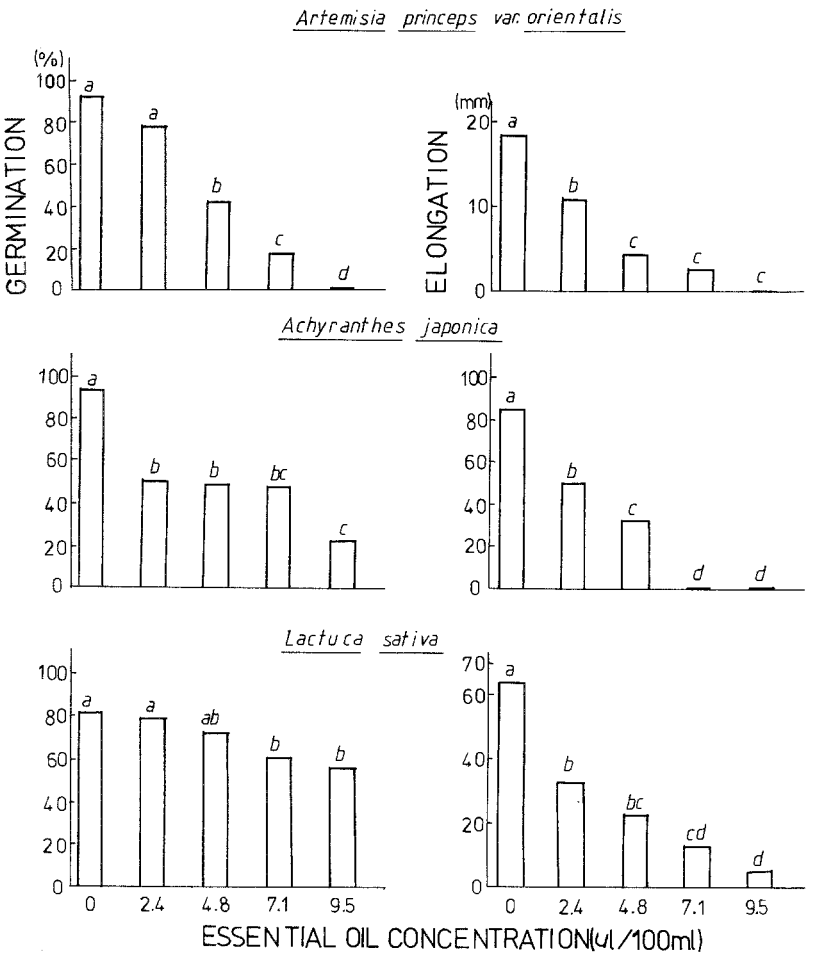

FIG. 3. Germination percentage (left) and mean seedling length (right) of receptor plants treated at different concentrations of wormwood leaf essential oil. Bars topped by different lowercase letters are significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

(Figure 4). On the other hand, concentrations above 10 ppm of the essential oil were highly toxic to *B. subtilis*. The growth of *B. subtilis* above 10 ppm was reduced to one tenth of the control.

Antimicrobial Potential: Antifungal Activity. As shown by colony diameter, the wormwood essential oil exhibited strong toxicity to the growth of the tested fungi (Figure 5). In general, the degree of toxicity was concentration-dependent. The growth of *P. ostreatus* was completely inhibited by 1000 ppm.

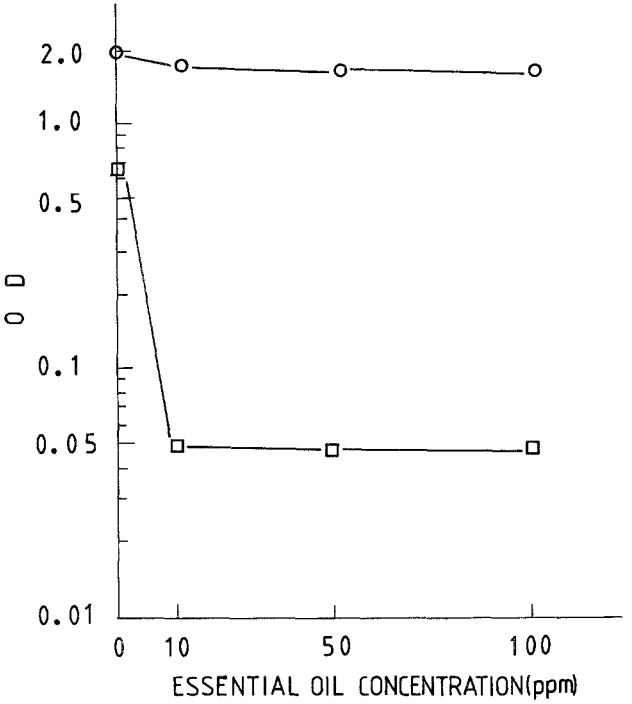

FIG. 4. Optical density (OD) of bacteria cultured at different concentrations of wormwood essential oil. Keys to species: ○, *Escherichia coli*; □, *Bacillus subtilis*.

DISCUSSION

Kil and Yun (1992) showed that water extract of wormwood suppressed seed germination and seedling growth of selected plants. Here we show that the volatile substances emitted from wormwood leaf also inhibited seed germination and radicle length of receptor plants. Water-distilled essential oil of wormwood leaf strongly inhibited germination and elongation of test species. The wormwood plant itself was inhibited by the volatile substances from leaves and essential oil of wormwood. Even the smallest dose of wormwood oil tested, 2.4 μl/100 ml, inhibited germination and elongation of the receptor species. Receptor plants had almost no radicle growth in 9.5 μl/100 ml essential oil. Therefore, it would be reasonable to assume that volatile compounds released from the wormwood plant acted as inhibitors to germination and growth. Furthermore, the degree of toxicity of released volatile substances was dependent on the receptor plant. This finding was in agreement with Halligan (1976), Heisey and Delwiche (1983), and Oleszek (1987).

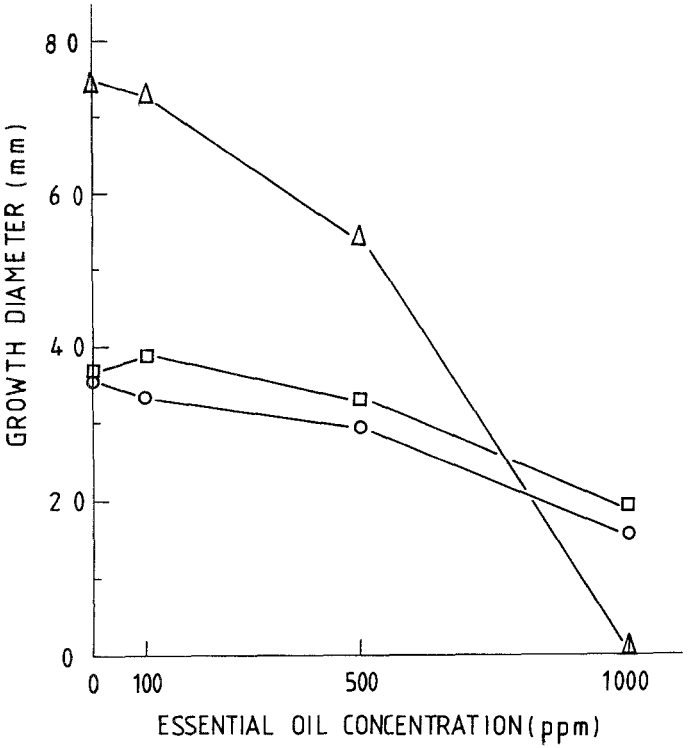

FIG. 5. Colony diameter of fungi taxa grown in complement medium at different concentrations of wormwood essential oil. Keys to species: △, *Pleurotus ostreatus*; ○, *Fusarium solani*; □, *Aspergillus nidulans*.

Zeringue and McCormick (1989) noted that phytoalexins are secondary metabolites produced by plants that hinder the invasion of microorganisms in host-plant tissues. It is known that some plants are capable of producing volatile compounds that have very effective antimicrobial activity. Hashidoko et al. (1989) suggested that sesquiterpenes had a specific and significant role of protection against microbial invasion if the tissue is wounded.

The essential oil of the wormwood plant exhibited antimicrobial activity by limiting the growth of *B. subtilis* to merely one tenth of the control at a concentration above 10 ppm. *P. ostreatus* was the most inhibited species among three fungi tested. There have been many reports on the antimicrobial properties of essential oils (Chalchat et al., 1985; Syed et al., 1986a,c; Dubey and Kishore, 1987; Inya-Agha et al., 1987). The fact that different species of the genus *Helichrysum* produce different secondary metabolites (phloroglucinols, acetophenones, flavonoids) as a biochemical defense mechanism against fungi and

bacteria is of some interest, since it indicates the use of different metabolic pathways in order to produce chemical barriers that exert a single ecological role of higher plant defense against different microorganisms (Tomas-Barberan et al., 1990).

The evidence from this study suggests that volatile substances from wormwood have an allelopathical effect on other plants and microorganisms.

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MALE-SPECIFIC VOLATILES FROM NEARCTIC AND AUSTRALASIAN TRUE BUGS (HETEROPTERA: COREIDAE AND ALYDIDAE)

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Abstract—Aeration and exocrine gland extracts were analyzed for three Coreidae and two Alydidae. Males of all the species studied emit volatile blends that are probably pheromones, but sexual communication in these insects evolved differently. In the alydids, *Riptortus serripes* and *Mirperus scutellaris*, the metathoracic scent glands are sexually dimorphic, and the dimorphisms are expressed chemically. Secretions from the male alydids contain high concentrations of esters or alcohols [e.g., (*E*)-2-hexenyl (*Z*)-3-hexenoate, (*E*)-2-hexenyl butyrate, and (*E*)-2-octenol], while females produce mainly acids and aldehydes [e.g., butyric and hexanoic acids, and (*E*)-2-hexenal]. In the coreids, *Amblypelta lutescens lutescens*, *Amblypelta nitida*, and *Leptoglossus phyllopus*, the metathoracic scent glands are not sexually dimorphic, but male- and species-specific volatiles are released, apparently from cells in the cuticular epidermis. The coreid male-specific volatiles are primarily monoterpenes and sesquiterpenes, including (−)-(3*R*)-(*E*)-nerolidol as the major component from *A. lutescens lutescens* (an Australasian species) and *L. phyllopus* (a Nearctic species). Only (+)-(3*S*)-(*E*)-nerolidol is commonly found

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in plants so (*E*)-nerolidol from these coreids is environmentally unique because of its chirality.

Key Words—Hemiptera, Coreidae, pheromone, attractant, scent glands, allomone, (*E*)-nerolidol, biocontrol, chirality, enantiomers.

INTRODUCTION

Attractant pheromones are chemically known for species in two of the seven infraorders of Heteroptera (Aldrich, 1988a; Schuh, 1986). Female mullein bugs, *Campylomma verbasci* (Cimicomorpha group: Miridae), release butyl and (*E*)-2-butenyl butyrates as a long-range attractant pheromone (Smith et al., 1991). In addition, attractant pheromones have been fully or partially identified from males of several predaceous (Aldrich, 1988b; Aldrich et al., 1986, 1991b; Kochansky et al., 1989) and phytophagous pentatomids (Pentatomomorpha) (Aldrich et al., 1989, 1991a, 1993; Oliver et al., 1992). Males of some other pentatomomorphan families (superfamily Coreoidea: Coreidae, Rhopalidae, and Alydidae) have glands associated with the genitalia that release species-specific secretions, but these exudates are apparently involved in short-range communication rather than attraction (Aldrich, 1988a). However, adult males of the bean bug, *Riptortus clavatus* (Alydidae), do attract conspecific adults (Leal and Kadosawa, 1992; Numata et al., 1990), as do males of the coreid *Leptoglossus australis* (Yasuda, 1990).

This report concerns our efforts to isolate and identify attractant pheromones of Alydidae and Coreidae. We investigated one Nearctic coreid species, *Leptoglossus phyllopus*, a polyphagous, occasional pest of fruit and nut crops in the southern United States (Ellis et al., 1992; Horton et al., 1992). Other coreids and alydids reported on here are Australasian species studied during a six-month sabbatical in Australia by one of us (J.R.A.). Fruit-spotting coreids in the genus *Amblypelta* are highly polyphageous, severely damaging insects (Brown, 1958). For examples, feeding by one *A. lutescens lutescens* bug per plant can stunt and distort a papaya fruit or collapse a shoot of macadamia nut, and unnoticed levels of *A. nitida* and *A. l. lutescens* can cause almost complete fruit drop in lychees (Brown, 1958; Waite, 1990). The Australian alydids examined are sometimes pests of beans: the pod-sucking bugs, *Riptortus serripes* and *Mirperus scutellaris* (Carver et al., 1991).

All these bugs are strong fliers able to migrate to a succession of crops, and their damage is initially inconspicuous (Miles, 1987). Synthetic attractants or repellents could be useful for managing these elusive and unpredictable pests.

METHODS AND MATERIALS

Insects. Amblypelta lutescens lutescens and *A. nitida* used in the study were from colonies started from adults and late-instar nymphs collected mainly on lychee (Tai So cultivar), *Litchi chinensis* (Sapindaceae), at the Maroochy Horticultural Research Station in October and November 1990. Adults of *R. serripes* and *M. scutellaris* were collected on ripening fruits of velvet bean trees, *Cassia tomentella*, and cockspur coral trees, *Erythrina crista-galli* (Papilionaceae), from November through January at Alexandra Headlands, Queensland, Australia. *Leptoglossus phyllopus* adults were collected in May 1987, on blueberry, *Vaccinium* sp. (Ericaceae), at the Southeastern Fruit and Tree Nut Research Laboratory, Byron, Georgia. The Australian species were reared on fresh green beans, sunflower seeds, and water at ambient temperature; wild *L. phyllopus* adults were maintained similarly, but at 26 ± 1 °C with a 16:8 (hr light–dark) photoperiod. Rearing procedures were as previously described (Aldrich et al., 1991a), except that sunflower seed was glued onto sheets of brown wrapping paper with wallpaper paste and cut into 10-cm squares that were discarded after depletion by the insects.

Extractions. Airborne extracts were prepared for all species except *M. scutellaris* by confining 20–50 insects in an 800-ml glass column, drawing air by vacuum (100 ml/min) through ca. 30 mg of activated charcoal inside a Swinney Luer-lock filter holder (13 mm; Thomas Scientific, Philadelphia, Pennsylvania, and extracting the filter with 200 μl of CH_2Cl_2 (Aldrich et al., 1989, 1991a). Extracts of the metathoracic scent glands of male and female *Amblypelta*, *Riptortus*, and *Mirperus* spp. were prepared by collecting the secretions in micropipets from the reservoirs of glands excised from CO_2-anesthetized bugs submerged in tap water, and dissolving the secretions in 0.5–1.0 ml of CH_2Cl_2. The contents of the two large nymphal dorsal abdominal glands were sampled in a similar manner in order to recheck the reported compositions of *Amblypelta* nymphal allomones (Baker et al., 1972).

Chemical Analysis. The Australian samples were analyzed initially by gas chromatography (GC) on a DB-5 column (0.25-μm film; 30 m × 0.25 mm ID; J&W Scientific) in a Hewlett-Packard 5890 GC/3392A Integrator with helium as carrier, programmed from 45°C for 2 min to 260°C at 15°/min, using a flame ionization detector (FID). Subsequently, the Australian samples and samples from *L. phyllopus* were analyzed in the United States on a DB-1 column (0.25-μm film, 30 m × 0.25 mm ID) in a Varian 3500 GC with helium as carrier (50 cm/sec), a temperature program from 50°C for 2 min to 235°C at 15°/min, an FID, and recording data using the Varian GC Star Workstation software on a Gateway 2000 386/25 computer. The GC traces presented in figures begin at 2.5 min (at left) so as to exclude the solvent peak and normalize

peaks to the most abundant natural product. Aeration samples of *A. lutescens lutescens* and *L. phyllopus* males were also analyzed on a Cyclodex-B chiral column (0.25-μm film; 30 m $\times$ 0.25 mm ID; J&W Scientific) using a Varian 3700 GC with helium as carrier (40 cm/sec), isothermally at 130°C in a split mode.

Electron impact mass spectra (EI-MS) were obtained for Australian samples at 70 eV using a Finnigan 1020 GC-MS with a 30-m DB-5 column (0.25-μm film $\times$ 0.25 mm ID), programmed from 40°C for 2 min to 260°C at 10°/min. EI-MS were obtained in the United States laboratory for samples of *L. phyllopus* and some Australian samples using either a Finnigan 4510 GC-MS equipped with an INCOS Data System, at 70 eV, and a 30-m DB-1 column, programmed from 60°C for 2 min to 250°C at 5°/min, or a Hewlett Packard 5971 GC-MS instrument at 70 eV, with a HP-5 column (0.11 μm film; 25 m $\times$ 0.2 mm ID), programmed from 50°C for 2 min to 250°C at 15°/min. Some extracts were also analyzed by chemical ionization mass spectrometry (CI-MS) with NH_3, ND_3, and isobutane as reagent gases using the Finnigan 4510 GC-MS.

In an effort to characterize the major male-specific component in *A. nitida* aeration samples, a 25-μl CH_2Cl_2 solution of hexanal and *cis-/trans*-limonene oxide (100 μg/μl and 50μg/μl, respectively) was treated with $NaBH_4$ by standard procedures, and then a 25-μl aliquot of an aeration extract was similarly treated for comparison.

Standards. Compounds identified by mass spectral data were cross-checked by GC comparison to authentic standards. Most compounds were commercially obtained from Aldrich Chemical (Milwaukee, Wisconsin) or Bedoukian Research (Danbury, Connecticut). Ocimene was obtained from International Flavors & Fragrances (Union Beach, New Jersey). (*E*)-4-Oxo-2-hexenal was synthesized according to Ward and VanDorp (1969), and esters not commercially available were synthesized by standard procedures. Synthetic standards of (−)-(3*R*)-nerolidol, (+)-(3*S*)-nerolidol, and β-*trans*-bergamotene were kindly supplied by Dr. David Cane, Brown University, Providence, Rhode Island (Cane et al., 1990); α-*trans*-bergamotene was supplied by Dr. Ted Turlings, USDA Insect Attractants Laboratory, Gainesville, Florida. The best standard of α-farnesene isomers was obtained by aeration of Granny Smith apples (Murray, 1969); a mixture of β- and α-farnesenes was also purchased from Bedoukian Research.

Field Test. A blend of four *A. lutescens lutescens* male-specific components was prepared as follows using available standards to mimic the proportions of these compounds observed in aeration extracts: 4.5 μl ocimene, 5.5 μl linalool, 243.5 μl farnesenes, and 307.3 μl nerolidol. The amounts blended were determined by multiplying the average percentage for each natural product (see Results) by the reciprocal of the purity for the correct isomer in each standard, and using double volumes of the chiral compounds (linalool and nerolidol) on the assumption that the insects produce the optically pure compounds. The

blended standards were dissolved in hexane to give a concentration of 0.1 μg/μl, and five traps were baited daily with 50 μl of test solution on 1-cm pieces of cigarette filter contained in open 0.3-ml plastic centrifuge tubes. Traps were made from 12-oz plastic cups coated with Stikem, and pairs of treated and untreated control traps were hung on known host trees of *A. lutescens lutescens* at the research center in Nambour, Australia, March 11–19, 1991.

RESULTS

Exocrine Glands. The metathoracic scent glands of the *Amblypelta* and *Leptoglossus* spp. are not sexually dimorphic. Their scent gland morphology conforms to the general coreoid pattern (Aldrich, 1988a): a pair of lateral gland tubules (also called accessory glands) each attached to the median reservoir by a duct. Surprisingly, the lateral glands of the methathoracic scent glands in *R. serripes* and *M. scutellaris* are distinctly sexually dimorphic, but in different ways. In *R. serripes*, the masses of lateral gland tubules are greatly hypertrophied in sexually mature males compared to females (Figure 1). However, in *M. scutellaris* the development of lateral gland tubules appears comparable in the sexes, but mature males possess a lateral gland reservoir at the base of each accessory gland apparently created by closure of a duct between the lateral glands and the primary reservoir (Figure 2). Secretion could be removed from the lateral gland reservoirs in micropipets without affecting secretion in the primary reservoir (Figure 2).

Males of *A. lutescens lutescens*, *A. nitida*, and *R. serripes* do not have ventral abdominal glands as occur in *Leptoglossus* spp. (Aldrich et al., 1979) and other coreoid bugs (Aldrich, 1988a). *M. scutellaris* males do have well-

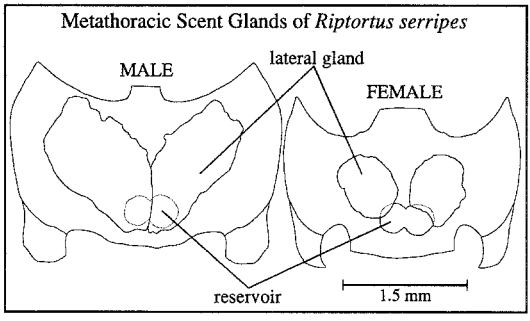

FIG. 1. *Riptortus serripes:* Diagram of the metathoracic scent glands of adults showing the hypertrophied lateral glands of a sexually mature male. The lateral glands, drawn here and in Figure 2 in outline form, are composed of masses of branched tubules.

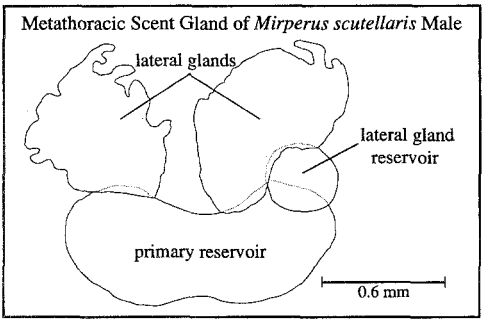

FIG. 2. *Mirperus scuterllaris:* Diagram of the metathoracic scent gland of a mature male showing a lateral gland reservoir at the base of one lateral gland; the secretion was removed from the other lateral gland reservoir in a micropipet without deflating the primary reservoir. The metathoracic scent gland of females (not shown) lack lateral gland reservoirs.

developed ventral abdominal glands opening between the seventh and eighth abdominal segments.

Coreid Chemistry. The metathoracic scent gland secretion from *A. lutescens lutescens* females (Figure 3) was not appreciably different from that of males (not shown). Hexanal is the major component in the metathoracic scent gland secretion, with intermediate amounts of hexyl and octyl acetates, and lesser amounts of hexanol, hexanoic acid, octanal, octanol, and hexyl hexanoate (Figure 3). Hexanal is also the major component in the dorsal abdominal gland secretion of nymphs, but other compounds identified from scent gland secretion of adults are absent; instead, (*E*)-4-oxo-2-hexenal is present in the nymphal secretion (Figure 3). The metathoracic and nymphal dorsal abdominal gland secretions of *A. nitida* (not shown) are chemically like those of *A. lutescens lutescens*.

Aeration extracts of *A. lutescens lutescens* males typically exhibit a set of compounds by GC that are never observed in aeration extracts of females (Figure 4, compounds **10–12**). Metathoracic scent gland components are present in aeration extracts of both males and females in variable amounts, depending on whether the bugs were disturbed or died during aeration (Figure 4). GC-MS analysis of *A. lutescens lutescens* male airborne extracts produced EI-MS for compounds **10** (R_t = 11.3 min) and **11** (R_t = 11.8 min) matching spectra in the computerized library for α-farnesene and nerolidol, respectively. Coinjection experiments with appropriate standards of geometric isomers showed that the natural product **10** is (*E*,*E*)-α-farnesene (Anet, 1970), and compound **11** from males is (*E*)-nerolidol (Figure 4). Standards of (−)-(3*R*)-(*E*)-nerolidol and (+)-(3*S*)-(*E*)-nerolidol eluted at 24.44 min and 24.74 min, respectively, at 130°C

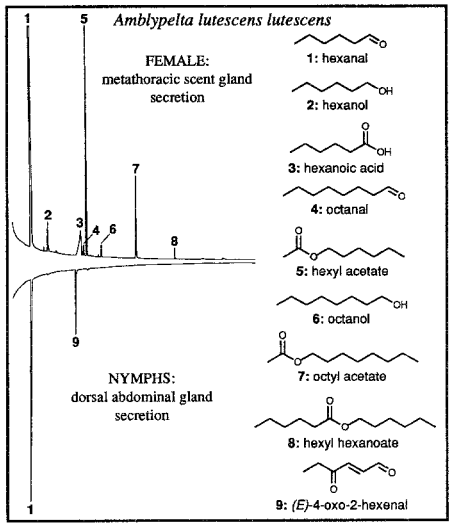

FIG. 3. *Amblypelta lutescens lutescens:* Gas chromatograms and chemistry of the metathoracic scent gland secretion (three mature females) and dorsal abdominal gland secretion (mixed anterior and posterior gland secretion from four fifth-instar nymphs). The GC traces shown here, and in the remaining figures, begin at 2.5 min (left) so as to exclude the solvent peak, attenuation was automatically adjusted to make the largest peak full-scale, and the bottom trace was inverted using Corel Draw (Version 3.0; Corel Systems Corp.) for easy comparison of the compositions of extracts.

on the chiral GC column, with overlapping elution such that the two peaks were resolved about halfway to the baseline. Coinjection of aeration extract from male *A. lutescens lutescens* with the optically pure standards of (*E*)-nerolidol showed that the insect natural product is (−)-(3*R*)-(*E*)-nerolidol. A close match for compound **12** (R_t = 12.2 min) was not found; EI-MS, *m/z*(%): 41(76), 43(76), 57(16), 59(53), 79(66), 80(82), 81(54), 85(43), 91(55), 93(74), 105(43), 119(100), 134(42), 159(7), and 187(2). CI-MS analyses for **12** indicated MW = 220, with no exchangeable protons; NH_3, *m/z*(%): 238(100, $[M + NH_4]^+$), 240(62), 255(7, $[M + NH_4(NH_3)]^+$), and 257(3); and ND_3, *m/z*(%): 242(100, $[M + ND_4]^+$), 246(30), 262(7, $[M + ND_4(ND_3)]^+$), and 266(2). Compound **12** remains unidentified. Other minor components (<5%) detected in male aeration samples analyzed in Australia (30-m DB-5 column), but not in samples from females, include β-ocimene (R_t = 7.5 min), linalool (R_t = 8.2 min), an unidentified compound (R_t = 8.7 min), and (*E*)-β-farnesene (R_t = 11.9 min).

Fewer adults of *A. nitida* were reared in the laboratory than for *A. lutescens lutescens*, but males of this species also clearly release volatiles not produced by females. Moreover, the blend of male-specific volatiles from *A. nitida* is

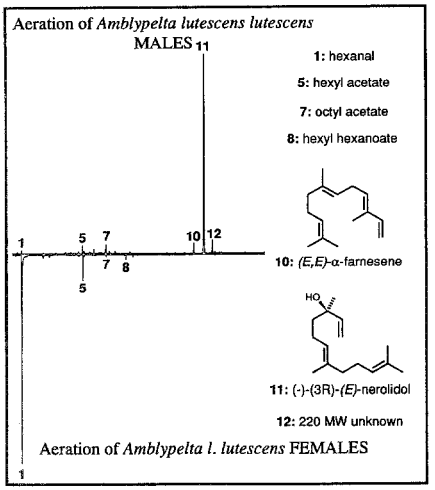

FIG. 4. *Amblypelta lutescens lutescens:* Gas chromatograms and chemistry of aeration extracts from males and females. Compounds **10–12** are male-specific volatiles; earlier-eluting compounds present in varying amounts are contaminants from the metathoracic scent glands. GC traces for this and ensuing figures were prepared with a DB-1 column (conditions in text), recording data using the Varian GC Star Workstation software on a Gateway 2000 386/25 computer.

distinct from that of *A. lutescens lutescens* males (Figure 5). Hexanal **(1)** and hexyl acetate **(5)** are metathoracic scent gland contaminants but compound **13,** identified as nonanal, is a male-specific volatile. While there is no trace of nerolidol in aeration extracts of *A. nitida* males, the minor component in extracts of *A. lutescens lutescens* males eluting at R_t = 8.7 min is the major component entrained from *A. nitida* males (Figure 5, compound **14**). A match for the EI-MS spectrum of **14** was not found; *m/z*(%): 43(27), 53(28), 67(10), 72(15), 77(24), 79(100), 81(45), 91(11), 93(12), 109(4), 119(1), 134(1), and 152(2). CI-MS analyses for **14** indicated MW = 152, with no exchangeable protons; NH_3, *m/z*(%): 153(16, $[M + H]^+$), 170(100, $[M + NH_4]^+$), 187(75, $[M + (NH_3)_2H]^+$); and ND_4, *m/z*(%): 154(8, $[M + D]^+$), 174(84, $[M + ND_4)^+$]), and 194(100, $[M + (ND_3)_2D]$). Compound **14** was unaffected by treatment with $NaBH_4$, suggesting that the natural product is an epoxide rather than an aldehyde or ketone. The following known 152 MW epoxides were eliminated as possible structures for compound **14** based on GC and MS data: limonene oxide, cineole, dehydro-1,8-cineole, 4,5-epoxy-1-*p*-menthene, and 1,2-epoxy-4-*p*-menthene. Compound **15** also has MW = 152 and may be a carbonyl-containing homolog of **14,** but a matching MS for **15** was not found; EI-MS, *m/z*(%): 43(100), 55(31), 67(27), 81(38), 91(36), 109(50), 119(20), 134(11), 137(9), 152(23,

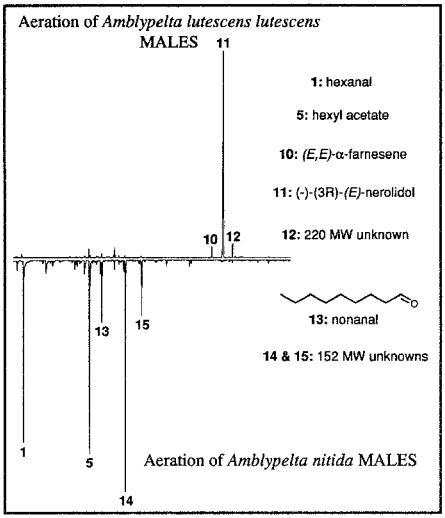

FIG. 5. *Amblypelta lutescens lutescens* versus *A. nitida:* Gas chromatograms and chemistry of aeration extracts from *Amblypelta* spp. males. Compounds **10–15** are male-specific volatiles; earlier-eluting compounds are metathoracic scent gland secretion contaminants.

M^+). Sufficient material was unavailable for further characterization of **14** and **15.** Finally, it was confirmed by GC-MS that the male-specific volatiles from *A. nitida* include a small amount (<1%) of compound **12,** which occurs more abundantly in aeration extracts of *A. lutescens lutescens* males (Figure 5).

Rechromatography of an aeration sample prepared in 1987 from *L. phyllopus* males produced a chromatogram like the original except that compound **17** was reduced in abundance, while **18** and **19** became more abundant (Figure 6). Hexanal **(1)** and hexyl acetate **(5)** are contaminants from the metathoracic scent glands but compound **16,** identified as decanal, and later eluting compounds are characteristic volatiles of males that are not produced by females of the species. Compound **18** is tentatively identified as a caryophyllene isomer based on the EI-MS. The EI-MS of **17** and **19** were nearly identical; e.g., **17,** *m/z*(%): 55(24), 69(48), 77(23), 79(27), 91(32), 93(100), 105(23), 107(28), 119(97), 133(8), 147(4), 161(7), 189(4), 204(5, M^+). Standards of *β-trans*-bergamotene and *α-trans*-bergamotene, but not *α-cis*-bergamotene, were available; *α-trans*-bergamotene coeluted with compound **19,** suggesting that **17** may be *α-cis*-bergamotene. As for *A. lutescens lutescens*, the major male-specific volatile from *L. phyllopus* was unequivocally identified as (−)-(3*R*)-(*E*)-nerolidol.

Alydid Chemistry. The sexual dimorphism observed for the metathoracic

scent glands of *R. serripes* is expressed chemically both in extracts of the glands (Figure 7), and aerations of live adults (not shown). Secretion from the scent gland reservoir of females simply consists of (*E*)-2-hexenal **(20)** and hexanoic acid **(3);** however, aeration extracts of females also contained a substantial amount (ca. 25% relative to hexenal) of (*E*)-2-hexenyl hexanoate **(25).** Scent gland secretion and entrained volatiles from *Riptortus* males are characterized by the presence of aliphatic esters not encountered in extracts of females, most

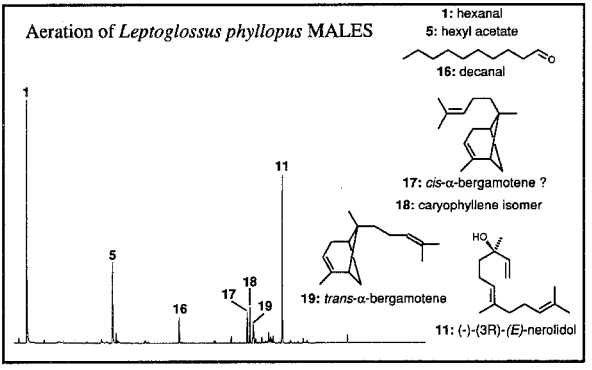

FIG. 6. *Leptoglossus phyllopus:* Gas chromatogram and chemistry of aeration extract from males. Compounds **11–19** are male-specific; earlier-eluting compounds are metathoracic scent gland secretion contaminants.

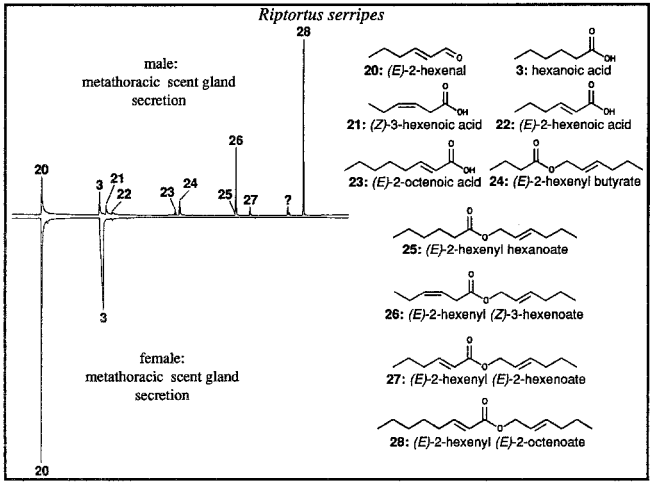

FIG. 7. *Riptortus serripes:* Gas chromatograms and chemistry of metathoracic scent gland secretion from a male and a female.

prominently (*E*)-2-hexenyl (*Z*)-3-hexenoate [**26;** EI-MS, *m/z*(%): 41(100), 55(90), 69(18), 83(22), 96(3), 114(12), 136(2), and 167(1)] and (*E*)-2-hexenyl (*E*)-2-octenoate [**28;** EI-MS, *m/z*(%): 41(53), 55(100), 67(19), 82(12), 97(6), 125(63), 167(3), 181(3), 195(4), and 224(2, M^+)] (Figure 7). The concentration of **26** in aeration extracts of male *R. serripes* was equal to or exceeded the concentration of **28.**

For *M. scutellaris* males, secretions from the primary and lateral scent gland reservoirs and from the ventral abdominal glands were analyzed separately (Figure 8). Secretion drawn from the primary reservoir of females contains butyric acid **(29),** (*E*)-2-hexenal **(20),** and (*E*)-2-hexenyl butyrate **(24)** while, in addition to these compounds, secretion from the primary reservoir of males contains (*E*)-2-octenal **(30)** as the major component. The lateral reservoirs of males contain the corresponding alcohols **31** and **32)** of the primary reservoir alk-2-enals, plus the major component (*E*)-2-hexenyl butyrate **(24)** and a trace of (*E*)-2-octenyl butyrate **(33).**

Field Test. Over the period March 12–18, 1991, no bugs were caught on

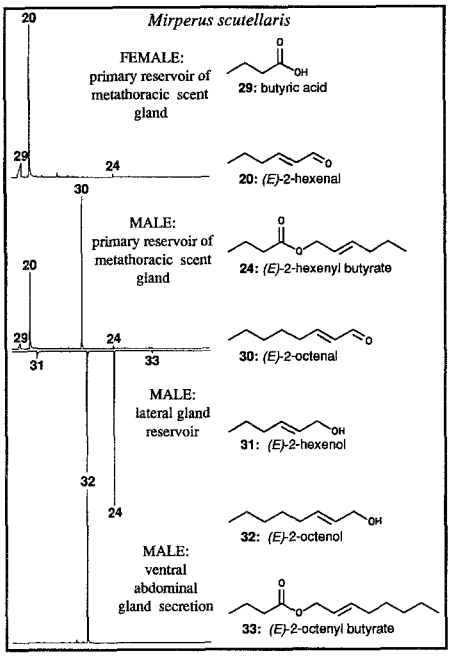

FIG. 8. *Mirperus scutellaris:* Gas chromatograms and chemistry of secretions from the primary metathoracic scent gland reservoir of a female, the primary metathoracic scent gland reservoir of a male, the lateral gland reservoir of the same male, and the ventral abdominal gland of a male.

traps, five *A. lutescens lutescens* adults were observed near treated traps (<30 cm), while one *A. lutescens lutescens* adult was nearby a control trap.

DISCUSSION

The coreid and alydid males studied here emit volatile blends that are probably attractant pheromones. However, sexual communication in these insects evolved differently. *Amblypelta* and *Leptoglossus* coreids lack consolidated glands from which sex pheromones can be selectively released. Rather, as for phytophagous pentatomids (Aldrich, 1988b; Carayon, 1981), coreid attractant pheromones apparently emanate from cells scattered throughout the abdominal epidermis. In the Australasian alydids, on the other hand, the metathoracic scent gland evidently evolved a sexual function secondarily. This interpretation is substantiated by the recent demonstration that in *R. clavatus* a blend of (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*Z*)-3-hexenoate, and myristyl isobutyrate acts synergistically to attract adults and nymphs (Leal and Kadosawa, 1992; Leal et al., 1993a,b; Leal, personal communication). Our research on the Australian bean bug, *R. serripes*, shows that the hypertrophied primary accessory glands of males are the source of *Riptortus* pheromones.

Sexual dimorphism of the metathoracic scent gland, although not common, is known for Heteroptera (Aldrich, 1988a). For example, males of the large milkweed bug, *Oncopeltus fasciatus* (Lygaeidae), release scent gland secretion fortified with esters because they contain secondary scent gland reservoirs like those of *M. scutellaris* (Games and Staddon, 1973). Curiously, the metathoracic scent glands of the two Nearctic alydids examined, *Alydus eurinus* and *Megalotomus quinquespinosus*, are not sexually dimorphic (Aldrich, unpublished data).

Amblypelta nitida and *A. lutescens lutescens* are extremely difficult to distinguish as adults, often being confused in older literature (Donaldson, 1983). We discovered that the chemistry of the adult and nymphal defensive secretions of *A. nitida* were confused as well. Baker et al. (1972) reported that "there is no major change of scent gland components at ecdysis of the final instar to the adult." To the contrary, we found that the allomones of nymphs and adults are distinctly different for both species (e.g., Figure 3).

Notwithstanding their similar appearance, *Amblypelta* males liberate species-specific volatile blends, while females are aromatically invisible. The only chemical hint of a common ancestry for *A. nitida* and *A. lutescens lutescens* is that some abundant components in one species occur as traces in the other species.

The discovery of (−)-(3*R*)-(*E*)-nerolidol as the major male-specific component of *A. lutescens lutescens* is intriguing. A computer-aided search of the

literature revealed only two reports of (−)-(3*R*)-(*E*)-nerolidol from plants (Picker et al., 1976; Rucker et al., 1981). Nevertheless, the antipode, (+)-(3*S*)-(*E*)-nerolidol, is common in plants (Cane et al., 1990), including native Australian *Eucalyptus* and *Melaleuca* spp. (Myrtaceae) reported as hosts of *Amblypelta* bugs (Bigger, 1985; Donaldson, 1983; Jones and Harvey, 1936; Sutherland et al., 1960). Thus, it appears that (*E*)-nerolidol from *A. lutescens lutescens* males is environmentally unusual because of its chirality. It is also possible that the pheromone made with racemic (*E*)-nerolidol was inactive due to inhibition by the unnatural enantiomer.

Identification of (−)-(3*R*)-(*E*)-nerolidol from *A. lutescens lutescens* was a coincidence for us because during an earlier, unpublished study of the North American coreid, *Leptoglossus phyllopus*, the major male-specific volatile isolated was identified as (*E*)-nerolidol. Reanalysis of an aeration sample from *L. phyllopus* males on the chiral GC column showed that this coreid also synthesizes purely the 3*R*-enantiomer of (*E*)-nerolidol. Various ants, beetles, and bugs produce (*E*)-nerolidol, but the chirality of nerolidol for these insects is presently unknown (Aldrich, 1988b; Burger et al., 1983; Jackson et al., 1990). One tachnid fly, *Trichopoda pennipes*, parasitizes *L. phyllopus* males significantly more than females (Aldrich et al., 1976, 1979), and it seems likely that (3*R*)-(*E*)-nerolidol is part of a kairomone that is exploited by this fly to find hosts. In fact, *T. pennipes* from Florida was introduced in Australia over 40 years ago for biocontrol of the southern green stink bug, *Nezara viridula* (Pentatomidae), but the colonies did not persist (Clausen, 1978). Perhaps *T. pennipes* reared from *L. phyllopus* would choose and successfully parasitize *A. lutescens lutescens*.

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MODIFICATION OF AN INHIBITION CURVE TO ACCOUNT FOR EFFECTS OF A SECOND COMPOUND

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Abstract—Three methods are proposed for modifying a logistic inhibition curve for a primary compound to account for the role of a second compound. The assumptions underlying each of the three resulting models—similar joint action, multiplicative, and modification—are discussed. The models are compared and model selection procedures are given. An example is presented in which the inhibiting effect of *p*-coumaric acid on the growth of morning-glory plants is modified by the addition of glucose.

Key Words—Inhibition curve, similar joint action, multiplicative model, logistic curve, *p*-coumaric acid, glucose.

INTRODUCTION

There are many models for describing the inhibitory action of a compound applied to experimental material. Prominent among these for growth and size measures is the family of logistic curves, which has been preferred because of its range-preserving properties, mathematical simplicity, and ease of parameter fitting and parameter identification. If a second compound is mixed with a primary compound, its presence may modify the effect of the primary compound on the experimental material. The purpose of this article is to present strategies for modifying the simple logistic model to account for the role of a second compound.

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Models for describing the joint inhibitory action of mixtures of compounds first appeared in the literature around 1940. A summary of the research is provided in a review by Morse (1987). The models proposed herein generalize the additive and multiplicative models discussed in that review.

METHODS AND MATERIALS

Models. Suppose that a compound C_1 is applied to experimental material at concentration X_1. Let Y denote the measured response of experimental material to C_i at this concentration. Suppose that the compound has an inhibitory effect on the experimental material and that the relationship between the measured response, Y, and the concentration, X_1, is well described by the three parameter logistic:

$$Y = \beta_1/(1 + \exp\{\beta_3[\ln(X_1) - \beta_2]\}), \quad \text{for } X_1 > 0 \tag{1a}$$

$$= \beta_1, \quad \text{for } X_1 = 0 \tag{1b}$$

where the parameters in the model have the following interpretation: β_1 is the response under control conditions ($X_1 = 0$), β_2 is the value of $\ln(X_1)$ required to achieve 50% inhibition, and β_3 controls the rate of descent of the curve.

Modification of the effect of the primary compound on the experimental material by the presence of a second compound may come about in a number of ways. Models will be presented that cover the following situations: (1) the second compound is similar in its action on the experimental material to the first, (2) the second compound has itself only a negligible effect on the experimental material but modifies the effective concentration of the primary compound, and (3) the second compound acts at a different site or by a different mechanism on the experimental material than the primary compound.

Similar Joint Action (SJA). In the first instance, the second compound C_2 is assumed to be similar in its action on the experimental material to the first. This means that the effect achieved by the primary compound when applied alone may also be realized by the second compound when applied alone, although at a different concentration. In this sense, the compounds enjoy a form of reciprocity in which in a mixture of the two compounds, one concentration of the primary compound may be substituted for a different concentration of the second compound with no change in effect on the experimental material. This concentration differential determines the relative potency of C_2 with respect to C_1. As a rule, this reciprocity is only valid over a limited range of concentration values. Such assumptions lead to the similar joint action model (Gerig et al., 1989). For this model the relationship between the measured response, Y, and the concentration X_1 and X_2 is described by:

$$Y = \beta_1/(1 + \exp\{\beta_3[\ln(Z) - \beta_2]\}), \quad \text{for } Z > 0 \tag{2a}$$

$$= \beta_1, \quad \text{for } Z = 0 \tag{2b}$$

where $Z = X_1 + \beta_4 X_2 + 2\beta_5(\beta_4 X_1 X_2)^{1/2}$, β_4 is the relative potency of C_2 with respect to C_1 and β_5 is the coefficient of synergy.

Using the methods of similar joint action analysis (Gerig et al., 1989), data may be used to estimate parameter values and test hypotheses using nonlinear regression (Gallant, 1975) and SAS PROC NLIN (SAS Institute Inc., 1988).

Modification (MOD). A second manner in which a second compound may modify the effect on the experimental material by the primary compound is by increasing or decreasing its effective concentration. This might occur if the presence of the second compound, which itself has no direct effect on the experimental material, in some way modifies the availability or effective concentration of the first compound. A model that encompasses this mechanism is described. The relationship between the measured response, Y, and the concentrations, X_1 and X_2 is:

$$Y = \beta_1/(1 + \exp\{\beta_3[\ln(Z) - \beta_2]\}), \quad \text{for } Z > 0 \tag{3a}$$

$$= \beta_1, \quad \text{for } Z = 0 \tag{3b}$$

where $Z = X_1 \exp(-\beta_4 X_2)$ and β_4 determines the degree of modification. Thus, when $\beta_4 = 0$, C_2 has no effect and when $\beta_4 < 0$ (or >0), increases in X_2 result in an increase (or decrease) in the effective concentration of C_1. In a previous paper, this parameter was referred to as a damping constant since it controls the degree to which C_2 modifies the action of C_1 on the experimental material (Gerig and Blum, 1991).

SAS computer code is presented in the Appendix as an illustration of the use of PROC NLIN (SAS Institute Inc., 1988) for analyzing data from this model.

Multiplicative (MLT). A third manner of modification occurs if the second compound itself has an effect on the experimental material but acts through a different mechanism or on a different site from the primary compound. It is also assumed that the two compounds do not interact. As in the other models, these assumptions may fail if either or both compounds exceed some maximum. Under these conditions the relationship between the measured response, Y, and the concentrations X_1 and X_2 is

$$Y = \beta_1/(1 + \exp\{\beta_3[\ln(X_1) - \beta_2]\})(1 + \exp\{\beta_5[\ln(X_2) - \beta_4]\}),$$

$$X_1 > 0, X_2 > 0 \tag{4a}$$

$$= \beta_1/(1 + \exp\{\beta_3[\ln(X_1) - \beta_2]\}), \quad X_1 > 0, X_2 = 0 \tag{4b}$$

$$= \beta_1/(1 + \exp\{\beta_5[\ln(X_2) - \beta_4]\}), \quad X_1 = 0, X_2 > 0 \tag{4c}$$

$$= \beta_1, \quad X_1 = 0, X_2 = 0 \tag{4d}$$

where parameters β_4 and β_5 play the same role for C_2 as β_2 and β_3 do for C_1.

SAS computer code is presented in the Appendix as an illustration of the use of PROC NLIN for analyzing data from this model.

RESULTS AND DISCUSSION

Comparisons between Models. In the modification model, the effect of increases in X_2 on the relationship between Y and X_1 can be seen by rewriting the model (3a and 3b) as

$$Y = \beta_1/(1 + \exp\{\beta_3[\ln(X_1) - (\beta_2 + \beta_4 X_2)]\}), \quad \text{for } X_1 > 0 \quad (5a)$$

$$= \beta_1, \quad \text{for } X_1 = 0 \quad (5b)$$

For $\beta_4 < 0$, increasing X_2 decreases the concentration of C_1 required to achieve 50% inhibition and, thus, shifts the inhibition curve to the left. The rest of the model is unchanged. For the multiplicative model, increasing X_2 causes β_1 to decrease and, therefore, results in a scaled down inhibition curve. In the similar joint action model, depending on the values of β_4 and β_5, increases in X_2 will result in a combination of a shift and a down scaling of the inhibition curve.

Of the three, the similar joint action model offers the most versatility, but at the same time imposes the stringent requirement that the two compounds show reciprocity. This is the only model that accounts for synergistic or antagonistic behavior. It is easily extended to more than two compounds (Gerig et al., 1989; Gerig and Blum, 1991).

Model Selection. There is no reason that all data of this structure should conform to one of these three models. Indeed, some data will not fit any of these models. For example, if each compound alone inhibits growth but when introduced into a mixture they react chemically to form a new compound with characteristics distinct from those of either constituent, then none of these models is likely to give an adequate fit.

Given data from an experiment, the preferred method for choosing among the three models (or possibly a fourth) is through an understanding of the underlying science. Lacking this, statistics can provide some direction. In particular, statistical tools may be employed to assess the fit of the data to a given model. Only consider models for which the estimated values of parameters are sensible. If parameter values are out of line, either the data do not fit the model or the model is misfit (e.g., the algorithm converged to a local minimum). For candidate models, form residual values by subtracting predicted values from observed values. These may be summarized in a histogram and plotted against predicted values and X_1 and X_2 (Draper and Smith, 1981). Such plots may point up inadequacies in the fit of the model and potential outliers. For statistics-based model selection, choose the model that has the more random-appearing residual plots, that has the smaller mean squared error and whose residuals have the

smallest inter-quartile range. Note that the R^2 measure of fit commonly used in linear regression does not generalize well to nonlinear regression.

Example. Consider the data from an experiment to study the effect of a primary compound, *p*-coumaric acid, on the growth of morning-glory plants as measured by shoot biomass. A second compound, glucose, was introduced as a C source. By itself, *p*-coumaric acid has been shown to have an inhibitory effect on the growth of morning-glory plants (Blum et al., 1993).

The data were fit separately to each of the three models using nonlinear regression. Table 1 contains the parameter estimates with their standard errors, root mean squared errors, and interquartile ranges for residuals for each model. Estimated parameter values are reasonable and in range for all three models. Note that for the SJA model, β_5 near zero indicates lack of synergy or antagonism for the joint action of *p*-coumaric acid and glucose. This accounts for the fact that the basic model parameters, β_1, β_2, and β_3 are similar in value for the three models. In the MOD model, the significant negative value of β_4 indicates that the presence of glucose enhances the effective concentration of *p*-coumaric acid. Plots showing the fit of each model indicate that the SJA and MLT models fit acceptably, but suggest that the MLT model is superior. The root mean squared errors and interquartile range measures support this choice. Figure 1, which shows such a plot for the MOD model, reveals that observations for

TABLE 1. PARAMETER ESTIMATES, STANDARD ERRORS, ROOT MEAN SQUARES, AND INTERQUARTILE RANGES FOR THREE MODELS[a]

	SJA model	MOD model	MLT model
β_1	0.15(0.01)[b]	0.12(0.01)	0.16(0.01)
β_2	4.17(0.15)	4.38(0.15)	4.12(0.09)
β_3	1.45(0.23)[c]	1.56(0.29)[c]	1.57(0.22)[c]
β_4	0.16(0.03)		
β_5	0.04(0.15)[d]		
β_4		−0.0023(0.0008)[c]	
β_4			6.34(0.43)
β_5			0.71(0.24)[c]
$\sqrt{\text{MSE}}$[e]	0.0173	0.0211	0.0163
Q_3–Q_1[f]	0.0186	0.0255	0.0157

[a] SJA = similar joint action. MOD = modification. MLT = multiplicative.
[b] Parentheses contain the standard error of the estimate.
[c] Significantly different from zero at the 5% level.
[d] Coefficient of synergy not significantly different from zero at the 5% level.
[e] MSE = root mean squared error for regression
[f] Q_3–Q_1 = interquartile range of the residuals

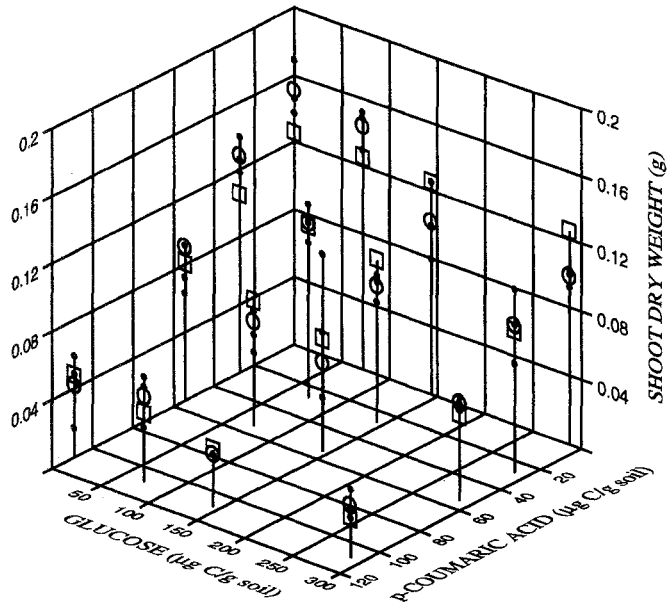

FIG. 1. Observed and fitted values for the MOD model. Observed = solid circles. Means = open circles. Predicted values = open squares.

which the *p*-coumaric acid concentration is zero fit poorly. These observations appear on the back face of the plot. This suggests that the modifying compound, glucose, is having an effect of its own which violates the assumption of the model. For the current experiment, statistical evidence points to the MLT model as superior; however, had the glucose levels been lower, the biology would have favored the MOD model (Blum et al., 1993).

APPENDIX

The full data set along with SAS code for fitting the multiplicative and modification models are given below.

```
DATA A;
INPUT Y X1 X2 REP @ @;
* Y = RESPONSE VARIABLE;
* X1 AND X2 = CONCENTRATIONS OF THE TWO COMPOUNDS;
* C = BLOCK OR REPLICATE NUMBER - 3 REPS IN EXAMPLE;
C1 = (REP = 1); C2 = (REP = 2); C3 = (REP = 3);
CARDS;
0.1457  0.000    0    1  0.1688  0.000    0    2  0.1370  0.000    0    3
```

```
0.1295  0.000   72   1  0.1482  0.000   72   2  0.1519  0.000   72   3
0.0795  0.000   144  1  0.0992  0.000   144  2  0.1253  0.000   144  3
0.1023  0.000   288  1  0.0921  0.000   288  2  0.0991  0.000   288  3
0.1184  27.233  0    1  0.1258  27.233  0    2  0.1392  27.233  0    3
0.0910  27.233  72   1  0.1029  27.233  72   2  0.1138  27.233  72   3
0.0824  27.233  144  1  0.0864  27.233  144  2  0.0706  27.233  144  3
0.1072  27.233  288  1  0.0632  27.233  288  2  0.0860  27.233  288  3
0.1154  54.467  0    1  0.0912  54.467  0    2  0.0629  54.467  0    3
0.0723  54.467  72   1  0.0428  54.467  72   2  0.0668  54.467  72   3
0.0532  54.467  144  1  0.0308  54.467  144  2  0.0695  54.467  144  3
0.0569  54.467  288  1  0.0603  54.467  288  2  0.0525  54.467  288  3
0.0589  108.933 0    1  0.0170  108.933 0    2  0.0483  108.933 0    3
0.0554  108.933 72   1  0.0321  108.933 72   2  0.0613  108.933 72   3
0.0306  108.933 144  1  0.0310  108.933 144  2  0.0305  108.933 144  3
0.0391  108.933 288  1  0.0227  108.933 288  2  0.0276  108.933 288  3
;
PROC NLIN;
TITLE1 'MLT: MULTIPLICATIVE MODEL';
* ---- Starting values for parameters ---- ;
PARMS
B11 = .15 B12 = .15 B13 = .15
B2 = 4
B3 = 1.6
B4 = 6.3
B5 = 0.76;
* ---- Model specification ---- ;
TOL = 0.0001;
T0 = B11*C1 + B12*C2 + B13*C3;
IF X1 < TOL THEN T11 = 0;
        ELSE T11 = EXP(B3*(LOG(X1) - B2));
T21 = 1/(1 + T11);
IF X2 < TOL THEN T12 = 0;
        ELSE T12 = EXP(B5*(LOG(X2) - B4));
T22 = 1/(1 + T12);
MODEL Y = T0*T21*T22;

PROC NLIN;
TITLE1 'MOD: MODIFICATION MODEL';
* ---- Starting values for parameters ---- ;
PARMS
B11 = .15 B12 = .15 B13 = .15
B2 = 4
```

```
B3 = 1.5
B4 = 0.0;
* ---- Model specifications ---- ;
TOL = 0.0001;
Z = X1*EXP(-B4*X2);
T0 = B11*C1 + B12*C2 + B13*C3;
IF Z < TOL THEN T1 = 0;
        ELSE T1 = EXP(B3*(LOG(Z) - B2));
T2 = 1/(1 + T1);
MODEL Y = T0*T2;
RUN;
```

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MODIFICATION OF ALLELOPATHIC EFFECTS OF *p*-COUMARIC ACID ON MORNING-GLORY SEEDLING BIOMASS BY GLUCOSE, METHIONINE, AND NITRATE[1]

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Abstract—Studies of allelopathy have emphasized primarily the identification and quantification of phytotoxins in soils, with only limited attention directed toward how organic (carbon) and inorganic constituents (nutrients) in the soil may modify the action of such phytotoxins. In the present study, increasing carbon (C) levels (up to 108 μg C/g soil) supplied as glucose, phenylalanine, or *p*-hydroxybenzoic acid did not alter morning-glory biomass, but similar C levels supplied as leucine, methionine, or *p*-coumaric acid were inversely related to morning-glory biomass. Similar joint action and multiplicative analyses were used to describe morning-glory biomass response to various C sources and to generate dose isolines for combinations of *p*-coumaric acid and methionine at two NO_3-N levels and for combinations of *p*-coumaric acid and glucose at one NO_3-N level. Methionine, glucose, and NO_3-N treatments influenced the inhibitory action of *p*-coumaric acid on biomass production of morning-glory seedlings. For example, results from the multiplicative analysis indicated that a 10% inhibition of morning-glory biomass required 7.5 μg *p*-coumaric acid/g soil, while the presence of 3.68 μg methionine/g soil the *p*-coumaric acid concentration required for 10% inhibition was only 3.75 μg/g soil. Similar response trends were obtained for *p*-coumaric acid and glucose. The higher NO_3-N (14 vs. 3.5 μg/g) treatments lowered the methionine and increased the *p*-coumaric acid concentrations required for 10% inhibition of morning-glory biomass. These results suggested that allelopathic interactions in soil environments can be a function of interacting neutral substances (e.g., glucose), promoters (e.g., NO_3-N), and/or inhibitors (e.g., methionine and *p*-coumaric acid) of plant growth.

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[1]The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of products named, nor criticism of similar ones not mentioned.

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Key Words—Morning-glory, *Ipomoea hederacea*, growth inhibition, *p*-coumaric acid, allelopathic interactions, leucine, methionine, glucose, nitrate.

INTRODUCTION

Soils contain unique mixtures of organic and inorganic constituents that act as promoter–inhibitor complexes of plant growth. These complexes, in conjunction with biotic, physical, and chemical soil characteristics influence the phytoactivity potential of any given organic constituent in the soil. Allelopathic studies, however, have emphasized primarily the identification and quantification of inhibitors in soils with only limited attention directed toward the way inorganic and other organic constituents in the soil may modify the action of phytotoxins.

Although a variety of phytotoxins have been identified in plant debris and soils, phenolic acids have been the most commonly cited allelopathic agents (Rice, 1984). Once identified, phenolic acids, usually in pure form, have been tested for toxicity on plant species of interest. However, attempts have been made to determine the phytotoxicity of individual phenolic acids under varying nutrient levels (Stowe and Osborn, 1980; Hall et al., 1983; Klein and Blum, 1990) and soil conditions (Blum et al., 1987, 1989) and in mixtures with other phenolic acids (Rasmussen and Einhellig, 1977; Williams and Hoagland, 1982; Blum et al., 1985, 1989; Einhellig, 1987; Gerig and Blum, 1991). How other organic molecules in soils (e.g., carbohydrates or amino acids) might influence the expression of phenolic acid toxicity has not been determined previously.

Microbial utilization of carbon compounds (e.g., carbohydrates, amino acids, phenolic acids, etc.) in high-carbon soils can lead to nitrogen immobilization (Broadbent and Tyler, 1962), a process by which microorganisms convert plant-available forms of nitrogen to forms that can not be used directly by plants. Depending on plant species and substrate, phytotoxicity of phenolic acids may (Stowe and Osborn, 1980; Hall et al., 1985) or may not (Klein and Blum, 1990) be influenced by nutrient levels in the substrate.

Such observations have led to the current work, which describes how various carbohydrates, amino acids, phenolic acids, and nitrate affect the biomass of morning-glory seedlings, as well as how glucose, methionine, and nitrate modify the inhibitory action of *p*-coumaric acid on biomass production of such seedlings. The carbohydrates, amino acids, and phenolic acids tested were chosen for differential rates of microbial utilization, different chemical characteristics, and/or potential phytotoxicity. Two previous studies provided the rationale for many of the initial choices. In one study, leaf expansion of cucumber seedlings grown in nutrient culture was inhibited by 0.5 mM glycine, leucine, methionine, phenylalanine, tyrosine, and tryptophan, but not by serine, proline, glutamic acid, arginine, and histidine (unpublished data). In a second study with

seven phenolic acids, *p*-coumaric acid, a cinnamic acid derivative, and *p*-hydroxybenzoic acid, a benzoic acid derivative, inhibited morning-glory radicle elongation (Blum et al., 1992).

METHODS AND MATERIALS

General Aspects.

Morning-glory seeds (*Ipomoea hederacea* L. Jacquin.: F&J Seed Service, Woodstock, Illinois) were germinated in the dark at 28–30°C in 155-ml cups (5 seeds/cup) containing 150 g of a soil–sand mixture (1:2 by weight) and 30 ml distilled water. Portsmouth B_1-horizon soil material (fine loamy, mixed, thermic Typic Unbraquaalts) was obtained from the NCSU Tidewater Research Station in Plymouth, North Carolina, sieved, air-dried, and adjusted to pH 5 using calcium hydroxide. River sand was thoroughly rinsed with deionized water and air-dried. After 24 hr, cups were inserted through holes in a plywood sheet that protected everything below the soil surface from direct irradiance of the light banks in the laboratory. Seedlings were grown at room temperature (21–30°C) with a 12-hr light period (140 μEinsteins/m^2/sec). Seedlings were thinned to one seedling per cup four or five days seeding. Seedlings were supplied with double-strength Hoagland's solution (pH 5; Hoagland and Arnon, 1950) every other day starting with day 4 or 5. Sufficient distilled water was added daily to bring the weight of a given cup (160 g initially for cup and soil) and its seedling to approximately 180 g. Combined cup, soil, and seedling weights were maintained above 165 g. For further details of the cup system, see Blum et al. (1987).

Treatments

Phytotoxicity of Various Carbon Sources. Cellulose (fibrous, medium; Sigma Chemical, St. Louis, Missouri) and potato starch (purified, powder; Fisher Scientific, Pittsburgh, Pennsylvania) were mixed with soil before seeding. Concentrations ranged from 0 to 6 mg/g soil. Aqueous solutions (pH 5) of glucose, leucine, methionine, *p*-coumaric acid, phenylalanine, or *p*-hydroxybenzoic acid (Sigma Chemical; 0–1 μmol/g soil) were added every other day starting with day 7 from seeding. Solution pH was adjusted to pH 5 with NaOH or HCl. Five milliliters of double-strength Hoagland's solution (pH 5), which supplied 14 μg NO_3-N/g soil, were added every other day starting with day 4. Seedlings were harvested when they were 17 days old, oven dried (105°C), and weighed.

Carbon and nitrogen contents of cellulose, starch, and Portsmouth B_1 soil material were determined with a Perkin-Elmer 2400 C, H, N analyzer (Perkin-Elmer, Norwalk, Connecticut). Carbon and nitrogen contents of other compounds were calculated from their structures.

Modification of Phytotoxicity by Nitrogen. Solutions (0–1 μmol/g soil) of glucose, leucine, methionine, or *p*-coumaric acid were added to cups every other day starting with day 6 from seeding. Five milliliters of double-strength Hoagland's solution supplying 3.5, 7, or 14 μg NO_3-N/g soil were added every other day starting with day 5. In addition, sets of seedlings not treated with organic compounds were given 5 ml double-strength Hoagland's solution supplying 0, 10.5, 17.5, or 21 μg NO_3-N/g soil. NO_3-N concentrations were adjusted by substituting KCl and $CaCl_2$ for KNO_3 and $Ca(NO_3)_2$. Seedlings were harvested when they were 16 days old, oven-dried, and weighed.

Methionine and/or p-Coumaric Acid. Solutions of methionine (0–0.5 μmol/g) or *p*-coumaric acid (0 to 1 μmol/g) or various combinations of these two compounds (0.032–0.25 μmol each/g; the maximum total mixture concentration applied was 0.5 μmol/g) were added every other day starting with day 6 from seeding. Five milliliters of double-strength Hoagland's solution supplying 3.5 or 14 μg NO_3-N/g soil were added every other day starting with day 5. Seedlings were harvested when they were 16 days old, oven dried, and weighed.

Glucose and/or p-Coumaric Acid. Solutions of glucose (0–4 μmol/g), *p*-coumaric acid (0–1 μmol/g), or combinations of these two compounds were added every other day starting with day 6 from seedling. Five milliliters of double-strength Hoagland's solution, supplying 7 μg NO_3-N/g soil, were added every other day starting with day 5. Seedlings were harvested when they were 16 days old, oven-dried, and weighed.

Experimental Design and Statistical Analyses

A randomized complete block design was used in all experiments. Plants were distributed under three light banks (blocks). The design for each experiment was as follows (compounds × concentrations × NO_3-N × blocks): Experiment 1—$8 \times 5 \times 1 \times 3 = 120$; experiment 2—$4 \times 3 \times 3 \times 3 = 108$ and $1 \times 1 \times 7 \times 3 = 21$; experiment 3—(5 methionine, 5 *p*-coumaric acid, 1 control, 9 combinations) $\times 2 \times 3 = 120$; and experiment 4—(3 glucose, 3 *p*-coumaric acid, 1 control, 9 mixtures) $\times 1 \times 3 = 48$.

Data were analyzed utilizing SAS (1988) procedures such as PROC ANOVA and PROC GLM. The similar joint action analysis, multiplicative analysis, and logistic analysis used for this study are described by Gerig et al. (1989) and Gerig and Blum (1993).

RESULTS AND DISCUSSION

Normally, concentrations of organic compounds used in bioassays are expressed in microgram or micromoles per gram of soil. Here we have chosen to express concentrations of the various carbohydrates, amino acids, and phe-

nolic acids in terms of their carbon content (micrograms of carbon per gram of soil). This provided a common basis by which the action of these compounds are comparable in terms of carbon quality, quantity, and C/N ratios.

The percentages of carbon in the compounds and soil material were as follows: cellulose (42.59%), glucose (40.00%), leucine (45.77%), methionine (40.25%), *p*-coumaric acid (65.83%), *p*-hydroxybenzoic acid (60.88%), phenylalanine (65.43%), potato starch (37.89%), and Portsmouth B_1 soil material (excluding sand; 0.49%). The percentages of nitrogen (N) in the amino acids and soil material were as follows: leucine (10.68%), methionine (9.39%), phenylalanine (8.48%), and Portsmouth B_1 soil material (excluding sand; 0.04%). The amount of NO_3-N supplied by the Hoagland's solution varied with experiment, but 5 ml of double-strength Hoagland's solutions supplied 2.1 mg NO_3-N (14 μg/g soil).

Phytotoxicity of Various Carbon Sources. The initial C/N ratios (i.e., before addition of Hoagland's solution) for cellulose- and starch-amended soils (each applied once) ranged from 12.25 (no cellulose or starch added) to 31.4 for cellulose and 29.3 for starch. Both Iritani and Arnold (1960) and Harmsen and Van Schreven (1955) have suggested that a C/N ratio of 20 is the approximate threshold between mineralization and immobilization of N in soils by microorganisms. In this study, however, these C/N ratios were not entirely applicable because Hoagland's solution, which provided a total of 14.7 mg NO_3-N, was applied uniformly from days 4 to 16 (14 μg NO_3-N/g soil every other day). The magnitude of N immobilization is determined by the availability of C and N to soil microbes. The importance of readily available forms of soil C to microbes for the process of nitrogen immobilization was supported by our experiments in that only starch C, the readily available C source, was inversely related to root biomass of morning-glory (Table 1). Cellulose C had no detectable effect on morning-glory biomass.

Since C and N sources were applied every other day for the other treatments, a representative C/N ratio could be calculated for the entire experimental period. C/N ratios (excluding native soil C and N) ranged from 0 (no C or NO_3-N added) to 5.1 for glucose, 0–2.2 for leucine and methionine, 0–7.8 for *p*-coumaric acid, 0–6 for *p*-hydroxybenzoic acid, and 0–4 for phenylanine. The ratios for the amino acids include the N from these amino acids. These ratios suggested that the additions of C by these compounds and the N by Hoagland's solution (NO_3-N) and/or amino acids resulted in systems that were not nitrogen limited. This conclusion was supported by the findings that increasing C levels of glucose (up to 72 μg/g), phenylalanine (up to 108 μg/g), and *p*-hydroxybenzoic acid (up to 84 μg/g) did not modify morning-glory biomass (Table 1). Since the C levels added by leucine (up to 60 μg/g), methionine (up to 60 μg/g), and *p*-coumaric acid (up to 108 μg/g) were similar to that added by glucose, phenylalanine, and *p*-hydroxybenzoic acid, the inverse relationships between C sup-

TABLE 1. PARTIAL REGRESSION COEFFICIENTS, p, AND R^2 VALUES FOR DRY WEIGHTS (g) AND ROOT SHOOT RATIOS OF 17-DAY-OLD IVY-LEAVED MORNING-GLORY SEEDLINGS GROWN IN PORTSMOUTH B_1 SOIL AND TREATED WITH VARIOUS CARBON SOURCES[a]

Source of carbon	Variable	Intercept	Linear	p	R^2
Single treatments[b]					
Cellulose				NS[c]	
Starch	Shoot			NS	
	Root	0.0889	-1.5850×10^{-5}	0.0066	0.40
	Plant	0.2673	-3.6602×10^{-5}	0.0344	0.30
	R/S ratio			NS	
Multiple treatments[d]					
Glucose				NS	
Leucine	Shoot			NS	
	Root	0.1093	−0.0009	0.0001	0.75
	Plant	0.2795	−0.0011	0.0001	0.79
	R/S ratio	0.6508	−0.0048	0.0023	0.52
Methionine	Shoot	0.1726	−0.0014	0.0001	0.71
	Root	0.1060	−0.0014	0.0005	0.62
	Plant	0.2785	−0.0028	0.0002	0.68
	R/S ratio	0.6096	−0.0049	0.0006	0.61
p-Coumaric acid	Shoot	0.1759	−0.0008	0.0033	0.50
	Root			NS	
	Plant	0.2751	−0.0011	0.0187	0.36
	R/S ratio			NS	
Phenylalanine				NS	
p-Hydroxybenzoic acid				NS	

[a] Five milliliters of double-strength Hoagland's solution (14 μg NO_3-N/g soil) were added to cups every other day starting with day 4 from seeding.
[b] Cellulose and starch (0–6 mg/g soil) were mixed with soil before seeding. Carbon added ranged from 0 to 2555 μg/g for cellulose and 0 to 2273 μg/g for starch. Carbon added was used as the independent variable in these models.
[c] $p > 0.05$.
[d] Compounds (0–1 μmol/g soil) were added every other day starting with day 7 from seeding. Carbon added ranged from 0 to 72 μg/g for glucose, 0 to 60 μg/g for leucine and methionine, 0 to 108 μg/g for p-coumaric acid and phenylalanine, and 0 to 84 μg/g for p-hydroxybenzoic acid. Carbon added was used as the independent variable in these models.

plied by leucine, methionine, and p-coumaric acid and morning-glory biomass were thus due to some type of phytotoxicity (Table 1). Additions of 50 μg C/g by soil leucine, methionine, and p-coumaric acid resulted in 20, 50, and 20%, respectively, lower plant biomass for morning-glory. The effects of leucine, however, were on root biomass, while methionine altered root and shoot biomass, and p-coumaric acid altered shoot biomass. Root–shoot ratios were

reduced only by leucine and methionine. These results suggested that the sites and/or modes of action of these compounds are different.

Modification of Phytoxicity by Nitrogen. The lower than expected biomass values in Figure 1A for the 3.5-μg NO_3-N treatment were a result of procedural error since they received only the last three N treatments compared to the five applied to all other treatments. Maximum biomass was achieved with every-other-day applications of 14 μg NO_3-N/g soil. NO_3-N concentrations greater than that had a negligible or negative effect on biomass. Plants treated with 14 μg NO_3-N/g soil were 1.9, 2.7, and 2.4 $\times$ greater in root, shoot, and plant biomass, respectively, compared to the 0 μg/g NO_3-N treatment. Root–shoot ratios, although highly variable, were at their lowest around 10–12 μg/g NO_3-N/g soil (Figure 1B).

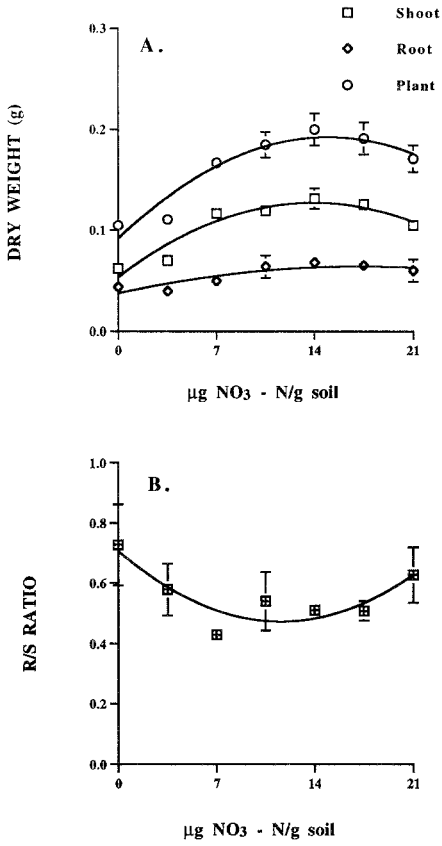

FIG. 1. Effects of NO_3-N on biomass and root–shoot ratio of morning-glory seedlings (means $\pm$ standard error).

The C/N ratios ranged from 0 to 21 for glucose, from 0 to 3.4 for leucine and methionine, and from 0 to 31 for *p*-coumaric acid treatments. The effects of leucine C and *p*-coumaric acid C were independent of NO_3-N applications (Tables 2 and 3). General linear model procedures for effects of leucine C within each NO_3-N treatment, however, resulted in a nonsignificant fit of models. Interactions for phenolic acid and nitrogen treatments on plant growth have been inconsistently expressed (Stowe and Osborn, 1980; Hall et al., 1983; Blum and Dalton, 1985; Klein and Blum, 1990).

Significant interactions between glucose C or methionine C and NO_3-N were observed on morning-glory biomass (Table 2). No consistent pattern for the interactions of glucose C and NO_3-N on morning-glory biomass occurred (Table 3). For methionine-treated morning-glory seedlings, plant biomass was very similar at concentrations greater than 30 μg C/g soil for all NO_3-N levels (Figure 2). This level of methionine C suppressed plant biomass by 16%, 45%, and 48% for the 3.5, 7, and 14 μg NO_3-N levels, respectively. Thus in the presence of higher nitrogen levels methionine inhibition was greater. Nitrogen mediated release of sulfur (a potential maximum of 4.84 mg S/treatment) from

TABLE 2. *p* VALUES FROM ANALYSES OF VARIANCE FOR MORNING-GLORY SEEDLING DRY WEIGHTS AND ROOT-SHOOT RATIOS

		p		
Source of carbon	Variable	Carbon (C)[a]	Inorganic N (IN)[b]	C × IN
Glucose	Shoot	NS[c]	0.0001	0.0357
	Root	NS	0.0004	NS
	Plant	NS	0.0001	NS
	R/S	NS	0.0009	0.0307
Leucine	Shoot	NS	0.0006	NS
	Root	0.0282	0.0449	NS
	Plant	0.0284	0.0005	NS
	R/S	NS	NS	NS
Methionine	Shoot	0.0001	0.0001	0.0001
	Root	0.0001	0.0058	0.0438
	Plant	0.0001	0.0001	0.0002
	R/S	NS	NS	NS
p-Coumaric	Shoot	0.0001	0.0001	NS
	Root	0.0002	0.0001	NS
	Plant	0.0001	0.0001	NS
	R/S	NS	NS	NS

[a] See Table 1 for range of carbon added.
[b] 3.5, 7, and 14 μg N/g soil in the form of NO_3^-.
[c] $p > 0.05$.

TABLE 3. PARTIAL REGRESSION COEFFICIENTS, p, AND R^2 VALUES FOR DRY WEIGHTS (g) AND ROOT–SHOOT RATIOS OF 16-DAY-OLD IVY-LEAVED MORNING-GLORY SEEDLINGS GROWN IN PORTSMOUTH B_1 SOIL AND TREATED WITH VARIOUS LEVELS OF NO_3-N AND CARBON SOURCES[a]

Nitrogen (μg/g)	Variable	Intercept	Linear	Quadratic	p	R^2
Glucose						
3.5	R/S ratio	0.5504	0.0036	1.3142×10^{-5}	0.0395	0.66
7	Shoot	0.1212	−0.0004		0.0351	0.49
Methionine						
3.5	Shoot	0.0729	−0.0002		0.0292	0.52
	Root	0.0406	−0.0004		0.0026	0.75
	Plant	0.1135	−0.0006		0.0002	0.87
	R/S ratio	0.5701	−0.0038		0.0406	0.47
7	Shoot	0.1166	−0.0021	1.9119×10^{-5}	0.0001	0.95
	Root	0.0501	−0.0015	1.8271×10^{-5}	0.0135	0.76
	Plant	0.1668	−0.0036	3.7389×10^{-5}	0.0008	0.91
14	Shoot	0.1321	−0.0031	3.4101×10^{-5}	0.0012	0.89
	Root	0.0641	−0.0007		0.0009	0.81
	Plant	0.1998	−0.0046	4.6054×10^{-5}	0.0004	0.92
p-Coumaric acid						
3.5	Shoot	0.0806	−0.0005		0.0090	0.65
	Root	0.0412	−0.0002		0.0104	0.63
	Plant	0.1219	−0.0008		0.079	0.66
7	Shoot	0.1317	−0.0008		0.0001	0.90
	Root	0.0646	−0.0003		0.0074	0.66
	Plant	0.1963	−0.0011		0.005	0.84
14	Shoot	0.1406	−0.0006		0.0041	0.71
	Root	0.0670	−0.0002		0.0411	0.47
	Plant	0.2077	−0.0008		0.0034	0.73
	R/S ratio	0.4426	0.0079	-5.770×10^{-5}	0.0265	0.70

[a] See Table 1 for range of carbon added.

methionine by soil microbes may help to explain why methionine was more toxic at higher nitrogen levels. The release of sulfur could lead to the formation of an anaerobic soil environment and/or the acidification of the soil. At the end of the experiment, however, the bulk soil pH was not significantly different from the bulk soil pH of the starting soil material. The oxygen status of the soil was not monitored. An odor characteristic of hydrogen sulfide, methyl mercaptan, and/or methyl sulfide, however, was noted 24 hr after methionine treatment. This odor was less distinct with each subsequent treatment.

Methionine and/or p-Coumaric Acid. Whether the sites or modes of action for two phytotoxic compounds are similar or different determines how a plant will respond as well as the statistical or modeling tools required to characterize

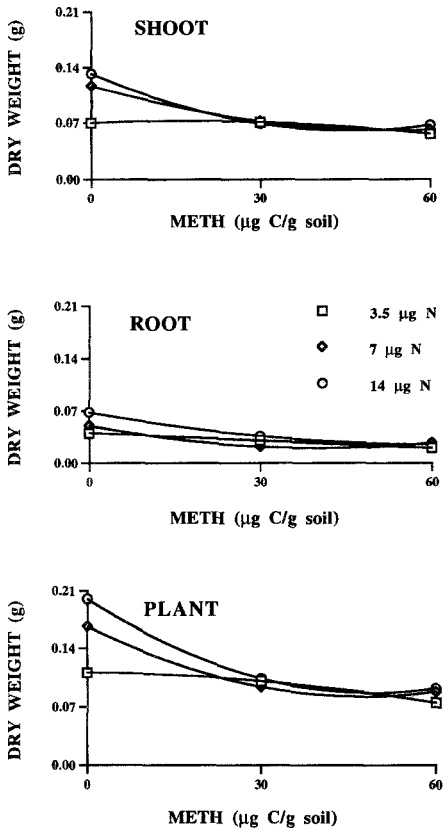

FIG. 2. Modification of the effect of methionine C on the biomass of morning-glory seedlings by NO_3-N (means ± standard error).

this response. Similar joint action analysis (Gerig et al., 1989) may be used if the sites and/or modes are similar and the compounds are substitutable. Multiplicative analysis (Gerig and Blum, 1993) may be used if the sites and/or modes are different and noninteracting. The model for similar joint action analysis allows for antagonistic, synergistic, or similar joint (additive) action of compounds. This model also allows for different potencies (toxicities) of the compounds. It further assumes that the effects achieved by a given compound in the mixture may be realized by substitution of an appropriate amount of the other compound. The model for multiplicative analysis is identical to the similar joint action analysis when the dose is zero. This model also allows for different potencies and concentrations of compounds.

We present both analyses here, but a graphical display only for the mul-

tiplicative analysis (Figure 3) since the displays for both analysis were essentially similar. The actual sites and/or modes of action for *p*-coumaric acid and methionine are not known, but plant response, as noted earlier, suggested that the sites and/or modes of action of the foregoing compounds were different. Additional evidence for different sites and/or modes of action for these compounds was obtained in a preliminary study (unpublished data) using aerated nutrient culture (with 0–1 mM concentrations of the inhibitors) where we observed that both net phosphorus uptake and water utilization of morning-glory seedlings were inhibited by *p*-coumaric acid, but not by methionine. Inhibition of net phosphorus uptake and water utilization by *p*-coumaric acid and other phenolic acids has also been observed for other Species (Glass and Dunlop, 1974; McClure et al., 1978; Blum et al., 1985; Lyn et al., 1990; Holappa and Blum, 1991; Booker et al., 1992).

The C/N ratios for *p*-coumaric acid treatments ranged from 0 to 31 for the low N (3.5 μg/g) and from 0 to 7.7 for the high N (14 μg/g) levels. The C/N ratios for methionine ranged from 0 to 1.7 for low N and from 0 to 1.1 for high N treatments. Although the underlying assumptions of the two analytical processes were different, the general conclusions about morning-glory responses were similar (Tables 4 and 5). Methionine suppressed morning-glory root biomass more than did *p*-coumaric acid. The 50% inhibition doses for methionine were three to five times lower than for *p*-coumaric acid. Effects of both compounds on shoot biomass were very similar because the doses were not significantly different. Effects of the mixtures on morning-glory biomass were either additive or multiplicative; no clear choice between the additive or multiplicative effects can be made on the basis of the present data.

Methionine $\times$ NO_3-N interactions observed in the "modification of phytotoxicity by nitrogen" experiment were eliminated here by reducing the maximum concentration of methionine from 60 μg C/g soil to 30 μg C/g soil. Higher NO_3-N levels when compared to lower NO_3-N levels, however, resulted in a higher *p*-coumaric acid 50% inhibition dose for shoot biomass (Table 4, similar joint action), and an increased rate parameter (slope) for plant biomass (Table 5, multiplicative analysis). Interactions for *p*-coumaric acid and NO_3-N were not observed in the absence of methionine in the "modification of phytotoxicity by nitrogen" experiment.

Isolines describing doses required for given fixed levels of inhibition were generated by both modeling procedures. In order to generate these values for the similar joint analysis, the coefficient of synergy was set to zero, because this coefficient was not significantly different from zero for *p*-coumaric acid and methionine treatments. The shape of the isolines, and therefore the dose for a given percent inhibition, were different for the two models (Figures 4 and 5). The differences in the shape and/or slopes of the isolines resulted from differ-

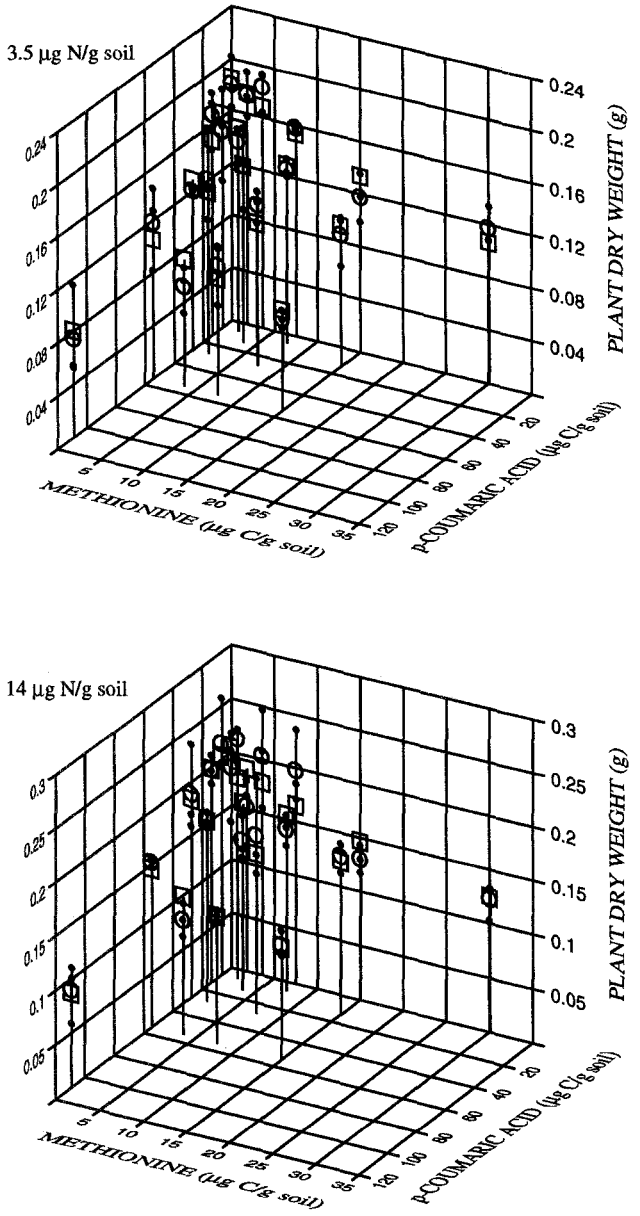

FIG. 3. Display of observed (solid dots), means (open circles), and predicted values (open squares) for seedling biomass from the multiplicative analyses for methionine and/or *p*-coumaric acid treated morning-glory seedlings supplied with 3.5 or 14 µg NO_3-N/g soil.

TABLE 4. SUMMARY OF JOINT ACTION ANALYSIS FOR EFFECTS OF *p*-COUMARIC ACID AND METHIONINE ON MORNING-GLORY SEEDLINGS GROWN UNDER TWO DIFFERENT NO_3-N LEVELS

	NO_3-N (μg/g soil)					
	Shoot		Root		Plant	
	3.5	14	3.5	14	3.5	14
B_1 (control values; g dry weight)	0.13	0.13	0.05	0.06	0.18	0.20
B_2 (dose [μg C/g soil] required for 50% inhibition)						
p-Coumaric acid C	58.86	113.58	158.91	96.67	80.55	107.43
Methionine C	54.57	51.88	29.32[a]	21.48[a]	46.03	36.92[a]
B_3 (rate parameter)	0.87[b]	1.33[b]	1.19[b]	1.72[b]	0.96[b]	1.48[b]
B_4 (relative potency)	1.08	2.19	5.42[c]	4.50[c]	1.75	2.91[c]
B_5 (coefficient of synergy)	0.13[d]	0.27[d]	0.96[d]	−0.13[d]	0.42[d]	0.11[d]

[a] Methionine dose is significantly different from *p*-coumaric acid dose at 5% level.
[b] Significantly different from zero at 5% level.
[c] Significantly different from 1.
[d] Not significantly different from zero at 5% level.

TABLE 5. SUMMARY OF MULTIPLICATIVE MODEL ANALYSIS FOR EFFECTS OF *p*-COUMARIC ACID AND METHIONINE ON MORNING-GLORY SEEDLINGS GROWN UNDER TWO DIFFERENT NO_3-N LEVELS

	NO_3-N (μg/g soil)					
	Shoot		Root		Plant	
	3.5	14	3.5	14	3.5	14
B_1 (control values; g dry weight)	0.13	0.14	0.06	0.07	0.18	0.20
B_2 (dose [μg C/g soil] required for 50% inhibition)						
p-Coumaric acid C	65.32	98.32	124.96	79.93	77.18	90.40
Methionine C	44.45	89.96	21.90[a]	25.18[a]	41.36	55.42
B_3 (rate parameter)[b]						
p-Coumaric acid C	0.81[c]	1.33[c]	0.79[c]	2.70[c]	0.80[c]	1.76[c]
Methionine C	1.21[c]	0.68[c]	0.81[c]	0.82[c]	0.94[c]	0.70[c]

[a] Methionine dose is significantly different from *p*-coumaric acid dose at 5% level.
[b] Methionine rate is not significantly different from *p*-coumaric acid rate at 5% level.
[c] Significantly different from zero at 5% level.

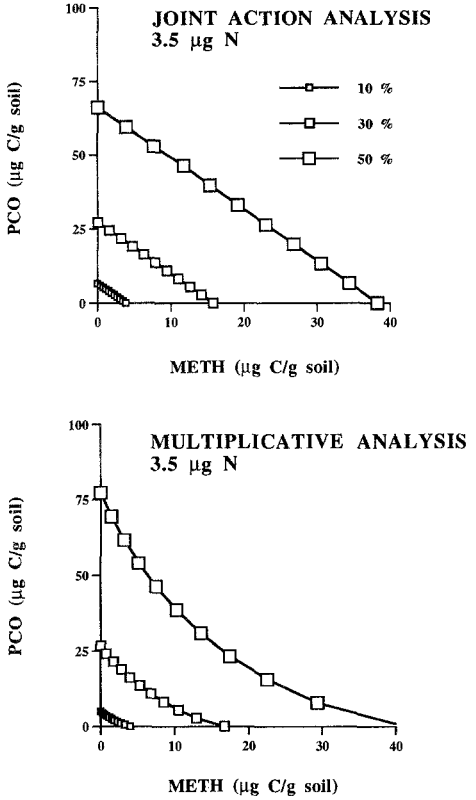

FIG. 4. Isolines for 10, 30, and 50% inhibition of morning-glory seedling biomass treated with *p*-coumaric acid C (PCO), methionine C (METH), and 3.5 µg/g NO_3-N. Values for isolines generated by joint action or multiplicative analysis.

ences in the structures of the models. The differences were accentuated for the higher NO_3-N levels.

Methionine and NO_3-N influence the inhibitory action of *p*-coumaric acid on biomass production of morning-glory seedlings. For example, results from the multiplicative model (choice based on plant biomass responses and nutrient uptake), indicated that the concentration of *p*-coumaric acid required to inhibit morning-glory plant biomass by 10% was 7.5 µg/g for plants treated with 3.5 µg NO_3-N/g soil, whereas the required concentration was reduced by 50% with the addition of 3.68 µg methionine/g (Table 6). Further, a 10% reduction in plant biomass required 7.5 µg/g and 39.51 µg/g soil of *p*-coumaric acid for plant grown in the presence of the 3.5 and 14 µg NO_3-N/g soil levels, respec-

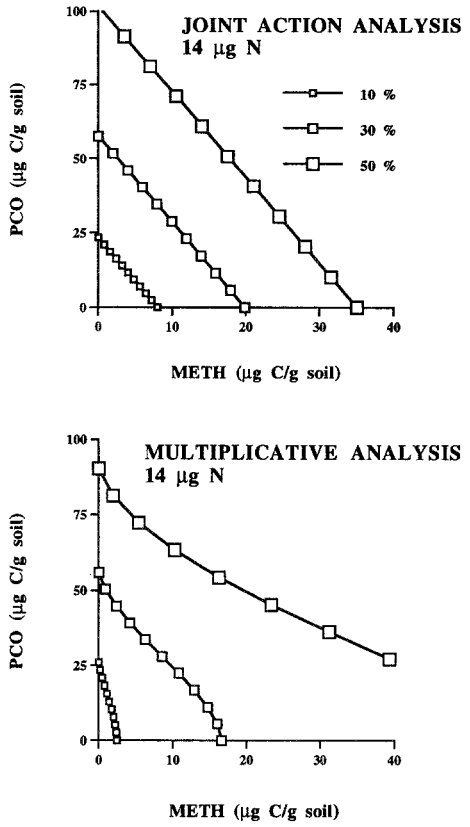

FIG. 5. Isolines for 10, 30, and 50% inhibition of morning-glory seedling biomass treated with *p*-coumaric acid C (PCO), methionine C (METH), and 14 μg/g NO_3-N. Values for isolines generated by joint action or multiplicative analysis.

tively. Thus, there was a fivefold increase in the requirement of *p*-coumaric acid relative to a fourfold increase in added NO_3-N.

The concentration of methionine required to inhibit morning-glory plant biomass by 10% was 9.81 μg/g soil for plants treated with 3.5 μg NO_3-N/g soil, but the requirement was reduced to 3.68 μg/g soil with the addition of 3.75 μg *p*-coumaric acid/g soil. A 10% suppression of plant biomass required 9.81 μg/g soil and 6.06 μg/g soil of methionine for plants treated with 3.5 and 14 μg NO_3-N/g soil, respectively. This was consistent with the higher toxicity of methionine at higher NO_3-N levels observed in the "modification of phytotoxicity by nitrogen" experiment.

Glucose and/or p-Coumaric Acid. The effects of *p*-coumaric acid on morning-glory plants may also be influenced indirectly by nitrogen immobilization,

TABLE 6. PERCENT INHIBITION DOSES FOR PLANT BIOMASS OF MORNING-GLORY SEEDLINGS TREATED WITH *p*-COUMARIC ACID (PCO) AND/OR METHIONINE (METH), AND *p*-COUMARIC ACID AND/OR GLUCOSE (GLU)

			Similar joint action analysis				Multiplicative analysis			
			10%		20%		10%		20%	
Compound	NO_3-N (μg/g)	PCO (%)	μg C/g	μg/g	μg C/g	μg/g	μg C/g	μg/g	μg C/g	μg/g
PCO	3.5	0	0	0	0	0	0	0	0	0
METH			3.83	9.52	8.96	22.26	3.95	9.81	9.40	23.36
PCO		50	3.31	5.08	7.74	11.76	2.47	3.75	6.81	10.34
METH			1.92	4.77	4.48	11.13	1.48	3.68	3.27	8.12
PCO		100	6.62	10.06	15.48	23.52	4.94	7.50	13.62	20.69
METH			0	0	0	0	0	0	0	0
PCO	14	0	0	0	0	0	0	0	0	0
METH			8.04	19.97	13.85	34.41	2.44	6.06	7.72	19.18
PCO		50	11.64	17.68	20.05	30.46	13.01	19.76	20.60	31.29
METH			4.02	9.99	6.93	17.22	1.42	3.53	4.21	10.46
PCO		100	23.29	35.38	40.11	60.93	26.01	39.51	41.19	62.57
METH			0	0	0	0	0	0	0	0
PCO	7	0	0	0	0	0	0	0	0	0
GLU			121.52	303.80	204.90	512.25	23.72	59.30	92.66	231.65
PCO		50	8.92	13.55	15.04	22.85	9.06	13.76	14.88	22.60
GLU			60.76	151.90	102.45	256.12	11.64	29.10	42.38	105.95
PCO		100	17.85	27.12	30.09	45.71	18.13	27.54	29.76	45.21
GLU			0	0	0	0	0	0	0	0

microbial formation of toxins, and/or the creation of anaerobic soil conditions by the addition of a C source such as glucose. For this experiment, the C/N ratios for *p*-coumaric acid treatments ranged from 0 to 15 and the C/N ratios for glucose ranged from 0 to 41. As in the "methionine and/or *p*-coumaric acid" experiment, similar joint action analysis and multiplicative analysis resulted in similar conclusions about morning-glory responses (Tables 7 and 8). Here also we present a graphical display only for the multiplicative model (Figure 6), since the displays for the two analyses were essentially identical. *p*-Coumaric acid reduced shoot and root biomass of morning-glory more than glucose did. The 50% inhibition doses for glucose (excluding roots) were 6–13 times greater than those for *p*-coumaric acid. Glucose essentially had no effect

TABLE 7. SUMMARY OF JOINT ACTION ANALYSIS OF *p*-COUMARIC ACID AND GLUCOSE ON MORNING-GLORY SEEDLING BIOMASS

	Shoot	Root	Plant
B_1 (control values; g dry weight)	0.15	0.08	0.23
B_2 (dose [μg C/g soil] required for 50% inhibition)			
p-Coumaric acid C	64.55	107.70	75.30
Glucose C	403.44[a]	53850.00[a]	537.86[a]
B_3 (rate parameter)	1.45[b]	2.40[b]	1.54[b]
B_4 (relative potency)	0.16[c]	0.002[c]	0.14[c]
B_5 (coefficient of synergy)[d]	0.04	3.70	0.04

[a] Glucose dose is significantly different from *p*-coumaric acid dose at 5% level.
[b] Significantly different from zero at 5% level.
[c] Significantly different from 1.
[d] Not significantly different from zero at 5% level.

TABLE 8. SUMMARY OF MULTIPLICATIVE MODEL ANALYSIS FOR EFFECTS ON *p*-COUMARIC ACID AND GLUCOSE ON MORNING-GLORY SEEDLING BIOMASS

	Shoot	Root	Plant
B_1 (control value; g dry weight)	0.16	0.09	0.25
B_2 (dose [μg C/g soil] required for 50% inhibition)			
p-Coumaric acid C	61.63	82.09	69.13
Glucose C	566.27[a]	5942.41	947.10[a]
B_3 (rate parameter)			
p-Coumaric acid C	1.57[b]	2.02[b]	1.64[b]
Glucose C	0.71[ab]	0.37	0.60[ab]

[a] Glucose dose is significantly different from *p*-coumaric acid dose at 5% level.
[b] Significantly different from zero at 5% level.

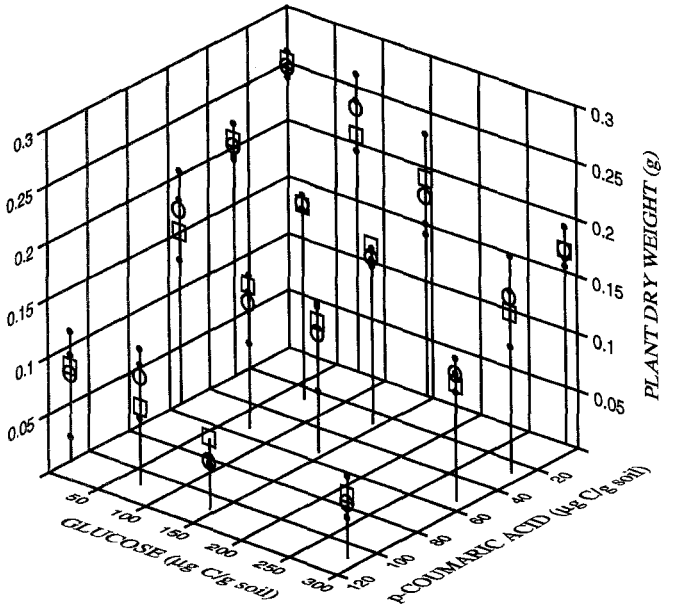

FIG. 6. Display of observed (solid dots), means (open circles), and predicted values (open squares) for seedling biomass from the multiplicative analysis for glucose and/or *p*-coumaric acid treated morning-glory seedlings supplied with 7 μg NO_3-N/g soil.

on morning-glory root biomass. Shoot biomass was more sensitive to *p*-coumaric acid than root biomass. Effects of the mixtures of *p*-coumaric acid and glucose on morning-glory were either additive or multiplicative. Again, no clear choice can be made between an additive or multiplicative effect on the basis of the present data.

Morning-glory plant biomass isolines for doses required for specific percentages of inhibition were also generated for this experiment. The coefficient of synergy for the similar joint action analysis was again set to zero. The shape of the isolines, and thus the dose for a given inhibition, were different for the two models (Figure 7). The differences in shape and/or slope of the isolines resulted from differences in the structures of the models.

Glucose, unlike methionine, would probably only indirectly affect morning-glory biomass (e.g., through nitrogen immobilization). A 10% inhibition (multiplicative analysis) of morning-glory plants grown in the presence of 7 μg NO_3-N/g soil treatments required 27.54 μg *p*-coumaric acid/g soil, but the addition of 29.10 μg glucose/g soil reduced this concentration by 50% (Table 6). The level of C added in the later system was below that required for nitrogen immobilization (C/N ratio = 3). A 10% inhibition of morning-glory biomass required

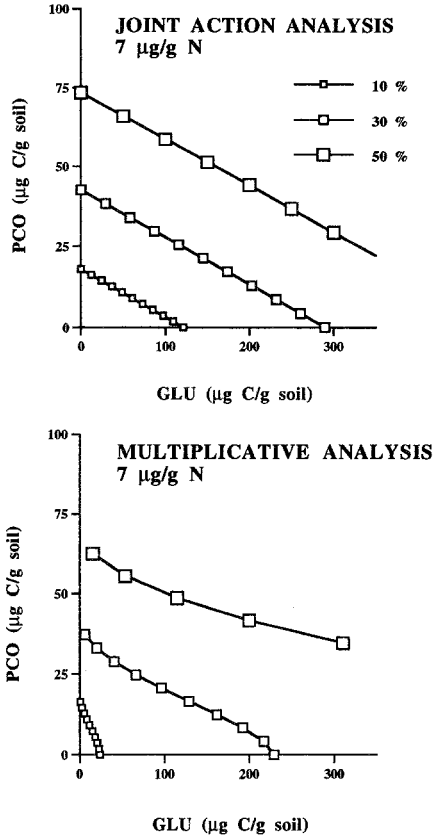

FIG. 7. Isolines for 10, 30, and 50% inhibition of morning-glory seedling biomass treated with *p*-coumaric acid C (PCO), glucose C (GLU), and 7 µg/g NO_3-N. Values for isolines generated by joint action or multiplicative analysis.

59.30 µg glucose/g soil (CN ratio = 3), but the addition of 13.76 µg *p*-coumaric acid/g reduced the required inhibitory concentration of glucose to 29.10 µg/g soil (C/N ratio = 3). These low C/N ratios suggest that glucose, in these experiments, may not be modifying morning-glory biomass accumulation by N-immobilization.

Concluding Remarks. The data clearly show that soil NO_3-N levels modified the concentrations of methionine (higher levels of NO_3-N reduced) and *p*-coumaric acid (higher levels of NO_3-N increased) required for inhibition of morning-glory biomass accumulation and that the concentrations of individual compounds (i.e., glucose, methionine, or *p*-coumaric acid) required for a given level of inhibition of morning-glory biomass accumulation were reduced when

a second C source (i.e., glucose, methionine, or *p*-coumaric acid) was added. For methionine and *p*-coumaric acid, both growth inhibitors, it is suggested that their sites and/or modes of action are different and thus their effects multiplicative. Similarly, we suggest that the sites and/or modes of action for inhibitory concentrations of glucose, above 72 μg glucose C/g soil, and *p*-coumaric acid are different and thus their effects multiplicative. For glucose concentrations below 72 μg glucose C/g soil and *p*-coumaric acid, neither the additive or multiplicative analysis appear to be entirely appropriate. We hypothesize that the addition of glucose below 72 μg C/g soil reduced the microbial utilization and/or sorption of *p*-coumaric acid in the soil and thus increased the effective concentration of *p*-coumaric acid. If this hypothesis is correct, then the inverse relationship between seedling biomass and *p*-coumaric acid in the presence of glucose would be more appropriately described by a logistic equation that adjusts the inhibitory concentrations of *p*-coumaric acid for different concentrations of glucose present. Unfortunately, since the experimental design for the "glucose and/or *p*-coumaric acid" experiment included only two noninhibitory glucose concentrations, this hypothesis could not be adequately tested (Gerig and Blum, 1993). Experiments are now in progress to test this hypothesis.

In a previous study we had observed that the concentrations of individual phenolic acids in mixtures of phenolic acids required for a given level of inhibition were reduced as the number of phenolic acids in a mixture increased (Gerig and Blum, 1991). Such findings, in conjunction with the findings reported here, suggest that allelopathic interactions in soil environments can be a function of interacting neutral substances (e.g., glucose), promoters (e.g., NO_3-N), and/or inhibitors (e.g., methionine and *p*-coumaric acid) of plant growth.

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PHOTOTOXICITY OF *CITRUS JAMBHIRI* TO FUNGI UNDER ENHANCED UV-B RADIATION: ROLE OF FURANOCOUMARINS

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Abstract—Extracts of *Citrus jambhiri* foliage exposed to and shielded from UV-B radiation were assayed for phytochemical changes and phototoxicity against four fungal pathogens, two of which (*Fusarium solani* and *F. oxysporum*) are causative agents of root rots and two of which (*Penicillium italicum* and *P. digitatum*) are associated with fruit rots. Conidial pigment mutants of these four fungal species were assayed to determine whether pigments play a role in protecting fungi against plant photosensitizers. Exposure to 10.2 kJ/day UV-B radiation for 95 days significantly reduced phototoxicity of leaf extracts to fungi. Although furanocoumarin levels were reduced by UV-B, analysis of covariance revealed that variation in phototoxicity of the extracts cannot be attributed entirely to variation in furanocoumarin content; thus, the possibility exists that nonfuranocoumarin phototoxic constituents, as yet unidentified, respond to UV-B exposure and contribute to overall phototoxic defense of *C. jambhiri* against pathogens. Root rot fungi were substantially more sensitive to furanocoumarin phototoxicity than were fruit rot fungi, a pattern consistent with the amount of light exposure normally experienced by these fungi when associated with phototoxic plants. Although pigmented strains of all four species displayed greater resistance to phototoxicity of pure furanocoumarins, no strain differences were detected in assays of foliar extracts; this finding also suggests that nonfuranocoumarin constituents may be involved in the phototoxic defense of *C. jambhiri* against pathogens.

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Key Words—*Citrus jambhiri*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium digitatum*, *Penicillium italicum*, fungi, bergapten, psoralen, furanocoumarin, phototoxicity, ultraviolet light.

INTRODUCTION

Effects of short-wave ultraviolet (UV-B) radiation on host–pathogen interactions are complex and highly variable (Carns et al., 1978). Plants are known to undergo biochemical changes (enhancement or decrease in secondary chemicals) under conditions of increased UV-B radiation. Among these changes is induced biosynthesis of UV-absorbing anthocyanins and other flavonoids and phenolics (Tevini et al., 1983). These changes, in turn, could alter susceptibility to pathogen attack. Plants in the family Rutaceae possess prooxidant or phototoxic chemicals, including flavonoids and furanocoumarins, that can protect against pathogen and herbivore attacks (Martin et al., 1966). Concentrations of such phototoxic chemicals may increase or decrease under the stress of enhanced UV-B, with corresponding increased or decreased inhibitory effects on associated populations of pathogenic microorganisms. In addition to changes in concentration, plant phototoxins may increase in toxicity under elevated UV-B conditions. Furanocoumarins in the presence of ultraviolet light can react with macromolecules such as DNA to cause toxicity. Alternatively, they can react with ground-state oxygen to form reactive oxygen species that can damage biologically important structures such as the cell membrane (Downum et al., 1982).

Citrus species are rutaceous plants widely distributed in tropical, subtropical, and semitropical areas, where the level of UV-B radiation striking the earth is appreciably higher than in temperate regions (Caldwell, 1981). The foliage of *Citrus* as well as other rutaceous plants is phototoxic to a variety of plants and animals, including humans (Israel, 1985), presumably due to its furanocoumarin content. Defense against pathogenic microorganisms of *Citrus* has also been attributed to its furanocoumarin content (Martin et al., 1966). *C. jambhiri* is used frequently as a root stock in commercial citriculture because of its desirable disease-resistance properties (Menge et al., 1989). This study was designed to examine the phototoxicity of UV-B-treated and untreated *C. jambhiri* plants against fungal pathogens and to determine the contribution of furanocoumarins to that phototoxicity.

The efficacy of UV-A and UV-B in photoactivation of furanocoumarins against fungi was also examined in this study. Furanocoumarins in *Citrus* are photoactivated by UV-B against insects (McCloud and Berenbaum, 1994). Even the relatively low concentrations of furanocoumarins that are found in *Citrus* under normal light conditions may thus be rendered toxicologically more effec-

tive against fungi under elevated UV-B conditions. To assess phototoxic properties of these compounds, pure samples of bergapten and psoralen were obtained. Their effect on four fungal pathogens (*Fusarium oxysporum*, *F. solani*, *Penicillium italicum*, and *P. digitatum*) was assessed to evaluate the protective role of fungal pigments against *Citrus* furanocoumarins. Previously, a related furanocoumarin, 8-methoxypsoralen (xanthotoxin), was tested for phototoxicity against the same fungal species, to assess the role of pigments in affording protection against xanthotoxin damage (Asthana and Tuveson, 1992). The investigations described here with bergapten and psoralen, therefore, served not only as a means to gain insight into the ecological significance of these compounds as phototactive constituents of plants but also served to provide comparative data relative to the action of xanthotoxin. Further investigations on oxygen involvement in furanocoumarin damage production were carried out utilizing an array of tester strains of *Escherichia coli*.

METHODS AND MATERIALS

Plant Samples. Propagation and UV-B irradiation of three clones of *C. jambhiri* plants were carried out at the Plant Biology greenhouse, University of Illinois at Urbana-Champaign (McCloud et al., 1992). After establishment of the cuttings (in about three weeks), 10 plants each of three clones similar in growth characteristics were selected. Five plants from the set of 10 in each clone were assigned as the experimental group and the remaining five were assigned to the control group. In total, the experimental design included 15 plants from within each clone that were treated as experimental and 15 that were the control group. Extracts taken from six plants, one from each clone by treatment combination, were used for fungal sensitivity assays.

UV-B Light System and Treatment Conditions. The UV-B light source in the greenhouse was a setup of banks of Westinghouse FS-72 fluorescent bulbs. The daily integrated fluence of the UV-B irradiation was 10.2 kJ BE_{300} (biologically effective radiation normalized to 300 nm), which was equivalent to that received at Champaign, Illinois, with a 15% stratospheric ozone reduction during clear sky conditions on the summer solstice. Bulbs were on for 8 hr centered in the 14-hr photoperiod of the experiment. The bench was divided into two (experimental and control) sides by suspending a sheet of Mylar (type S; Gar-Ron Plastics, Baltimore, Maryland) between the two light banks; this sheet also absorbed any scattered UV-B radiation, thus shielding the control side. On the experimental side of the bench were plants exposed to UV-B, as the bulbs were filtered with a 3-mil cellulose acetate film (Folex Inc., Palmyra, New Jersey) that allows transmission of wavelengths up to 280 nm but filters out shorter wavelengths (Mirecki and Teramura, 1984). The control side of the

bench had a similar setup of plants with bulbs filtered by Type S Mylar film, which effectively blocks all wavelengths below 320 nm. Supplemental visible light was provided by eight HID metal halide lamps suspended above the UV-B bank of bulbs. An estimated 600–1000 μ-mol/m^2 of photosynthetically active radiation was available to the plants depending on variable cloud cover. Bench sides were periodically switched and plants were randomized within treatments at intervals to minimize edge and shield effects. The exposure of *C. jambhiri* was carried out for a duration of 95 days through the spring and summer of 1990; for details of maintenance and execution of treatments, see McCloud et al. (1992).

Preparation of Extracts from UV-B-Exposed C. jambhiri. Upon termination of exposure time, the aboveground mass of the plants was harvested. The material comprising the leaves and the stems was grouped into four tissue classes. The leaves were designated as young, intermediate, and old, based on visual qualitative parameters. The young leaves were soft and bright green and not fully expanded or had just undergone expansion. They were apical and typically ended one node proximal to the fully expanded leaves. Intermediate leaves were fully expanded and had begun to harden and were a light green shade. Older leaves were clearly distinguishable by their dark green color and toughness of the lamina. Material remaining after detachment of the leaves comprised the stem class.

Harvested tissues were kept frozen at −25°C until further processing. The frozen tissue was freeze-dried, ground in a Wiley mill to pass through a 40-mesh sieve, and then assayed for furanocoumarin content. The samples remaining after HPLC analysis for furanocoumarins (for details see McCloud et al., 1992), were evaporated and redissolved in methanol. The methanol extracts thus prepared were then used for detection of phototoxicity in the fungal system.

Fungal Bioassays. Four species of fungi pathogenic on *Citrus*—two species associated with root rot diseases and two associated with fruit rot diseases—were used in this study. These species were selected to contrast the responses of pathogens that normally encounter dramatically different light environments on the same host. The two root rot fungi—*Fusarium oxysporum* Schlechtendahl, ATCC 36576, and *Fusarium solani* (Martius) Saccardo—are the causative agents of dry root rot and fusarium wilt, respectively. The two fruit rot fungi—*Penicillium italicum* Wehmer, ATCC 48814, and *P. digitatum* Saccardo, ATCC 10030—are the causative agents of blue mold and green mold of *Citrus*, respectively (Whiteside et al., 1988). In a previous study, conidial pigment mutants of these four species were isolated (Asthana and Tuveson, 1992); these mutants were used in this experiment. In contrast with wild-type *Fusarium* species, which produce orange–yellow pigments when exposed to light, A.*F.o. 1* and A.*F.s. 1* mutant strains appear white in color and produce no visible pigment. In both species of *Penicillium*, A.*P.i. 2* and A.*P.d. 2*, white mutant strains

were isolated; in addition to these white strains, a rust-color mutant was isolated in *Penicillium digitatum* (A.*P.d. 1*) and a brown mutant was isolated from *P. italicium* (A.*P.i. 1*). The use of these mutant strains allowed us to ascertain the contributions of pigments in protecting against UV-activated phototoxins in these fungi.

Cultures of the fungi were inoculated onto potato dextrose agar (PDA Difco) supplemented with 0.5% yeast extract (Difco PDA + YE), and incubated at 25°C. The *Fusarium* species were incubated under two 40-W Cool White fluorescent lamps that provided continuous illumination for up to a week to allow maximum expression of the orange carotenoid pigment(s). New cultures were initiated by streaking a loopful of mycelial strands and conidia from single conidial colonies onto fresh PDA + YE plates. To assess viability, conidia were plated on yeast agar glucose (YAG) medium containing 5 g yeast extract (Difco), 12 g agar (Difco), and 20 g glucose. Additionally, 400 mg of sodium desoxycholate was added to restrict the size of fungal colonies for ease of counting.

Conidia were harvested from 7-day-old cultures of the fungal species. Conidial suspensions were obtained by pouring 9.9 ml phosphate buffer (K-K, 0.067 M, pH 7.0) and 0.1 ml of 0.1% of Tween-80 (a wetting agent) over the culture and rubbing the surface with a sterile metal dallying rod. The suspension was filtered through sterile cotton wool to remove mycelial fragments. The resulting suspension was vortexed to break up conidial chains. Conidial density was estimated from hemacytometer counts.

A 1-ml sample was withdrawn from the conidial suspension and kept in the dark as a control for light-independent toxicity. Viability of this sample was assessed after all manipulations were completed with the light-treated samples. Light-treated samples (0.1 ml) were plated after appropriate dilution to assess viability. The treated conidia were incubated for 2 to 3 days at 30°C to allow colony formation and then counted. The viable counts at each sampling point (N) were averaged and divided by the average viable counts for untreated cells (N_0) to yield a surviving fraction. The means and standard deviation (SD) at various sampling points were then calculated. The SD was divided by the mean at each sampling point to give a fractional deviation from the mean. All fractional deviations were averaged and their SD calculated to obtain a measure of the variation among the surviving fraction at each sampling point. Total survival curves (plotting surviving fraction versus fluence) were prepared for each strain tested so that the shape of the curves could be assessed allowing for the comparison of the relative sensitivity of the strains under investigation. Bergapten and psoralen were dissolved in 95% ethanol at a concentration of 1 mg/ml of solvent.

Cell suspensions were plated in a room provided with KEN-RAD 40-W "gold" fluorescent lights to prevent possible photoreactivation.

Bacterial Bioassays. A series of isogenic *Escherichia coli* K12 strains has

been developed to deduce the inactivating mechanism(s) of UV-A versus UV-C (Tuveson, 1987). Strains RT7h–RT10h carry all four possible combinations of genes controlling DNA repair (*uvrA6* versus *uvrA6*$^+$) and catalase proficiency (*katF* versus *katF*$^+$). The strains carry the revertible *his-4* locus (Kato et al., 1977). Using these strains, it is possible to deduce whether inactivation caused by a particular agent is based on an oxidative versus a nonoxidative mechanism and to deduce the involvement of various repair mechanisms of DNA. Since carotenoids are deposited in the *E. coli* membrane, further support for the membrane as a possible target for attack can be obtained using the strain carrying the plasmid allowing for the expression of carotenoids (HB101pPL376 or LE392pPL376) and its noncarotenoid-producing relatives.

The complex medium used in bacterial assays was Luria-Bertani (LB) containing 10 g tryptone (Difco), 5 g yeast extract (Difco), and 10 g NaCl per liter. Plating medium was semienriched medium (SEM), which consisted of minimal A medium, supplemented with the nutritional requirements of the particular strains as well as casamino acids (Difco, 0.4 ml of a 10% solution per liter; Kato et al., 1977).

Cells were grown at 37°C with shaking in side-arm flasks (Belco) containing 50 ml of LB. Growth was monitored by measuring the change in absorbance using a Klett-Summerson colorimeter equipped with a green filter. A 5-ml sample of stationary phase cells (2.5 h after entering the transition from exponential to stationary growth phase) was removed and chilled on ice, washed three times with 0.067 M, pH 7.0 phosphate buffer (K-K) to remove residual medium, diluted in cold buffer (ice bath temperature) to approximately 5.0×10^8 cells/ml, and placed in a 16 × 160-mm test tube with a magnetic stirring bar at the bottom. Cells were irradiated and samples taken at predetermined intervals. At each sampling point, three 0.1-ml samples were plated after appropriate dilution to assess viability. The samples were plated on SEM plates and surviving colonies counted after 24–48 hr of incubation at 37°C. Calculations for the surviving fraction, mean, standard deviation, and fractional deviation from the mean were carried out in a manner similar to that used for the fungal experiments.

Assessing Phototoxicity of Plant Extracts. For preliminary testing of the presence of light-activated molecules in the leaf extracts, an agar disk diffusion bioassay (conducted according to the methods of Tuveson, 1987) was employed with the test organisms. Leaf extracts of *Citrus jambhiri* were spotted onto a filter paper disk (6 mm in diameter) and placed on an agar plate seeded densely (10^8 conidia/ml) with the particular test organism. The extract was allowed to diffuse out into the agar layer for 1 hr and then exposed to UV-A for an additional hour. The plates were incubated at 37°C overnight and the diameter of the resulting zones of inhibition measured to give a preliminary estimate of the degree of phototoxicity of extracts. Zones of inhibition greater than 6 mm in

diameter were considered positive for phototoxicity. Conidial suspensions of *Fusarium oxysporum* and *F. solani* were used with the foliar extracts to obtain quantitative estimates of phototoxicity. The fruit-rot pathogens of *Penicillium* species displayed a lack of photosensitivity in the disk-diffusion assay and were not used in the quantitative assays.

Quantitative assessment of the degree of sensitivity of fungal pathogens was carried out in a manner similar to that previously described for the fluence–response curves used with other photosensitizers (Asthana and Tuveson, 1992). The log-transformed reciprocal of the surviving fraction (N/N_0) was used to measure fungal toxicity of extracts. Mean toxicity was expressed as the average of all the observations of toxicity of crude extracts against both strains in both *Fusarium* species.

Statistical Analysis. Variation in the furanocoumarin concentrations of crude extracts from *Citrus* leaves and in toxicity to fungal pathogens was analyzed by three-way analysis of variance with a SAS statistical package [Statistical Analytical System (SAS) Institute, 1982]. Toxicity of extracts to fungi was scored as the log transformed reciprocal of the surviving fraction. Furanocoumarin ratios were compared by the nonparametric, distribution-free Kruskal-Wallis one-way analysis of variance (Sokal and Rohlf, 1982). The relationship between furanocoumarin concentrations in the foliar extracts and the toxicity to the fungi was examined using a linear regression. Analyses of covariance (ANCOVA) were used to determine the effect(s) of the independent variable(s), when corrected for total furanocoumarin content, individual furanocoumarin content (psoralen and bergapten), and the ratio of the two furanocoumarins in the assay.

Chemicals. Bergapten was obtained from Aldrich (Milwaukee, Wisconsin) and psoralen was obtained from Sigma Chemical Co. (St. Louis, Missouri); both were used without further purification. The absorption spectrum of bergapten has a maximum at 290 nm, which supported the use of UV-B radiation as suitable activating wavelengths; based on the first law of photochemistry it is generally acknowledged that a substance must absorb in a particular wavelength in order for that wavelength to be photoactivating (von Sonntag, 1987; Hader and Tevini, 1987).

RESULTS

Phototoxicity of Citrus *Extracts.* Fungal toxicity of the *C. jambhiri* extracts varied significantly with UV-B treatment and with the age of the tissue type (young, intermediate, and old leaves and stem). The effect of tissue type on toxicity also varied with fungal species (Table 1). UV-B exposure resulted in differing mean toxicity of these extracts; the UV-B-treated plants had a significantly lower mean (0.81) than did the UV-B-untreated samples (1.04). When

tissue classes were compared with respect to fungal toxicity, they segregated into two distinct groups. The young and the intermediate leaf age classes comprised one group, while the second group contained the old leaf and stem tissue classes. The young and intermediate leaf class was more toxic (Table 1) than the old leaf and stem class, irrespective of UV-B treatment.

Only two furanocoumarins, bergapten and psoralen, were present in detectable quantities in foliage extracts. Mean levels of total furanocoumarins (bergapten + psoralen, micrograms per gram dry weight) in extracts from UV-B-treated and untreated plants were significantly different; the treated samples had a significantly lower furanocoumarin concentration than the untreated ones (Table 2). This finding differs from an earlier report on a larger sample (McCloud et al., 1992) that UV-B exposure has no effect on furanocoumarin content of *C. jambhiri* foliage. This absence of overall effect may be due to between-subsample response differences; in other words, plants respond individualisti-

TABLE 1. MEAN PHOTOTOXICITY (LOG-TRANSFORMED RECIPROCAL OF SURVIVING FRACTION) OF *Citrus jambhiri* EXTRACTS AGAINST *Fusarium* spp.[a]

Tissue class	UV+	UV−
Young	1.3a	2.1a
Intermediate	1.5a	1.7a
Old	0.2b	0.2b
Stem	0.2b	0.1b

[a] Three-way analysis of variance reveals a significant main effect of UV ($P = 0.042$) and tissue class ($P = 0.0001$) as well as significant UV by tissue class ($P = 0.34$) and fungal species by tissue class ($P = 0.0003$) interactions. Means within a column followed by the same letter are not significantly different at $P = 0.05$, Tukey's studentized range (HSD) test.

TABLE 2. MEAN LEVELS OF FURANOCOUMARINS IN DIFFERENT TISSUE CLASSES OF *Citrus jambhiri* EXPOSED TO OR PROTECTED FROM UV-B FOR 95 DAYS[a]

	UV-B+		UV-B−	
Tissue class	Psoralen	Bergapten	Psoralen	Bergapten
Young	44.4a	33.3a	66.1a	41.2a
Intermediate	33.8a	29.1a	48.8a	38.7a
Old	7.9b	14.7b	10.8b	16.8b
Stem	5.3b	6.1b	6.7b	5.9b

[a] Means within a column followed by the same letter are not significantly different at $P = 0.05$, Tukey's studentized range (HSD) test. Means are micrograms per gram dry weight.

cally to UV-B and overall effects may be influenced by the number and identity of plants sampled.

Analysis of covariance (ANCOVA) was used to examine other effects on toxicity of extracts when corrected for total furanocoumarins (psoralen + bergapten), psoralen, bergapten, and the ratio (psoralen–bergapten). In this manner, evidence for phototoxic constituents other than furanocoumarins contributing to the toxicity of extracts was sought. UV-B treatment and the interaction between UV-B and tissue class had a significant effect on the total furanocoumarin content of the samples. In addition, there was a significant three-way interaction between fungal species, age of tissue, and UV-B radiation (Table 3, A). The analysis of

TABLE 3. EFFECT OF PLANT TISSUE, UV-B, AND FUNGAL SPECIES ON PHOTOTOXICITY OF *Citrus jambhiri* EXTRACTS TO *Fusarium* spp.

Source	*df*	*ss*	*F*	*P*
A. Analysis of covariance with total furanocoumarin content as covariate				
Furanocoumarins	1	46.1406	168.82	0.0001
UV	1	1.3682	5.01	0.0282
Tissue	3	6.1368	7.48	0.0002
UV × tissue	3	2.7997	3.41	0.0216
Species × UV × tissue	6	7.2337	4.41	0.0007
Error	75	20.4987		
B. Analysis of covariance with psoralen content as covariate				
Psoralen	1	43.7770	161.43	0.0001
UV	1	0.2005	0.74	0.0163
Tissue	3	6.4717	7.95	0.0001
UV × tissue	3	3.0613	3.76	0.0142
Species × UV × tissue	6	7.2337	4.45	0.0007
Error	75	20.3386		
C. Analysis of covariance with bergapten content as covariate				
Bergapten (5-MOP)	1	48.9458	185.99	0.0001
UV	1	0.0164	0.06	0.1660
Tissue	3	4.1903	5.31	0.0010
UV × tissue	3	2.1850	2.77	0.0472
Species × UV × tissue	6	7.2355	4.58	0.0005
Error	79	20.7894		
D. Analysis of covariance with the psoralen–bergapten ratio as covariate				
Ratio	1	20.2559	75.19	0.0001
UV	1	0.0513	0.19	0.0120
Tissue	3	30.5606	37.81	0.0001
UV × tissue	3	2.7766	3.44	0.0211
Species × UV × tissue	6	7.2337	4.48	0.0006
Error	75	20.2049		

covariance, when performed with psoralen (Table 3, B) and with the ratio of psoralen–bergapten (Table 3, D) as the covariate, showed UV-B treatment and tissue class to have significant effects on toxicity against the fungal species tested. However, when corrected for bergapten, the UV-B effect was no longer significant (Table 3, C). Since the furanocoumarins, individually and collectively, as well as their ratio, followed such similar patterns in the analyses, their degree of correlation was determined by Pearson's correlation test ($P < 0.0001$ for all pairs). All of the classes of furanocoumarins were highly correlated and were therefore pooled for analysis of heterogeneity of slopes in which total furanocoumarin content was the independent variable. Interestingly, the relationship between the furanocoumarin content of leaf extracts and their toxicity to fungi varied significantly with the fungal species ($P = 0.035$); there was no significant effect of tissue class ($P = 0.078$) on the relationship between furanocoumarin content and toxicity. Strain never had any significant effect, either as a main effect or as part of an interaction term.

The results of the ANCOVAs and heterogeneity of slopes analyses suggest that other constituents in the leaf extracts are toxic or phototoxic and vary with UV-B irradiation and age of plant tissues. Alternatively, toxicity of extracts not accounted for by furanocoumarin content alone may be attributable to other constituents of the crude foliar extracts, which may interact synergistically with the furanocoumarins (e.g., Berenbaum and Neal, 1985).

Fungal Fluence–Response Curves. Contrary to expectation, virtually no fungal inactivation was obtained for bergapten in the presence of UV-B. In contrast, UV-A was effective at activating bergapten. Thus, UV-A was used as the appropriate light source in the experiments with phototoxicity of furanocoumarins.

Exposure to the UV-A-activated bergapten resulted in sensitization of all four fungal species tested (Figure 1A–D). In the root-rot fungus, *Fusarium oxysporum*, both strains (wild-type and the mutant A.*F.o. 1*) were inactivated to approximately a 10^{-5} survival level. There was partial protection observable in the case of the carotenoid-producing wild type, which became more pronounced at increasing fluences (Figure 1A and B). The inactivation of the white mutant A.*F.s. 1* in the species *Fusarium solani* when compared to the wild type producing carotenoids was considerably increased (Figure 1A and B). The wild type was at least two orders of magnitude less sensitive than the white mutant strain A.*F.s. 1* and displayed this protection even at shorter fluences. No in vivo bleaching of the conidial carotenoid pigment was seen in either of the *Fusarium* species in these experiments at the end of the UV-A and phototoxin exposure.

In the fruit-rot pathogens, both *Penicillium* species showed distinct patterns of sensitivity to UV-A-activated bergapten. In *P. italicum*, the wild type and the mutant with altered brown coloration (A.*P.i. 1*) were similar in their response and minimally inactivated, while the white mutant (A.*P.i. 2*) was extremely

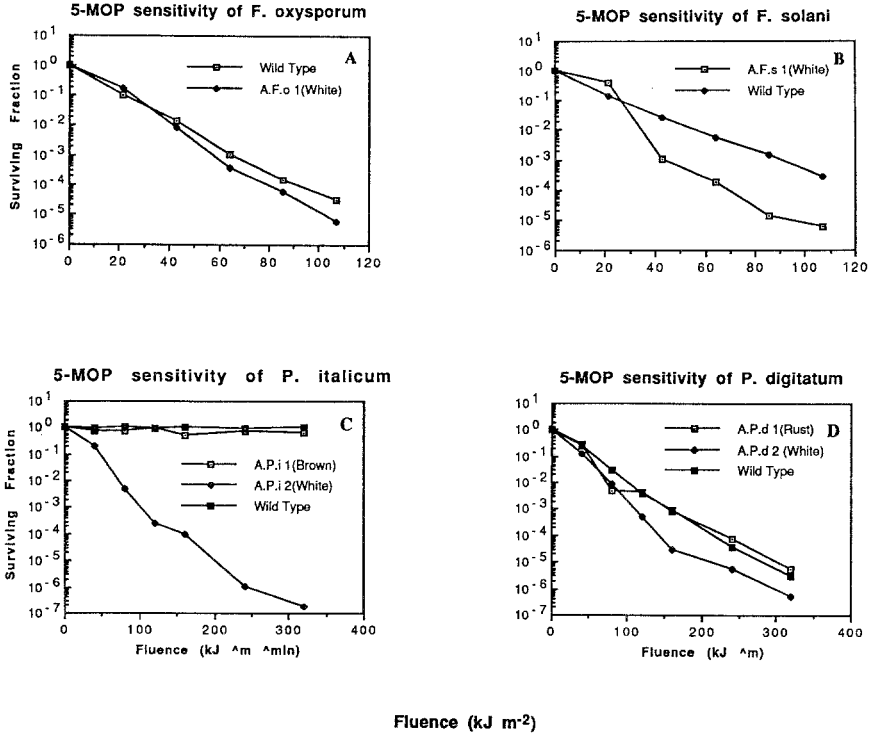

FIG. 1. Fluence–response curves of plant pathogenic fungi when exposed to bergapten (5-MOP) in the presence of UV-A. (A) *Fusarium oxysporum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.268. The mean fractional deviation for all plate counts was 0.085 ± 0.079. (B) *Fusarium solani:* The range of fractional deviations of the mean individual plate counts was 0.0–0.187. The mean fractional deviation for all plate counts was 0.114 ± 0.118. (C) *Penicillium italicum:* The range of fractional deviations of the mean individual plate counts was 0.011–0.172. The mean fractional deviation for all plate counts was 0.064 ± 0.044. (D) *Penicillium digitatum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.212. The mean fractional deviation for all plate counts was 0.066 ± 0.056.

sensitive (Figure 1C). Both the wild type and the brown mutant (A.*P.i. 1*) were quite resistant. In *P. digitatum*, the wild type and the rust mutant (A.*P.d. 1*) were also similar in their response, being inactivated to an equivalent level of survival (surviving fraction of 10^{-5}), while the white mutant (A.*P.d. 2*) again was extremely sensitive (Figure 1D). The white mutants (lacking pigmentation) in both species of *Penicillium* were inactivated equivalently (survival level about 10^{-6}), exhibiting extreme sensitivity to bergapten in the presence of UV-A. The wild-type strain in both *Penicillium* species appeared to be adapted to the del-

eterious consequences of simultaneous exposure to foliar furanocoumarins and UV-A, as compared to the highly sensitive strains (A.*P.i. 2* and A.*P.d. 2*), which are totally lacking in pigmentation. In the case of *P. italicum*, the wild type and the strain with altered pigments afforded near total protection, while those in *P. digitatum* were less efficient.

Inactivation of all four fungal pathogens with the phototoxin psoralen activated by UV-A was observed to varying degrees (Figure 2A–D). The response in all four species was similar to that observed with bergaptan and UV-A with respect to inactivation kinetics. *Fusarium* species were inactivated to comparable

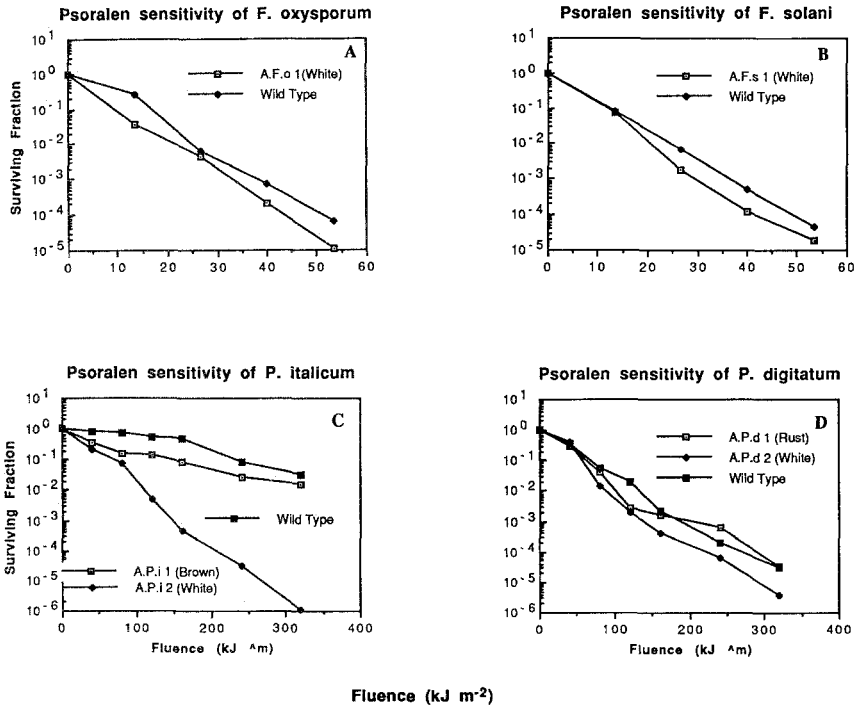

FIG. 2. Fluence–response curves of plant pathogenic fungi when exposed to psoralen in the presence of UV-A. (A) *Fusarium oxysporum:* The range of fractional deviations of the mean individual plate counts was 0.006–0.111. The mean fractional deviation for all plate counts was 0.039 ± 0.032. (B) *Fusarium solani:* The range of fractional deviations of the mean individual plate counts was 0.030–0.161. The mean fractional deviation for all plate counts was 0.1 ± 0.058. (C) *Penicillium italicum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.260. The mean fractional deviation for all plate counts was 0.099 ± 0.093. (D) *Penicillium digitatum:* The range of fractional deviations of the mean individual plate counts was 0.0–0.266. The mean fractional deviation for all plate counts was 0.056 ± 0.072.

survival levels when treated with psoralen and UV-A, while UV-B, when used as the wavelength for activation, failed to have a discernible effect on survival of either of the two species. Partial protection by pigment-producing wild-type strains in both *Fusarium* species was observed (Figure 2A and B). The amount of protection offered by carotenoids appeared very similar in the wild-type strains of the two species. The white mutants (A.*F.o. 1* and A.*F.s. 1*) in both *Fusarium* species were also inactivated to the same level of survival.

Comparison of the response of *Penicillium italicum* and *P. digitatum* to psoralen in the presence of UV-A also revealed a pattern very similar to that observed with bergapten and UV-A. *P. italicum* wild-type and brown mutant (A.*P.i. 1*) strains were similar in response, being very resistant, while the white mutant (A.*P.i. 2*) was severely inactivated (Figure 2C). The wild type and the rust mutant (A.*P.d. 1*) of *P. digitatum* responded similarly and were inactivated equivalently, but to a lesser extent than the white mutant (A.*P.d. 2*) (Figure 2D). The *P. digitatum* white mutant strain was inactivated, as was the white mutant of *P. italicum*, to a survival level of about 10^{-6}. Once again, the lack of pigmentation in the white mutants of the two *Penicillium* species resulted in increased damage to the conidia. The *P. italicum* brown mutant was much more resistant to the effects of psoralen in the presence of UV-A than the rust-colored mutant of *P. digitatum* under identical conditions. *P. digitatum* was only slightly effective in protecting against the damage induced by psoralen and UV-A by virtue of its pigments in wild-type and rust strains.

Bacterial Fluence–Response Curves. The *E. coli* strains that lack the ability to repair DNA damage (*uvrA6*; RT7h and 9h) and the strain that is DNA-repair-proficient but catalase-deficient (*katF*; RT8h) were all equivalent to the "wild-type" RT10h, which is proficient for both DNA repair and catalase activity when exposed to bergapten (Figure 3). That inactivation kinetics of all strains are identical suggests that this reaction probably does not produce catalase as a damaging species.

Inactivation and mutagenesis by psoralen in the presence of UV-A in the *E. coli* strains used in these investigations have been observed and reported by Tuveson et al. (1986). In that study, differential psoralen phototoxicity to the four tester strains indicated that the mode of action of psoralen involves cycloaddition to DNA.

DISCUSSION

Furanocoumarins have long been known to occur in leaves of rutaceous plants (Murray et al., 1982). Furanocoumarins were found consistently in the leaves of *Citrus jambhiri* regardless of UV-B treatment or leaf age. The young and intermediate leaf age class contained higher concentrations of furanocou-

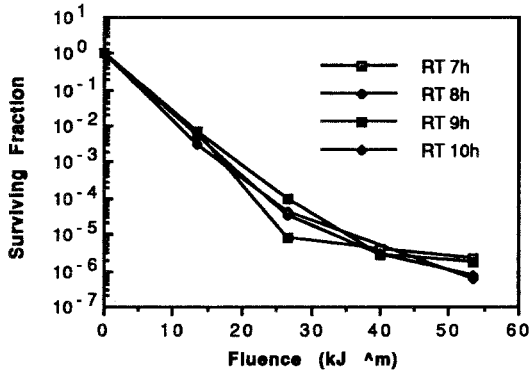

FIG. 3. Fluence–response curves for *Escherichia coli* strains differing in repair and catalase proficiency when exposed to bergapten (5-MOP) in the presence of UV-A. The range of fractional deviations of the mean individual plate counts was 0.0–0.361. The mean fractional deviation for all plate counts was 0.143–0.099.

marins when compared to the older leaves and the stem class. According to Zobel and Brown (1990a,b), furanocoumarins are higher in concentration in the leaf blade of newly unfolded leaves and are more concentrated on the surface. In general, the highest concentrations of furanocoumarins in the leaves of rutaceous plants occur in the epidermal tissues on the leaf surface and are transported from the interior to the exterior of the leaf (Zobel and Brown, 1988, 1989, 1990a,b). In the fruit of *Citrus*, furanocoumarins are generally localized in oil glands in the peel (Fisher and Trama, 1979). This association of furanocoumarins with aerial plant parts and, specifically, in epidermal tissues suggests that their phototoxicity may be most effective in external regions of the plant, where they are readily exposed to solar radiation. Desjardins et al. (1989) showed that fungal pathogens derived from infected plants were tolerant of and could metabolize furanocoumarin precursors as well as furanocoumarins; strains derived from soil were tolerant only of the furanocoumarin precursors and not of the end product, implying lack of adaptation. Similarly, in this study, soil-inhabiting *Fusarium* species showed greater sensitivity to furanocoumarins in the presence of UV than did fruit-inhabiting *Penicillium* species (Figure 1A–D), suggesting that fungi normally exposed to phototoxins in the presence of sunlight have evolved more effective resistance mechanisms against their hosts' defenses.

Results of the analyses of covariance (Table 3, A–D) suggest that other, as yet unidentified, phototoxic components, which also vary with UV-B treatment and leaf age, might exist in the extracts. It is interesting that, in contrast to bacterial toxicity of *C. jambhiri* extracts, which is attributable entirely to

furanocoumarin content (McCloud et al., 1992), our results support the contention that cooccurring nonfuranocoumarin components in the extract account in part for fungal toxicity. *Citrus* plants produce phototoxins that are effective against *E. coli*, a bacterium that is normally resident in vertebrate guts, but not against fungal pathogens of plants. Since fungi pathogenic to *Citrus* are ecologically more relevant in this context, it is notable that we were able to detect the presence of other putative phototoxins in the extracts. Furanocoumarin-based defense mechanism(s) in plants might be compromised by UV-B irradiation, while other components of the plant's natural defense systems are not. Whether or not the furanocoumarins work synergistically with other phototoxic compounds remains to be investigated further. One such candidate compound is citral, a terpene aldehyde, recently demonstrated to display UVA-activated oxygen-dependent phototoxicity to the four fungal strains examined in this study (Asthana et al., 1992). Limonene, another terpene, is known to occur in *Citrus* leaves and is toxic to some organisms, including humans (Karlberg et al., 1991), but it does not have an appreciable absorption in the UV-A range. However, it is possible that it can react with biological molecules in the presence of other photosensitizers (presumably with their photosensitized products). Furanoquinoline alkaloids in Rutaceae are also reported to be phototoxic to fungi (Towers et al., 1981; Pfyffer et al., 1982), to cooccur with furanocoumarins in many rutaceous species (Pfyffer et al., 1982), and to be fungicidal (Pfyffer et al., 1982; Towers et al., 1981).

Significant differences in the strains within each *Fusarium* species (pigment-containing wild type; *F. oxysporum* and *F. solani* and the unpigmented mutant; A.*F.o. 1* and A.*F.s. 1*) were not detected in the assay with foliar extracts. The contribution of pigments in protecting against furanocoumarins in *Citrus* foliar extracts was thus not very important. Alternatively, any distinction between pigmented and nonpigmented strains may have been obscured by their response to components other than furanocoumarins in the crude foliar extracts. It is known that furanoquinolines function by covalent photobinding to DNA (Pfyffer et al., 1982), a mechanism against which carotenoids would be ineffective.

All furanocoumarins examined in this study absorb maximally in the UV-B range (Murray et al., 1982); however, these wavelengths proved ineffective in photoactivating them to a toxic state when tested with either the fungal or the bacterial systems. This anomaly could conceivably result from the excited states of furanocoumarins entering into photobinding reactions being either the triplet or singlet after exposure to UV-A or to UV-B. Since these wavelengths are energetically different, from the kinetic standpoint they may result in differential orbital excitations of the photosensitizer atoms and cause them to enter triplet or singlet excited states. The predominance of either photosensitizer excited state could result in a different action within the cell (Song, 1982). This apparent oddity might serve to illustrated that there exists a real boundary between UV-A

and UV-B wavelengths and their biological effects, when considering both their direct and indirect effects. Additionally, the belief that UV-B, even at lower intensities, could be potentially more damaging to organisms than UV-A may not be universally true. What this study does determine is the efficacy of UV-A, rather than UV-B, wavelengths in photoactivation of *Citrus* furanocoumarins against fungi.

Joshi and Pathak (1983) tested reactive oxygen formation by several linear and angular furanocoumarins in vitro and found that they produce singlet oxygen and superoxide radical with variable efficacy. It was postulated that both forms of active oxygen contribute to the in vivo phototoxicity of these agents, possibly at the level of the cell membrane. Purified furanocoumarins [including xanthotoxin (Asthana and Tuveson, 1992)] were much more phototoxic at equivalent concentrations than the *Citrus* extracts, suggesting that their effect may have been partially masked in the crude leaf extracts. The pigment-containing strains in *Fusarium solani* and those in *F. oxysporum*, to a lesser degree, showed protection against bergapten-induced damage (Figure 1A and B), supporting the involvement of oxidative damage. Support for the generality of this contention was sought using bacterial strains proficient and deficient in catalase, but no conclusion to this effect could be reached, as all strains were equally sensitive (Figure 2) in contrast with earlier studies on psoralen toxicity (Tuveson et al., 1988), in which *E. coli katF* strains are more sensitive. Although DNA cycloadduct formation by furanocoumarins has generally been cited as the principal phototoxic mechanism, damage by furanocoumarins might result from dual or even multiple actions involving oxygen that essentially compete with each other within a cell. The furanocoumarins might damage the cell membrane oxidatively (by the mediation of reactive species) or reach the DNA in a nonoxidative manner (mono- or bifunctional cross-linked adducts) depending upon the conditions external or internal to the cell. Such conditions might include solubility, permeability, or concentration of phototoxin, its access to DNA, or the pH of the cell. If the conditions fluctuate, as might very well be the case within the milieu of a living cell, one or the other effect may predominate. Both of these effects could play an important role in the overall pattern of furanocoumarin phototoxicity in cells.

It is interesting to note that addition of the methoxy group on the 5-position of psoralen (bergapten) decreases the toxicity by an order of magnitude when compared to the parent compound. The addition of the methoxy group on the 8-position (xanthotoxin), however, enhances the phototoxicity for both eukaryotic and prokaryotic systems investigated here. Desjardins et al. (1989) also observed that methoxylation at the 8-position of linear furancoumarins rendered them more toxic to fungal species than the unsubstituted ones. There may be evolutionary significance to the fact that *C. jambhiri* biosynthesizes and accu-

mulates the more potent form, psoralen, rather than bergapten in greater abundance as part of its allelochemical defense system.

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EVIDENCE FOR SEX PHEROMONES PRODUCED BY MALES AND FEMALES IN *Blatta orientalis* (DICTYOPTERA, BLATTIDAE).

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Abstract—Males and females of *Blatta orientalis* produce a volatile sex pheromone attractive at a distance. The male initiates courtship behavior by adopting a calling posture while emitting his sex pheromone. He exposes the anterior region of his tergites by stretching his abdomen horizontally. Morphological observations show the existence of male tergal glands on the anterior region of tergites 1 to 8. Behavioral assays of females utilizing methylene chloride extracts of various male body parts reveal that extracts of the anterior region of tergites are highly attractive and induce more positive responses that any other body part. Once near the male, the attracted female opens widely her genital atrium thus exposing her atrial glands. Behavioral assays reveal that these atrial glands are certainly the site of production of the female sex pheromone.

Key Words—Sexual behavior, calling posture, pheromonal gland, *Blatta orientalis*, cockroach, Dictyoptera, Blattidae.

INTRODUCTION

The mating behavior of the oriental cockroach, *Blatta orientalis*, has been described in detail and often compared with that of closely related *Periplaneta* species (Rau, 1924; Quadri, 1938; Roth and Willis, 1952; Barth, 1970; Simon and Barth, 1977a). All the precopulatory sequences classically described in many cockroach species were observed: attraction of the sexual partner, male and female encounter, antennal contacts, male wing-raising, female mounting

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and feeding on male's dorsum, and finally copulation with male and female facing in the opposite direction.

The existence of sex pheromones attractive at a distance has been clearly demonstrated in several species of cockroaches (Roth and Willis, 1952; Barth, 1968; Wright, 1977; Breed, 1983), and probably these attractants are produced by many other species. However, for most cockroaches, the chemical nature and site of production remain unknown (reviewed in Schal and Smith, 1990; Schal *et al.*, 1992; Abed *et al.*, 1993). In *B. orientalis*, the possible existence of a female sex pheromone was first investigated by Roth and Willis (1952) and Barth (1970). They concluded that females did not produce a volatile sex pheromone. Furthermore, Barth (1970) pointed out that "males rarely show signs of sexual motivation until they have accidentally contacted the body or antennae of a virgin female." Subsequently, from results obtained with conditioned filter papers, Simon and barth (1977b) demonstrated the existence of a female pheromone and concluded that it appeared to be volatile. On the basis of earlier studies in *Periplaneta americana* (Persoons *et al.*, 1974, 1979), Warthen *et al.* (1983) investigated *B. orientalis* in a similar manner. They tried to elucidate both the chemical nature of the pheromone and its site of production. They published the mass spectrum of a compound showing analogies with periplanone B, the female sex pheromone of *P. americana* and concluded that this new compound probably originated from the female crop, esophagus, and proventriculus.

In this work, we reinvestigate in detail the sexual behavior of the oriental cockroach in order to establish clearly the existence of a female or a male sex pheromone. The origins of the male and female sex pheromones and their emission, functions, and specificity are discussed.

METHODS AND MATERIALS

Large colonies of insects were reared in a series of glass aquaria (50 × 40 × 30 cm) at 25 ± 1°C with a relative humidity of 70%. Food (dry dog food) and water were supplied *ad libitum*. Insects were kept under a 12:12 hr light–dark photoperiod, the scotophase beginning at 6:00 am. Nymphs of the last two instars were collected from the large colonies and sexed. Male and female nymphs were reared in separate rooms in glass aquaria (28 × 14 × 10 cm). Newly emerged adults were separated daily and reared in groups of 10 or 20 in plastic boxes (18 × 15 × 7 cm), according to their sex. Males and females were kept separate until used for tests.

Behavioral Observations

According to Simon and Barth (1977a), the observations were first made during the first 6 hr of the scotophase. However, we determined that maximum activity of this species occurred in the second half of the scotophase. Conse-

quently, all our behavioral experiments were performed during this last period. Tests were carried out under a red dim light in glass aquaria (28 × 14 × 10 cm), which were washed thoroughly with methylene chloride after each test.

Mating Behavior. All the tests were conducted as follows: a virgin female was placed in the test aquarium for at least 10 min before observations were made. A virgin male was introduced, and the behavior of the insects was noted during a 10-min period. The behavior of the females, in the presence of a mature virgin male, was noted in relation to their age, from the imaginal molt to age 30 days. For each age, the observations were replicated 10 times. The occurrence of significant behaviors was recorded for quantification: males displaying wing-raising posture, females climbing and feeding on the male's back, females opening the genital atrium, unsuccessful copulatory attempts, and mating. The same observations were made in relation to the age of males in the presence of a mature virgin female. As for females, the observations were replicated 10 times for each age.

Male Calling Behavior. In order to describe male calling behavior, 15- to 20-day-old sexually mature virgin males were observed. In a first experiment, five males were placed in the aquarium 1 hr before the beginning of tests and the behavior of each male was noted for 30 min. To determine a possible effect of isolation on this behavior, we studied and compared the calling activity of isolated and grouped mature virgin males during a 24-hr period (two observations per hour). Twenty isolated males and two groups of 10 males were observed.

Female Behavior. We focused our attention on a specific behavior observed during male–female and female–female confrontations: the opening of genital atrium. In a first experiment, the behavioral sequences (opening of genital atrium, aggressivity) of three grouped 8- to 15-day-old virgin females were noted for 30 min. Then a mature virgin male was introduced into the container and the behavior of each insect was recorded. The test was replicated 10 times. An effect of isolation was envisaged as for males, so in a second experiment, 10 females were isolated after the imaginal molt and each kept in separate boxes (9 × 6 × 4 cm). On the ninth day, these females were sexually mature and were observed, in absence of a male, for 24 hr (two observations of 10 min per hour).

Conditioned Papers. Filter paper disks (55 mm ID) were placed for 15 days on the floor of boxes containing, respectively, 20 mature virgin males and 20 mature virgin females. The activities of these conditioned papers were tested (three replicates) in aquaria containing 10 males or 10 females. An impregnated and a control paper were placed at each end of the aquarium. The following behaviors were noted during a 10-min period: number of insects attracted (visits), time spent near the paper, and responses elicited (wing-raising for males, opening of genital atrium for females). The results were tested by chi-square test.

Bioassays (Y-Maze Olfactometer)

Olfactory stimuli were tested on virgin males and females in a Y-maze olfactometer. It consisted of a glass tube (3 cm ID) with a 40-cm-long common arm, A, connected to two 27-cm-long-choice arms, B and C (the angle between B and C is 70°). The samples to be tested (three living insects or papers impregnated with male extracts) were placed in a box (9 × 6 × 5 cm) at the ends of arms B or C. An airstream controlled by a flowmeter (10 ml/min) flowed across the boxes and arms B and C to channel the chemical stimuli through the common arm to the "starting box" where the insects to be tested were placed. Each of the insects to be tested was isolated in separate starting boxes (9 × 6 × 5 cm) for 1 hr before the beginning of the test. The box is connected to the common arm at the beginning of the experiment and a wire screen door allowed the insect to enter the olfactometer. Fifteen virgin males or females (unless stated otherwise) were used for each test. During the 5-min period following the entrance of the insect into the olfactometer, the following parameters were noted: first choice of the insect and number of visits and time spent in each arm. Control tests were undertaken in order to verify that insects were not attracted more by either arm when no stimulus was present. The olfactometer was cleaned with methylene chloride after each test. The results were analyzed by Monte Carlo test (Vaillant and Derridj, 1992).

Morphology

For light microscopy studies, abdomens from mature males and females were dissected, fixed in alcoholic Bouin's, embedded in paraffin, sectioned at 7 μm, and stained with Masson's trichrome (Goldner Variant) (Martoja and Martoja-Pierson, 1967). For scanning electron microscopy, the pieces were placed into 95% alcohol, dried, coated with gold, and examined with a Stereoscan 250 MK2 (Cambridge SA).

Preparation of Extracts

The same procedure of extraction was used for males and females. Dissected pieces were pooled in 30 ml distilled methylene chloride and extracted for 1 hr at room temperature. The extracts were filtered through glass wool and concentrated to 500 μl by the Kuderna-Danish method (Marquardt and Luce, 1961), then concentrated to 100 μl under a gentle nitrogen flow and stored at −20°C until used.

With the males, an initial extraction was carried out on two hundred forty 15- to 30-day-old virgin males. Separate samples were made using tergites 1 to 10 and sternites 1 to 9. A second extraction was carried out on one hundred 15- to 20-day old virgin males. The following samples were made: anterior part of

tergites 1 to 8; posterior part of tergites 1 to 8; sternites 1 to 9. For the females, four samples of one hundred 9- to 15-day-old virgin females were made: tergites 8 to 10; sternite 7; genitalia and other tissues from the genital atrium, and finally the midgut.

Papers (2 cm^2) impregnated with these male or female extracts were tested under similar conditions as mentioned above on five females (10 replicates) and five males (six replicates), respectively. The results were tested by chi-square test.

Chemical Analysis

Gas-chromatographic analyses (GC) were carried out with a Girdel 31 gas instrument with a flame ionization detector. One microliter of each sample was injected via a split–splitless injection system, operating with a split flow of 20 ml/min and a septum purge of 3 ml/min. The split and purge were closed during injection and were opened 30 sec after injection. The capillary column was a CPWax 58CB fused silica column (30 m × 0.25 mm ID) (Chrompack) with temperature programming from 40 to 240°C at 3°C/min. Injector and detector temperatures were, respectively, 260°C and 280°C. Helium was used as the carrier gas (2 ml/min). The GC traces were recorded by a CR3A Shimadzu integrator.

Gas chromatographic–mass spectrometric (GC-MS) analyses were carried out with a Nermag R10-10C quadrupole-mass spectrometer coupled to a Girdel 31 gas chromatograph. The GC conditions were the same as described above.

Identifications were tentatively made by comparison of the spectra of the unidentified compounds to those of the EPA-NIH spectral data base (Heller and Milne, 1980) and the mass spectra from the library of the Laboratoire de Recherches sur les Arômes de 1'I.N.R.A. (Dijon).

RESULTS

Morphology of Glands

Male Tergal Glands. In *B. orientalis*, nymphs and adults of both sexes possess tergal glands (Roth and Stahl, 1956; Platnner *et al.*, 1972; Brossut and Roth, 1977). In nymphs of both sexes (Figure 1a) and adult females (Figure 1b), we described cercal glands and tergal glands located on tergites 6 and 7, and their proteinaceous sticky secretion is currently being studied in our laboratory (Abed, 1992). In males, we observed that all tergites possess tergal glands. Our observations reveal that these glands are not associated with regional cuticular modifications and are simply perforated areas with abundant glandular open-

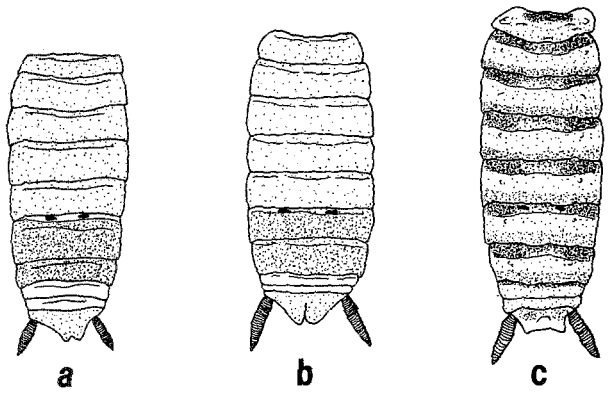

FIG. 1. *Blatta orientalis:* localization of tergal glands in nymph (a), female (b), and male (c). The glandular zones are simply perforated areas not associated with cuticular modifications. The density of glandular openings varies considerably. In nymphs of both sexes and adult females, tergites 6 and 7 and the cerci produce a sticky proteinaceous defensive secretion. In males, the anterior part of tergites 1 to 8 has many more glandular openings than the posterior part.

ings (Figure 1c). More numerous glandular openings are visible on the anterior part of tergites 1 to 8, which are normally hidden by the preceding segment (Figure 2A). On the first tergite, the glandular openings form a median area, whereas on the tergites 2 to 8, they are distributed transversally as a narrow band and delineate laterally riddled areas. We counted a mean of 3000 glandular openings per square millimeter in the median area of all the tergites. Laterally, the glandular openings are much more numerous, with an increasing gradient from tergite 8 (5000 openings/mm^2) to tergite 2 (13,000 openings/mm^2), and about 11,000 openings/mm^2 are visible on tergite 1. On the posterior part of the tergites 1 to 8, the glandular areas form a uniform transverse band with a mean number of 3500 glandular openings per square millimeter (Figure 2B).

Histological sections from tergites revealed important morphological differences between the anterior and the posterior parts of tergites. These glandular epithelia are made up of glandular units of class III according to Noirot and Quennedey (1974), into which glandular secretion is emitted outside through a cuticular duct. In the anterior part of the tergites, the glands consist of clusters of crowded glandular cells with dense content. In the posterior part, the glandular epithelium consists of a simple layer of glandular cells filled with clear vacuoles.

Female Atrial Glands. We described the presence of a paired atrial gland in females of *P. americana* (Abed *et al.*, 1993). The females of *B. orientalis* possess similar glands, which are apparent only during the calling posture when the genital atrium is widely open. The genital atrium is limited dorsally by the paraprocts and the pygidium (tergite 10) and ventrally by the intersternal fold

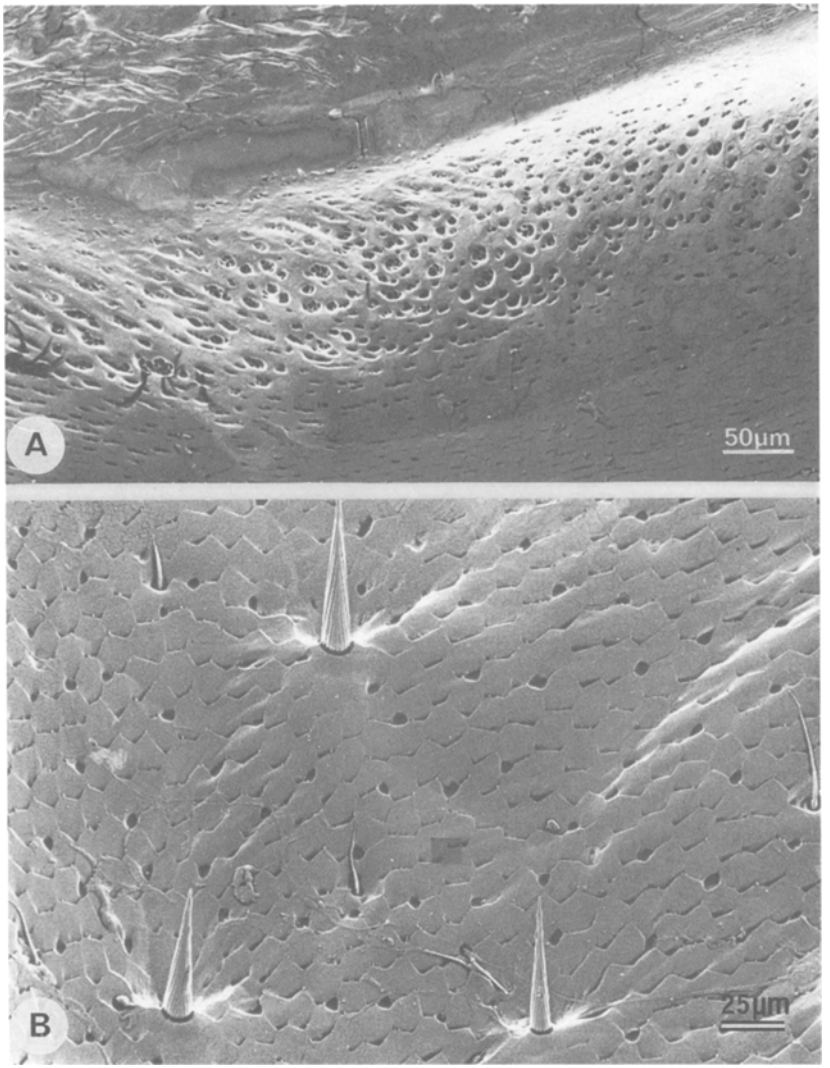

FIG. 2 *Blatta orientalis:* Morphology of the male tergal glands. Tergites 1 to 8 possess specialized glandular areas. The anterior part of the tergites produces the male sex pheromone; the posterior part produces aphrodisiacs. Anterior part of tergite 5 (A) and posterior part of tergite 5 (B) showing glandular openings.

and sternite 7. The gland is normally invaginated at the base of the paraprocts and can be observed only when it is evaginated by hemolymph pressure. The wall of the gland consists of a secretory epithelium of glandular units of class I according to Noirot and Quennedey (1974), where secretion crosses directly the glandular cuticle to be released (Abed, 1992).

Sexual Behavior

Male and Female Courtship Behavior. Our description of these sequences is made on the basis of the observation of 50 successful matings and numerous unsuccessful attempts.

The male typically initiates the sexual behavior, adopting a calling posture. Choosing a horizontal area, the male stretches its hindlegs, slightly raising its abdomen, which is parallel to the ground. At the same time, the abdomen is

FIG. 3. *Blatta orientalis:* Courtship behavior. Calling posture of male: the extended abdomen fully exposes the tergites (A). After contact with a male, the female adopts a calling posture. The genital atrium is opened widely (B). The two turgescent whitish structures are the atrial glands.

extended, revealing the brightly colored anterior part of the tergites (Figure 3A). From time to time, the phallomere is extruded and retracted. While assuming this calling posture, the male is motionless or walks slowly. The mature virgin female rapidly detects the presence of a calling male, displaying increased antennal waving and a specific stilted gait. Once near the male, antennal contacts notwithstanding, the female opens her genital atrium (Figure 3B). The opened atrium triggers an immediate reaction from male which, in a very excited state, displays the wing-raising posture after brief antennal contact with the female's body. The wings are raised to an angle of about 45°, following which the male pushes its abdomen under the head of the female, which then climbs onto the male's back and "licks" its tergites. The "feeding" by the female on the male tergal secretions releases a backward movement on the part of male, which simultaneously raises its wings to an angle of 90°. This backward movement places the head of the female at the level of the first segment of the abdomen. The female, which is now quite motionless, is then in a proper position for mating. The male extends its abdomen and protudes its phallomere in order to grasp the genitalia of female. The genital atrium of the female has been open since her initial response to the calling male. The female then turns 180° and mating continues in the typical position of opposition. Copulation lasts about 40 min (mean 41.27 min, SE 10.85 min, $N = 50$).

Our observations showed that 42% of the males omitted the calling posture and went directly to females, 14% of the females moved directly to males, and 44% of the encounters occurred at random. In most cases, the male first antennated the female abdominal segments, then walked around the female, eventually moving to a position in front of her. After brief antennal fencing, the male displayed the typical wing-raising posture. As soon as the male approached, the female opened her genital atrium; physical contact is not necessary. Eighty-eight percent of the females kept their genital atria open during the following sequences of precopulatory behavior. The time spent between the first encounter and mating varies from 2 to 565 sec (mean 164 sec; SE 145.21 sec; $N = 50$). Each sequence can be repeated a large number of times. For example, one male displayed 34 times before the female climbed on its back, and mating occurred after six attempts. Of the 50 successful matings observed, we counted an average of 6.76 wing-raisings per male. In *B. orientalis*, wing-raising lasts from 20 to 30 sec and if the female failed to respond promptly, the male resumed a normal posture. It raised its wings a few seconds later when it was close to the same female again.

Successful mating depends on female behavior and mainly on the female genital atrium opening. Females could climb onto the male's back, feed on his tergal secretions, but refuse to mate. Unreceptive females discouraged males by various behavioral postures: keeping the genital atrium closed, raising the tip of the abdomen to avoid mating attempts, and escaping or kicking the male with

their hindlegs. With an unreceptive female, the male reacted according to his degree of excitation: he withdrew or, on the contrary, increased his wing-raising, switching from normal wing-raising to quick wing-fluttering.

Male Calling Activity. In large colonies, during the entire scotophase, motionless males can be observed, displaying the typical calling posture. Most of the males called near females, and their abdomens were more extended than they were during experimental confrontations with females. Calling activity lasted from 20 min to 1 hr, being interrupted by resting periods of a few minutes.

Continuous observations of two groups of 10 virgin males during a 24-hr period showed that calling behavior mainly occurred during the second half of the scotophase (Figure 4). Males started calling at the end of the day, and most of them were calling from the seventh to the ninth hour of the scotophase. The only period in which no calling males were observed was during the 3 hr of the mid-photophase. The presence of calling males had a stimulating effect on other males. This effect is clearly demonstrated in Figure 5, which compares the percentage of calling in two groups of 10 males to those of 20 isolated males.

Periodicity of Mating Behavior. Figure 6 compares the males' wing-raising activity, the percentages of females climbing onto the males' backs, the copulatory attempts, and the matings observed during the first and the second halves of the dark period. Our results clearly demonstrate that sexual activity was much higher during the second half of the scotophase, and this is especially true for the females. Only two unsuccessful mating attempts were observed during the first 6 hr of the dark period.

Maturation of Sexual Behavior. Males started courting females about one

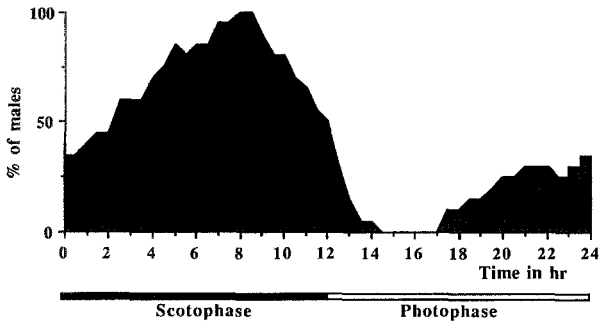

FIG. 4. Calling activity of 20 virgin adult males during a 12:12 hr light–dark cycle. Males were reared in two groups of 10 immediately after their imaginal molt. Twenty days later, their calling activity was recorded for 24 hr. The maximum (up to 80%) number of males displaying the calling posture was observed during the last 6 hr of the dark period (all males called at the 8th hr of the scotophase).

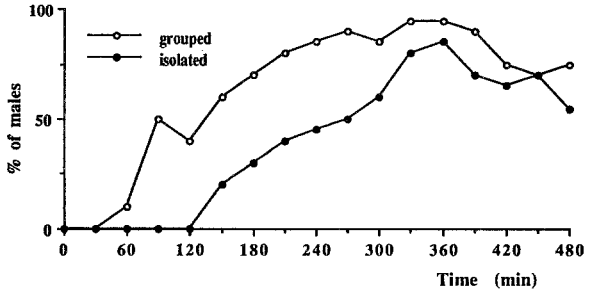

FIG. 5. Effect of isolation on male calling behavior. Calling activity was recorded during the last 8 hr of the dark phase. Each of 20 males was isolated two days before the beginning of the observation period and kept in separate boxes. Twenty males were reared in two groups of 10 after the imaginal molt.

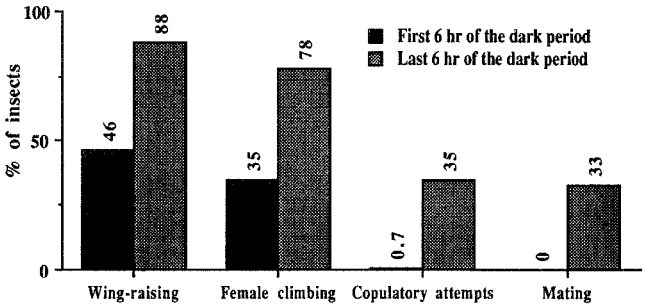

FIG. 6. Sexual behavior of *Blatta orientalis* during the scotophase. Mating behavior of males and females was recorded during the first 6 hr and the last 6 hr of the scotophase (males 15–20 days old, females 8–15 days old, receptivity at its maximum). Male wing-raising posture, female climbing, copulatory attempts, and mating were recorded. Ten females at each age were tested.

week after their imaginal molt. Eighty percent of them wing-raised when they were 6 days old, and all males exhibited this behavior at 13 days of age. As shown in Figure 7a, teneral females can elicit the wing-raising posture of males, and their efficiency in releasing this behavior does not significantly vary during the 30 following days. In the same way, 60% of the very young females (6–24 hr old) climbed on the back of wing-raising males. This precocious behavior did not reflect their receptivity or their ability to mate, and 60% of the matings occurred when females were 9 or 10 days old (Figure 7b). Few matings occurred during the maturation and deposition period of the ootheca (from day 14 to 19). Then, matings were again observed after the deposition of the first ootheca.

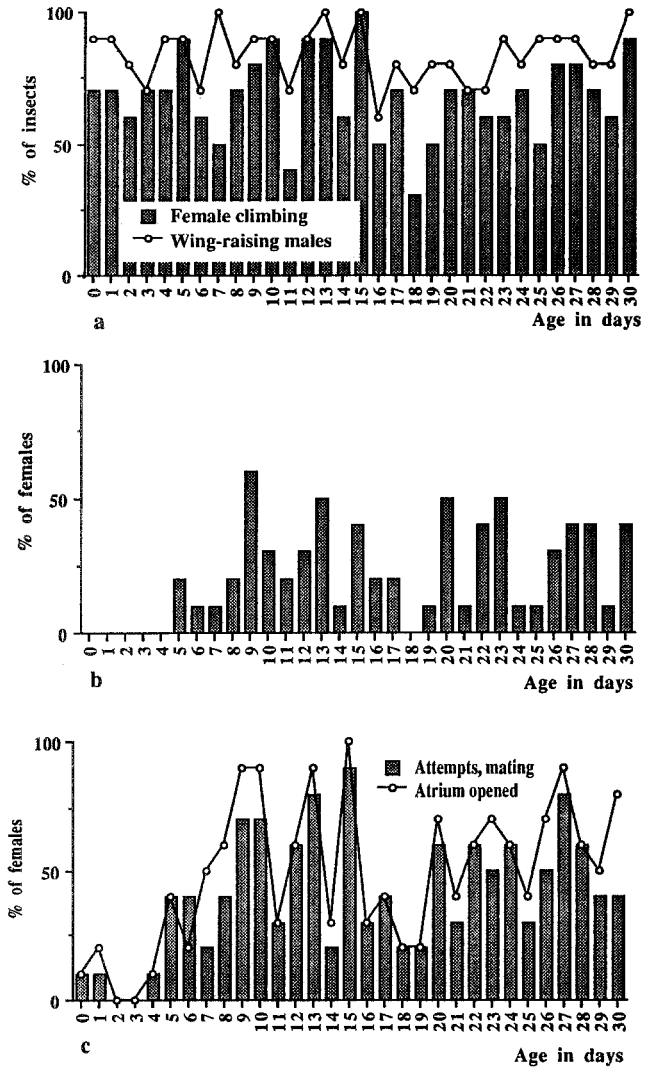

FIG. 7. Sexual behavior of females during the second part of the scotophase. A virgin female was placed in the presence of a mature male (15–20 days old) for a maximum of 10 min. The behavior of the male (wing-raising) and the female (climbing onto male's dorsum, opening of the genital atrium), copulatory attempts, and mating were recorded. Ten females at each age were tested. (a) Male wing-raising behavior elicited by females at different ages and climbing response of these females; (b) mating; (c) female calling posture, copulatory attempts, and mating.

Figure 7c reveals that the opening of the genital atrium is correlated with mating success and thus reflects the receptivity of the female.

Releasers Involved in Mating Behavior

The first releaser involved in mating behavior is the sex pheromone emitted by calling males. Y-maze olfactometer experiments demonstrate that 77% of the virgin females were attracted by calling males (Table 1). Females did not discriminate "noncalling" mature males from immature males. When given a choice between calling males and noncalling mature males, only 53% of the females chose calling males. However, they spent significantly more time in the arm containing calling males (142.9 ± 14.3 sec/vs. 59 ± 4.53 sec).

The second releaser is emitted by females when they open their genital atria, everting their atrial glands. Seventy-three percent of the tested males chose "calling" females when they were given a choice between them and males. Sixty percent spent significantly more time in the arm containing calling females compared to noncalling females or to males. Observations of isolated females reveal that they never spontaneously adopt this posture, but it is possible that the opening of the genital atrium could also result from mutual excitation between conspecifics. This releaser clearly acts as a female sex pheromone, and its release is generally triggered by the perception of the male sex pheromone.

The observations of the female calling posture show that, after several mutual contacts, mature females briefly open their atrium. These calling females are aggressive towards other females, and so, results obtained from groups of three mature females are very variable (Figure 8). The introduction of a male into the area induced the opening of the genital atrium of three females in five groups; of two in four groups and of one female in one group. This behavior occurred immediately and without any physical contact between male and females. In each group, the male mated within a minute after his introduction.

Isolation, Extraction, and Chemical Analysis of Male and Female Sex Pheromones

Male Sex Pheromone. When 30 virgin females were given a choice between papers conditioned by males or by females, 97% of them were attracted by male-conditioned papers and spent more time near these papers (Table 2). All the females were attracted to the papers in the first 20 sec following their introduction and were sexually excited, as evidenced by increased antennal waving. However, only 14% of them opened their genital atria.

Extracts from male tergites attracted all the females (Table 3). Sixty-two percent of females were attracted by extracts of male sternites but spent little time near the papers and did not show the sexual excitation exhibited in presence of extracts of tergites. A second series of bioassays revealed that there is a

TABLE 1. OLFACTOMETRIC RESULTS: Y-MAZE OLFACTOMETER WAS USED TO TEST ATTRACTIVENESS OF MALES, FEMALES, OR MALE TERGAL EXTRACTS TOWARD ADULT MALES AND FEMALES[a]

		Arm B			Arm C			Results[b]	
Insects which made a choice	N	Stimulus (placed at the end of the arm B)	First choice % of insects	Time spent (sec) ($\bar{X} \pm$ SE)	Stimulus (placed at the end of the arm C)	First choice % of insects	Time spent (sec) ($\bar{X} \pm$ SE)	First choice	Time spent
Females	15	air	40	70.1 ± 12.35	air	60	88.7 ± 10.02	NS	NS
	30	calling males	77	162 ± 12.96	females	23	55.4 ± 7.46	*	*
	15	calling males	53	142.9 ± 14.30	noncalling males	47	59 ± 4.53	NS	*
	15	immature males	46	85.2 ± 12.31	mature males	54	84.2 ± 10.97	NS	NS
	15	extract of male tergites	47	125.6 ± 7	solvent	53	62.1 ± 14.36	NS	*
Males	15	air	47	72.5 ± 13.72	air	53	91 ± 15.11	NS	NS
	30	males	55	130.3 ± 15.50	females	45	80.8 ± 11.12	NS	NS
	15	males	27	81.1 ± 8.43	"calling" females	73	155.9 ± 14.37	*	*
	15	"noncalling" females	40	59.2 ± 7	"calling" females	60	94.7 ± 14.36	NS	*

[a] The stimuli were placed at the end of the choice arms, B and C. Adult males and females were given a choice between living males, living females, male tergal extracts,and air. The first choice and the mean time (±SE) spent in each arm were noted. N = number of test.

[b] MonteCarlo test for first choice and time spent: NS = not significant; $*P < 0.05$.

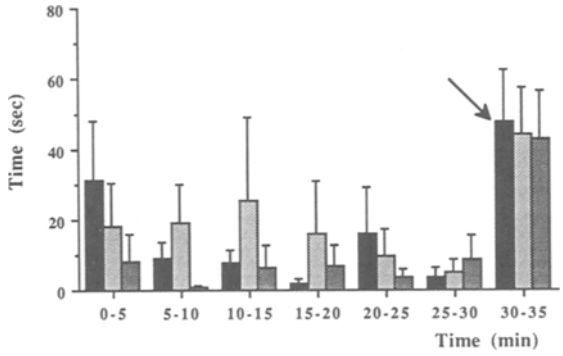

FIG. 8. Calling behavior of 10 groups of females (illustrating the duration of the opening of the genital atrium). The opening activity of female genital atrium was recorded during the second part of the dark period. Three mature virgin females (8–15 days old) were marked and placed in a test aquarium, and their behaviors were recorded for intervals of 5 min during a 30-min period. A mature male was then introduced (arrow). The time (in seconds) at which females opened their genital atria was measured during each interval of time. Each bar represents the mean of 10 females; vertical lines indicate SE. (female 1, ■; female 2, ▩; female 3, ▩).

TABLE 2. REACTIONS OF VIRGIN MATURE FEMALES TO FILTER PAPERS (Whatman No. 1) CONDITIONED BY CONSPECIFICS[a]

Treatment	N	Females attracted[b]	Visits ($\bar{X}$ + SE)	Mean time ± SE (sec) in contact with the paper	Genital atrium opening
Control	30	14a	15.33 ± 2.6	86.66 ± 12.17	0
Conditioned by males	30	29b	33.33 ± 6.66	264.66 ± 32.33	4
Conditioned by females	30	20c	26.33 ± 4.66	186.66 ± 55.85	0

[a]The filter papers to be tested were placed in rearing chambers of males or females for 15 days. A given filter paper was tested on 10 females for 5 mins. The test was replicated three times (N = 30 individuals). Attraction was evaluated in terms of number of insects attracted to contacting the conditioned paper, the mean number of visits, and the mean time of contact. Female calling posture (opening of the genital atrium) was also noted.

[b]Experiments followed by different letters are significantly different, $P < 0.05$, chi-square test.

significant difference between the attraction of the females by extracts from the anterior part of the tergites and the posterior part of the tergites. All the females were attracted by extracts from the anterior part and 28% opened their genital atria.

Female Sex Pheromone. All the males react instantly to the introduction

of a paper conditioned by females (Table 4). Ninety percent of them were attracted and manifested a strong sexual excitation (increased antennal waving and locomotion) as well as probing the paper with the palps and antennae for a long time. Papers conditioned by males are also attractive, but once located,

TABLE 3. REACTIONS OF VIRGIN FEMALES (8–30 DAYS OLD) TO EXTRACTS FROM VARIOUS PARTS OF MALE AND FEMALE ABDOMENS (METHYLENE CHLORIDE WAS TESTED AS CONTROL)[a]

	N	Females attracted[b]	Visits ($\bar{X} \pm$ SE)	Mean time $\pm$ SE (sec) in contact with paper	Genital atrium opening
Male body part					
Control (solvent)	50	23a	7.4 $\pm$ 1.16	38.4 $\pm$ 8.08	0
Tergites (1 to 10)	50	50b	28.4 $\pm$ 3.44	104.6 $\pm$ 2.15	7
Sternites (1 to 9)	50	31c	14.2 $\pm$ 2.15	54.2 $\pm$ 12.03	1
Tergites: anterior part	50	50b	25.6 $\pm$ 3.57	146.4 $\pm$ 33.7	14
Tergites: posterior part	50	31c	10 $\pm$ 1.87	52 $\pm$ 7.05	2
Sternites (1 to 9)	50	29c	8.8 $\pm$ 0.8	53.6 $\pm$ 7.48	2
Female body part					
Control	20	8d	4 $\pm$ 1.08	25 $\pm$ 8.44	0
Atrial glands	20	14c	12 $\pm$ 3.48	70.25 $\pm$ 11.22	0
Midgut	20	12c	6.5 $\pm$ 1.019	31 $\pm$ 4.6	0
Tergite 10	20	11c	5.75 $\pm$ 1.65	24 $\pm$ 4.37	0
Sternite 7	20	10c	4.75 $\pm$ 0.75	27.75 $\pm$ 7.6	0

[a] Females were reared in groups after the imaginal molt. Five females were used for each test. N = total number of individuals tested.

[b] Each experiment followed by the same letter are not significantly different. Experiments followed by different letters are significantly different, $P < 0.05$, chi-square test.

TABLE 4. REACTIONS OF VIRGIN MATURE MALES TO FILTER PAPERS (Whatman No. 1) CONDITIONED BY CONSPECIFICS[a]

Treatment	N	Males attracted[b]	Visits ($\bar{X} \pm$ SE)	Mean time $\pm$ SE (sec) in contact with the paper	Male wing-raising
Control	30	9a	6 $\pm$ 0.57	13	0
Conditioned by males	30	18b	11.67 $\pm$ 1.2	38.33 $\pm$ 1.66	0
Conditioned by females	30	27c	20.66 $\pm$ 0.66	111 $\pm$ 11.71	0

[a] The conditioned papers were obtained and tested on males as reported in Table 2. Male wing-raising posture was noted. N = total number of individuals tested.

[b] Experiments followed by different letters are significantly different, $P < 0.05$, chi-square test.

elicit little interest. It should be noted that during these bioassays we never observed a wing-raising posture.

Papers impregnated with extracts from the two last segments of the females' abdomens (5 female equivalents) attracted 84% of the males. After an active palpation of the paper, only one male wing-raised but it did not completely raise its wings. Extracts of atrial glands (0.5 female equivalents) attracted all the tested males that actively palpated the paper (Table 5). This extract was significantly more attractive than extracts from any other female body part and was the only one inducing the wing-raising posture of males (one male wing-raised four times). We must emphasize that the extract of female midgut attracted only 35% of the males. When tested on females, the glandular extract attracted 70% of the females but once located, elicited little interest (Table 3). It was not more active on females than the other female extracts.

Chemical Analysis. Extracts from the anterior part of male tergites, from male sternites, and also similar female extracts from tergites and sternites were analyzed and compared by GC. A typical GC trace is shown in Figure 9. All the chemical profiles are comparable. All the compounds detected after the retention time of 50 min are in equal concentration in the samples considered. Thus, we focused our attention on the compounds detected before the fiftieth minute. Eight compounds were tentatively identified on the basis of their mass spectra. For six compounds, tentative identification was made by coinjection with pure synthetic compounds. A male-specific compound (peak 8) found in extracts of tergites and sternites remains unidentified, while another unidentified compound (peak 6) is three times more abundant in male extracts. Peak 1

TABLE 5. REACTIONS OF 30 VIRGIN MALES (13–33 DAYS OLD) TO EXTRACTS FROM DIFFERENT PARTS OF FEMALE BODY (METHYLENE CHLORIDE WAS TESTED AS CONTROL)[a]

Female body part	N	Males attracted[b]	Visits ($\bar{X} \pm$ SE)	Meantime $\pm$ SE (sec) in contact with paper	Male wing-raising
Control	30	9a	1.5 $\pm$ 0.42	8.66 $\pm$ 2.12	0
Atrial glands	30	30b	13 $\pm$ 1.48	76.16 $\pm$ 6.94	1
Midgut	30	11c	3.5 $\pm$ 0.34	15.16 $\pm$ 2.21	0
Tergite 10	30	18c	9.66 $\pm$ 1.54	41.83 $\pm$ 7.56	0
Sternite 7	30	14c	5.17 $\pm$ 1.62	21.5 $\pm$ 2.95	0

[a] Males were reared in groups after the imaginal molt. Five males were used for each test. N = total number of individuals tested.

[b] Experiments followed by different letters are significantly different, $P < 0.05$, chi-square test.

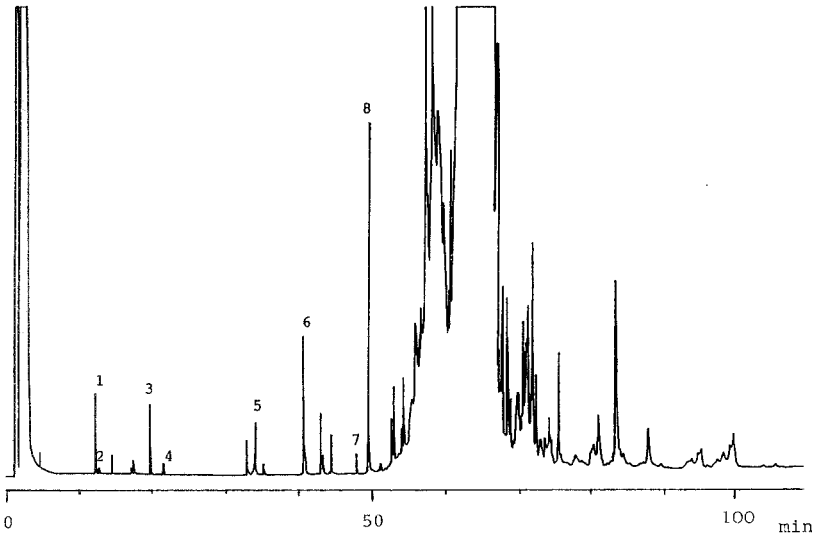

FIG. 9. Capillary gas chromatogram of tergal gland secretion from males of *Blatta orientalis*. Conditions: CPWax 58 CB fused silica column (30 m × 0.25 mm ID) (Chrompack); temperature 40–240°C (3°/min); carrier gas, helium at flow rate of 2 ml/min. Compounds: (1) 6-methyl-5-hepten-2-one, (2) hexanol, (3) benzaldehyde, (4) octanol, (5) hexyl benzoate, (6) unknown, (7) ethyl hexadecanoate, (8) unknown.

(6-methyl-5-hepten-2-one) is five times more abundant in male tergites than in female tergites but is present in considerably greater amounts in male sternites.

Extracts of whole abdomen and the last two abdominal segments from mature virgin females were analyzed to detect the presence of periplanone B or the presence of a compound with a mass spectrum matching those published by Warthen *et al.* (1983). Our analyses failed to reveal any traces of these compounds in our extracts.

DISCUSSION

In most species of cockroaches, the female produces a volatile sex pheromone that attracts conspecific males (Barth, 1968; Breed, 1983; Schal and Smith, 1990). During the emission of the pheromone, she can adopt a particular stance referred to as the calling posture. This behavior was first observed and described in several tropical species (Willis, 1970; Schal and Bell, 1985; Sreng, 1990; Abed, 1992) and in cosmopolitan species (Hales and Breed, 1983; Seelinger, 1984; Smith and Schal, 1991; Abed *et al.*, 1993) but has never been reported in males except in the subfamily Oxyhaloinae. In this subfamily, the males

produce the attractive pheromone and adopt a very characteristic calling behavior (Roth and Dateo, 1966; Sreng, 1990; Sirugue, 1992).

Earlier observations partly described the male sexual behavior of *B. orientalis* (Roth and Willis; 1952). These authors noticed that, during courtship, the male walked slowly around the female, his extended abdominal segments rendering the intersegmental membranes visible. They concluded that the female initiated the sexual behavior. We have clearly demonstrated here that it is the male of *B. orientalis* that adopts a calling behavior, attracts the female, and releases sexual behavior. This is the first report of a male sex pheromone in Blattidae. A calling posture associated with the emission of a male sex pheromone was recently found in three other species of Blattidae, *Eurycotis floridana*, *Eurycotis kevani*, and *Pelmatosilpha guianae* (Polyzosteriinae) (Farine, personal observations). Calling behavior is now described in a large number of species, either in males or in females, but the source of pheromonal production remains to be determinated for most of them. During the calling posture, the insect exposes structures that are normally hidden at rest (Willis, 1970; Hales and Breed, 1983; Schal and Bell, 1985; Abed, 1992). In male *B. orientalis*, the anterior part of the tergites that produce the sex pheromone is exposed during the calling posture. We must emphasize that a detailed observation of the calling behavior, when it exists, might point to the origin for the pheromone.

In *B. orientalis*, if the male plays the main part in the sexual encounter by emitting the attractive sex pheromone, the following sequences of mating behavior clearly depend on female receptivity. A receptive female, proximate to the male, exposes her genitalia and, in so doing, emits a pheromone that excites the male. This pheromone is the second releaser involved in mating behavior, not the first one as suggested earlier (Simon and Barth, 1977a,b; Warthen *et al.*, 1983). Barth (1970), who obtained negative results with males that had been exposed to filter papers conditioned by virgin females, concluded that females did not produce a volatile sex attractant. Subsequently, Simon and Barth (1977b) demonstrated the existence of a volatile female sex pheromone. Our experiments with extracts from male or female body parts and with papers conditioned by males or females confirmed the existence of two volatile sex pheromones. This is the first time that this phenomenon is described in cockroaches. Our observations are comparable to pheromonal communication in some moths. In many species of Noctuidae and Arctiidae, in response to the perception of the female sex pheromone, the male exposes its scent brushes or its coremata (Haynes and Birch, 1984).

Females of *P. americana* also adopt a calling posture. Although the chemical nature of the sex pheromone of this species was extensively studied (reviewed in Persoons *et al.*, 1990), its origin remained a matter of debate until recently. The observation of the female calling behavior (Seelinger, 1984) focused our attention on the atrial glands, which were found to be the source of periplanone

B (Abed *et al.*, 1993). Some differences characterize the calling posture of *P. americana* females and those of *B. orientalis* females. Isolated females of *B. orientalis* never spontaneously adopt this posture (it generally follows the perception of the male pheromone), whereas it is normal for females of *P. americana* to exhibit this posture in the absence of males. Females of *B. orientalis* never remain in this posture for more than one or two minutes, while those of *P. americana* can call for long periods, sometimes for hours (Abed *et al.*, 1993).

In most cockroach species, when the partners come into contact, their mutual recognition is based on antennal contact with the female contact sex pheromone, which triggers wing-raising in the male. The importance of this antennal fencing appears to vary with species. In *Blaberus craniifer* (Blaberidae, Blaberinae), antennal fencing is essential to trigger male wing-raising (Goudey-Perrière, 1987; Abed, 1992) whereas in *B. orientalis*, males can display after contacting any part of the female body. After perception of the female sex pheromone, the male is in such a high state of sexual excitation that only very brief antennal contact is required to trigger wing-raising. In *P. americana*, most of the males omit this behavior (Roth and Willis, 1952; Barth, 1970), and in this species the volatile female sex pheromone has a more prominent role (Barth, 1970).

In *B. orientalis*, the male tergal glands that produce the sex pheromone attractive at a distance are located on the anterior part of tergites 1 to 8, while the posterior part produces the "aphrodisiacs," secretions "licked" by females. This represents the only example of such a functional duality in cockroach tergal glands. In *E. floridana*, the male tergal glands also produce both semiochemicals, but the tergites involved are clearly separated: tergal secretions "licked" by females are produced by the first tergite (Brossut and Roth, 1977) and the male sexual pheromone by tergite 8 (Farine, unpublished results). In species of Blaberidae, in which males also produce these two signals (*Nauphoeta cinerea* and *Leucophaea maderae*), the male sex pheromone is produced by sternal glands, whereas the "aphrodisiacs" are synthesized by tergal glands (Brossut and Sreng, 1985; Sreng, 1990; Sirugue *et al.*, 1992).

Our GC-MS analyses showed that only one compound (peak 8) was specific to males. In *P. americana*, periplanone B was unambiguously identified in our laboratory in atrial glands by comparison of the mass spectra of a compound from biological samples to those of a pure synthetic compound. It was estimated that about 30 ng/gland was present (Abed *et al.*, 1993). Warthen *et al.* (1983) tried to identify the *B. orientalis* female sex pheromone, isolating a compound with a molecular weight of 232 and a mass spectrum showing some structural analogies with those of periplanone B. We did not find a compound with a similar mass spectrum in *B. orientalis*.

Paradoxically, we demonstrated that abdominal extracts from female *B.*

orientalis elicit more wing display in males of *P. americana* that in those of their own species (Abed, 1992; Abed *et al.*, 1993). Synthetic periplanone B attracts males of *B. orientalis* but does not trigger wing-raising. Male tergal gland extracts from *B. orientalis* are not attractive to females of *P. americana* (Abed, 1992). In many species of cockroaches, sexually excited males frequently display in front of females from other species, but successful interspecific matings never occur. In the Oxyhaloinae, male sex pheromones are very specific and can be of relatively simple structure. For example, in *L. maderae*, the volatile attractant is a single compound, 3-hydroxy-2-butanone, whereas in *N. cinerea*, the pheromone is a mixture of two compounds, 2-methylthiazolidine and 4-ethylguaiacol (Sirugue, 1992). Arrestants active on females at short range supplement the specificity of the attractive pheromone (Sirugue, 1992; Sirugue *et al.*, 1992).

In *B. orientalis*, the emission of two different volatile pheromones, first by the male, then by the female, must contribute to sexual isolation. Our current research suggests that this strategy, relying on the emission of successive chemical signals, is common to many species of Blattidae.

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SEX PHEROMONE COMPONENTS OF THE FRUIT-TREE LEAF ROLLER, *Archips argyrospilus* (WALKER) (LEPIDOPTERA: TORTRICIDAE), IN BRITISH COLUMBIA

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Abstract—In field experiments in the Okanagan Valley, British Columbia, the pheromone blend of (11*Z*)-tetradecen-1-ol acetate (*Z*11-14:OAc), (11*E*)-tetradecen-1-ol acetate (*E*11-14:OAc), (9Z)-tetradecen-1-ol acetate (Z9-14:OAc) and dodecan-1-ol acetate (12:OAc) at a 100:64:2:1 ratio (western FTLR blend) attracted significantly more male fruit-tree leaf roller (FTLR), *Archips argyrospilus* (Walker), than did the previously reported four-component blend and modifications thereof. Addition of (11Z)-tetradecen-1-ol (Z11-14:OH) to the western FTLR blend in a ratio of 4% relative to Z11-14:OAc further significantly enhanced attraction. Compounds were identified and their ratio determined by coupled gas chromatographic–electroantennographic (GC-EAD) and coupled GC–mass spectrometric analyses of female FTLR pheromone gland extracts and by retention index calculations of candidate pheromone components. Determination and use of geographically specific pheromonal blends may be required for optimal, semiochemical-based biorational control of FTLR and other lepidopteran orchard pests.

Key Words—*Archips argyrospilus*, fruit-tree leaf roller, Lepidoptera, Tortricidae, sex pheromone, (11Z)-tetradecen-1-ol acetate, (11*E*)-tetradecen-1-ol acetate, (9Z)-tetradecen-1-ol acetate, dodecan-1-ol acetate, (11Z)-tetradecen-1-ol.

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INTRODUCTION

The fruit-tree leaf roller (FTLR), *Archips argyrospilus* (Walker), is a widespread and important pest of apple, pear, and cherry in the Okanagan Valley of British Columbia (Madsen and Procter, 1982). Populations of FTLR in British Columbia have been controlled with insecticidal organophosphates for many years; however, localized development of resistance to organophosphates (Madsen and Carty, 1977; Vakenti et al., 1984; Cossentine and Jensen, 1991) has made control difficult. Implementation of an alternative management strategy, such as pheromone-based mating disruption, is needed for economical production of apples in some areas of British Columbia.

Four sex pheromone components have been identified in female FTLR: (11*Z*)-tetradecen-1-ol acetate (*Z*11–14 : OAc) and dodecan-1-ol acetate (12 : OAc) (Madsen et al., 1973), (11*E*)-tetradecen-1-ol acetate (*E*11–14 : OAc) (Roelofs et al., 1974) and (9*Z*)-tetradecen-1-ol acetate (*Z*9–14 : OAc) (Cardé et al., 1977). Both western and eastern male populations of FTLR are highly attracted by a four-component pheromone blend of *Z*11–14 : OAc, *E*11–14 : OAc, *Z*9–14 : OAc, and 12 : OAc in a 15 : 10 : 1 : 50 ratio (Cardé et al., 1977). However, recent experiments (Deland, 1992) suggest that populations of FTLR in British Columbia use additional pheromone components and/or different ratios of the known sex pheromone components. A pheromonal blend specific to western populations of FTLR could be more effective in a mating disruption program in British Columbia. We report the identification and field testing of candidate pheromone components in the Okanagan Valley, British Columbia.

METHODS AND MATERIALS

Laboratory Analysis. FTLR larvae were field collected near Kelowna (Okanagan Valley) and reared on apple leaves at a photoperiod of 16 : 8 hr light–dark. Two to 3 hr into the scotophase (Paradis and Leroux, 1965), abdominal tips of 2- to 3-day-old virgin female adults were removed and extracted for approximately 5 min in hexane. Aliquots containing one female equivalent of pheromone gland extract were subjected to coupled gas chromatographic-electroantennographic analysis (GC-EAD) (Arn et al., 1975) and coupled GC–mass spectrometry (MS) on two fused silica column (each 30 m × 0.25 or 0.32 mm ID) coated with either SP-1000 (Supelco, Bellefonte, Pennsylvania 168263) or DB-23 (J&W Scientific, Folsom, California 95630).

Synthesis of Candidate Pheromone Components. All candidate pheromone components were synthesized as previously described (Henrick, 1977). Synthetic compounds were >98% isomerically and chemically pure. None of the chemical contaminants elicited antennal responses in GC-EAD recordings.

Field Experiments. Six field experiments were conducted in three apple

orchards near Kelowna. Wing traps (Phero Tech Inc., Delta, British Columbia) were baited with rubber septa (Sigma, St. Louis, Missouri) impregnated with candidate pheromone components (167–182 μg in 100 μl of HPLC grade hexane), and pinned to the inner side of the trap top. All traps were suspended from trees 1.5 m above ground in the outer foliage and randomized in complete blocks with traps and blocks separated by 15 m.

The first four-treatment, 10-replicate experiment (June 25–26, 1992) tested attraction of male FTLR to: (1) the four-component pheromone blend as previously reported (reported FTLR blend) (Cardé et al., 1977); (2) the same four-components at ratios identified in pheromone gland extracts from western female FTLR (western FTLR blend); (3) a 10-candidate-pheromone-component blend; and (4) caged virgin females (modification of Proverbs et al., 1966). The second five-treatment, 10-replicate experiment (July 4–5, 1992) tested the western FTLR blend, the reported FTLR blend, and three ratio modifications of the latter. The third eight-treatment, 10-replicate deletion experiment (July 10–12, 1992) tested the 10-candidate-pheromone-component blend of experiment 1 in comparison with blends from which groups of compounds had been removed. The fourth eight-treatment, 15-replicate experiment (July 16–17, 1992) tested the western FTLR blend alone and in all binary, ternary, and quaternary combinations with the three alcohols (11*Z*)-tetradecen-1-ol (*Z*11–14:OH), (11*E*)-tetradecen-1-ol (*E*11–14:OH) and (9*Z*)-tetradecen-1-ol (*Z*9–14:OH). The fifth five-treatment, 10-replicate experiment (July 21–23, 1992) tested the western FTLR blend alone and in combination with either (4*E*,11*E*)-tetradecadien-1-ol acetate (*E*4,*E*11–14:OAc), (11*E*)-tridecen-1-ol acetate (*E*11–13:OAc), *Z*11–14:OH or the three alcohols *Z*11–14:OH, *E*11–14:OH, and *Z*9–14:OH. A final two-treatment, 17-replicate experiment (July 24–26, 1992) tested the western FTLR blend alone and in combination with *Z*11–14:OH.

In experiments 1–4 and 5–6, the number of male FTLR captured was recorded 24 and 48 hr after placement of traps in the field, respectively. In experiment 3, trap positions were rerandomized after the first count and captured males were recounted 24 hr later.

All data were transformed by $\sqrt{x + 0.375}$ and subjected to an analysis of variance (ANOVA) followed by a Student-Newman-Keuls' mean separation procedure ($\alpha = 0.05$) using the SAS statistical software (SAS Institute, version 6.07).

RESULTS

Laboratory Analysis. Analysis of female pheromone gland extracts GC-EAD (Figure 1) revealed 12 compounds that elicited responses by male FTLR antennae. Identical mass spectrometric and retention characteristics on DB-23

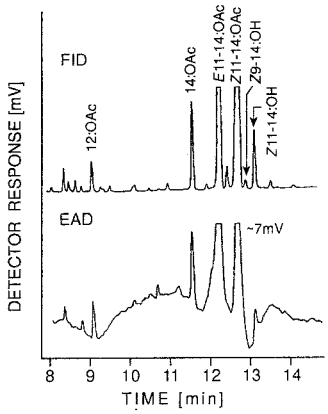

FIG. 1. GC-EAD of one aliquot of female FTLR pheromone gland extract. Not all EAD-active compounds are visible and/or separated in this GC-EAD trace. The antennal recording was carried out with a male FTLR antenna. DB-23 column (30 m × 0.32 mm ID): 1 min at 70°C, 20°C/min to 140°C, 2°C/min to 200°C.

and SP-1000 columns and comparable EAD activity of authentic standards and female-produced compounds identified the following eight-candidate pheromone components: *Z*11–14:OAc, *E*11–14:OAc, *Z*9–14:OAc, tetradecan-1-ol acetate (14:OAc), 12:OAc, *Z*11–14:OH, *E*11–14:OH, and *Z*9–14:OH. The following four compounds were only tentatively identified by comparing their retention indices on one fused silica column with those of authentic standards: (9*Z*)-dodecen-1-ol acetate (*Z*9–12:OAc), (9*E*)-dodecen-1-ol acetate (*E*9–12:OAc), *E*11–13:OAc, and *E*4,*E*11–14:OAc.

Field Experiments. Traps baited with two virgin female FTLR attracted significantly more male FTLR than did the reported FTLR blend and a 10-candidate-pheromone-component lure (Figure 2). The western FTLR blend was significantly more attractive then the reported FTLR blend and three modifications of the latter (Figure 3). Deleting both *Z*9–12:OAc and *E*9–12:OAc from the 10-component lure, and adding *Z*11–14:OH, *E*11–14:OH and *Z*9–14:OH to the western FTLR blend significantly increased attraction (Figure 4). Binary, ternary, and quaternary combinations of the western FTLR blend with the alcohols *Z*11–14:OH, *E*11–14:OH, and *Z*9–14:OH (experiment 4) did not result in statistically different trap catches but suggested that *Z*11–14:OH alone may synergize attraction to the western FTLR blend. In experiment 5, addition of *Z*11–14:OH to the western FTLR blend significantly enhanced attraction of male moths, whereas addition of either all three alcohols, *E*4, *E*11–14:OAc, or *E*11–13:OAc had no effect (Figure 5). The western FTLR blend combined

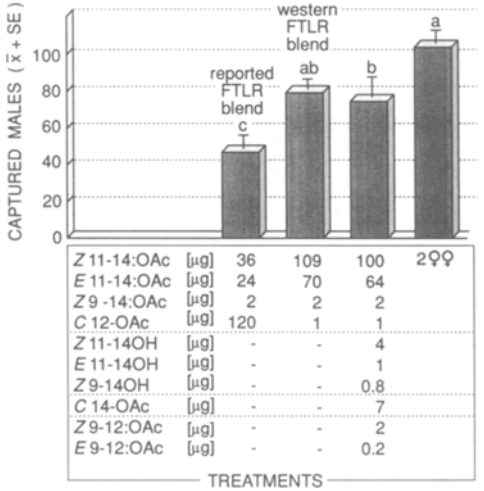

FIG. 2. Captures of male FTLR in traps baited with the western and previously reported FTLR pheromone blends, a 10-candidate-pheromone-component blend, and virgin females. Okanagan Valley, June 25–26, 1992; $N = 10$. Bars superscripted by the same letter are not significantly different ($P < 0.05$).

with *Z*11–14:OH was significantly more attractive than the western FTLR blend alone (Figure 6).

DISCUSSION

Male FTLR in British Columbia strongly responded to a pheromone lure containing *Z*11–14:OAc, *E*11–14:OAc, *Z*9–14:OAc, and 12:OAc at a ratio of 100:64:2:1. At this natural, gland-derived ratio (western FTLR blend), 167 µg of the four components exceeded attraction of the previously reported four-component blend (Cardé et al., 1977) when compared at an equivalent amount (Figure 3). While a large proportion of 12:OAc seems to compensate for as yet unknown pheromone components (Madsen et al., 1973), it seems unnecessary or even disadvantageous in more complete pheromonal blends (Figure 3) (Bjostad et al., 1985).

Addition of *Z*11–14:OH to the western FTLR blend in a ratio of 4% relative to *Z*11–14:OAc further enhanced attraction (Figures 5 and 6). This result contrasts with previous findings that the presence of *Z*- and *E*11–14:OH decreased attraction to the corresponding acetates (Roelofs et al., 1974). It suggests that, in western FTLR, the acetate–alcohol ratio is critical for optimal attraction, as has been shown for the eye-spotted bud moth, *Spilonota ocellana* (Denis and Schiffermüller) (McBrien et al., 1991).

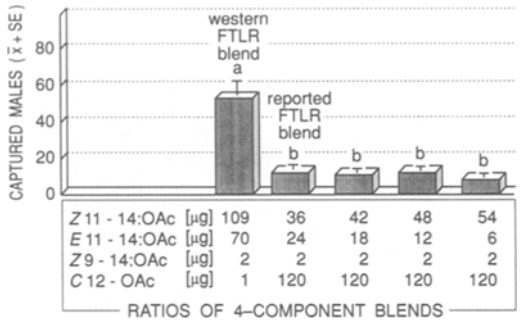

FIG. 3. Captures of male FTLR in traps baited with the western FTLR pheromone blend, the previously reported FTLR pheromone blend, and three ratio modifications thereof. Okanagan Valley, July 4–5 ,1992; $N = 10$. Bars superscripted by the same letter are not significantly different ($P < 0.05$).

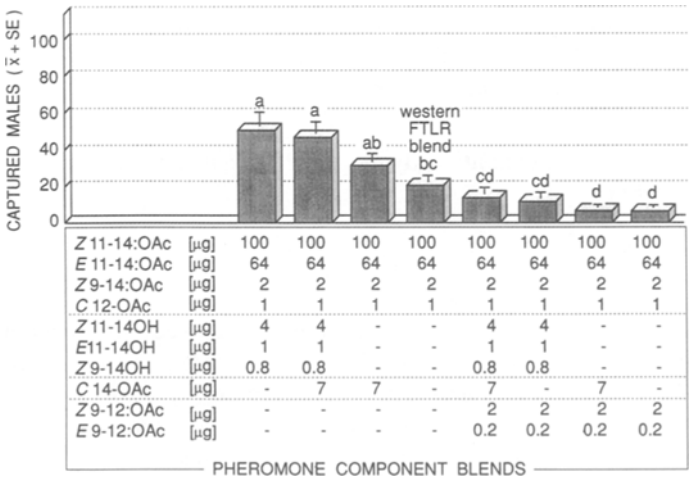

FIG. 4. Captures of male FTLR in traps baited with a 10-candidate-pheromone-component blend and other blends from which groups of compounds were removed. Okanagan Valley, July 10–12, 1992; $N = 15$. Bars superscripted by the same letter are not significantly different ($P < 0.05$).

In western North America, Z11–14:OAc is a common sex pheromone component of several sympatric leaf rollers including FTLR; the European leaf roller, *Archips rosanus* (L.) (Roelofs et al., 1976b); the oblique-banded leaf roller (OBLR), *Choristoneura rosaceana* (Harris) (Roelofs and Tette, 1970); and the three-lined leaf roller, *Pandemis limitata* (Robinson) (Roelofs et al.,

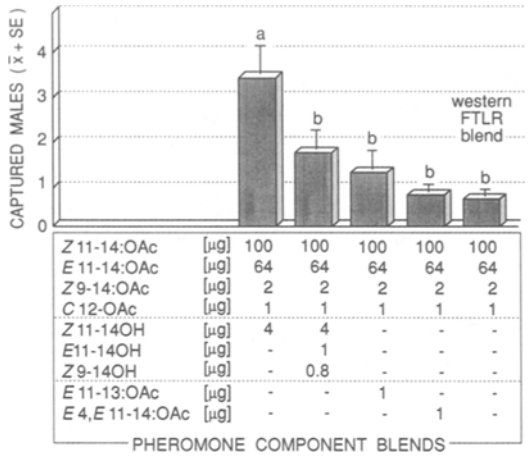

FIG. 5. Captures of male FTLR in traps baited with the western FTLR blend alone and in combination with candidate pheromone components. Okanagan Valley, July 21–23, 1992; $N = 10$. Bars superscripted by the same letter are not significantly different ($P < 0.05$).

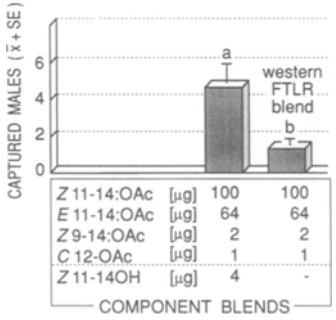

FIG. 6. Captures of male FTLR in traps baited with the western FTLR blend alone and in combination with Z11–14:OH. Okanagan Valley, July 24–26, 1992; $N = 17$. Bars superscripted by the same letter are not significantly different ($P < 0.05$).

1976a). In eastern North America, the red-banded leaf roller *Argyrotaenia velutinana* (Walker) (Roelofs and Arn, 1968) also produces Z11–14:OAc. Species specificity of pheromone communication channels seems to be maintained by the presence or absence of additional pheromone components. For example, E11–14:OAc, is an important synergistic component in FTLR (Roelofs et al., 1974), but when it represents more than 2 and 6% of the pheromone blend, repels *A. rosanus* (Vakenti et al., 1988) and *P. limitata* (Roelofs et al., 1976a),

respectively. Similarly, 12:OAc in the FTLR pheromone seems to inhibit *A. rosanus* (Roelofs et al., 1976b). *E*9–12:OAc and *Z*9–12:OAc, identified in the effluvia of female *A. velutinana* (Bjostad et al., 1985), reduced response by male FTLR to the western FTLR blend (Figure 4), suggesting that these two dodecenyl acetates are not present in or released from pheromone glands of female FTLR.

Alcohol components have been identified in many other western leaf-roller pheromones such as *C. rosaceana* (Hill and Roelofs, 1979), *A. rosanus* (Roelofs et al., 1976b), the tufted apple bud moth *Platynota idaeusalis* (Walker) (Hill et al., 1974), and *Argyrotaenia dorsalana* (Dyar) (Steck et al., 1977). The presence of these different alcohol components and/or different acetate–alcohol ratios may impart further specificity of pheromone communications channels among leaf rollers.

In both the Coleoptera and Lepidoptera the production of and response to pheromones has been shown to differ between geographically separated populations of the same species (Klun et al., 1975; Lanier et al., 1972; Miller et al., 1989; Thomson et al., 1991; Tòth et al., 1992). Western populations of *C. rosaceana*, for example, utilize (11*Z*)-tetradecenal as a synergistic pheromone component (Thomson et al., 1991), while the same compound appears lacking in the pheromonal blend of eastern OBLR populations. We will therefore test the most attractive pheromone blend of western FTLR in eastern North America to evaluate its attractiveness for eastern FTLR populations.

Identification of the complete pheromonal blend for important lepidopteran orchard pests in different geographic locations is necessary to compare and assess the efficacy of complete versus incomplete, and species-specific versus generic pheromone blends in mating disruption programs. Such an approach could lead to the development and implementation of more effective and/or economic management systems than those currently being used.

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SESQUITERPENES IN THE FRONTAL GLAND SECRETIONS OF NASUTE SOLDIER TERMITES FROM NEW GUINEA[1]

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Abstract—In five New Guinea *Nasutitermes* (*N. gracilirostris*, *N. novarumhebridarum*, *N. torresi*, *N. triodiae*, and an undescribed species *N.* sp.F), we have detected and identified 10 sesquiterpenes. Eight of these compounds (β-elemene, β-caryophyllene, α-humulene, α-muurolene, γ-selinene, β-selinene, germacrene-A, and γ_2-cadinene) were identified by GC and GC-MS (EI). Two uncommon sesquiterpenes, (5*R**, 7*R**, 10*S**)-selina-4(14),11-diene and (5*R**, 7*R**, 10*S**)-selina-3,11-diene, were identified by GC, GC-MS (EI, CI), GC-FTIR, and mono- and bidimensional NMR. Whereas in most species sesquiterpenes are present in low or trace amounts, in *N. novarumhebridarum* the sesquiterpenic fraction of soldier frontal gland secretion is equal to that of the monoterpenes.

Key Words—*Nasutitermes*, Isoptera, defense secretion, sesquiterpenes, β-elemene, β-caryophyllene, α-humulene, α-muurolene, γ-selinene, β-selinene, germacrene-A, γ_2-cadinene, (5*R**, 7*R**, 10*S**)-selina-4(14),11-diene; (5*R**, 7*R**, 10*S**)-selina-3,11-diene.

INTRODUCTION

Among termites, both mechanical and chemical weapons are used in defense, and some taxa have developed very sophisticated defensive mechanisms (Deligne

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et al., 1981; Prestwich, 1983, 1984). During their evolution, the soldiers of Nasutitermitinae have been subjected to different morphological modifications, reflected in the loss of mechanical weapons (regression of the mandibles) and the acquisition of very efficient chemical defenses (development of the frontal gland with nasus development in some genera) (Hare, 1937; Ahmad, 1950; Emerson, 1952, 1955; Sands, 1957; Sen Sarma, 1968; Krishna, 1970; Prestwich and Collins, 1981). These modifications have resulted in the well-known nasute soldiers of the highly evolved Nasutitermitinae, whose defense consists solely of a gluey secretion, elaborated by the frontal gland and squirted through the nasus onto adversaries. Besides its purely defensive role, various communicative effects have been assigned to the frontal gland secretion of several species (Moore, 1968; Maschwitz and Mühlenberg, 1972; Eisner et al., 1976; Vrkoč et al., 1978; Traniello, 1981; Roisin et al., 1990).

In *Nasutitermes*, the frontal gland secretion was described as mainly consisting of a mixture of diterpenoid "resins," derived from cembrane, in a solution of monoterpenic "solvents" (Prestwich, 1979a). Besides their role as solvents, the monoterpenes are somewhat toxic (Howse, 1975; Hrdý et al., 1977; Everaerts et al., 1988a,b) and, in some cases act as alarm pheromones (Moore, 1968; Maschwitz and Mühlenberg, 1972; Vrkoč et al., 1978; Roisin et al., 1990); the diterpenes only seem to be responsible for the viscosity of the secretion (Prestwich, 1979b). In addition, besides the mono- and diterpenes, a few sesquiterpenes were reported to be present in the defensive secretion of *N. octopilis* (Prestwich and Collins, 1981) and *N. nigriceps* (Valterovà et al., 1987), and in the related genus *Velocitermes* (Valterovà et al., 1988). Sesquiterpenes constitute more than 55% of the secretion of *Subulitermes baileyi* (Prestwich and Collins, 1981).

The frontal secretions of several New Guinea *Nasutitermes* were previously analyzed (Dupont et al., 1981; Braekman et al., 1984, 1986; Roisin et al., 1987; Everaerts et al., 1988b). While most species analyzed do not possess sesquiterpenes in their defensive secretions, sesquiterpenes are detected in five species and these constitute the focus of the present work.

METHODS AND MATERIALS

Insects. Five *Nasutitermes* species were analyzed: *N. gracilirostris* (Desneux), *N. novarumhebridarum* (N. & K. Holmgren) (designated as *N.* sp.C in Dupont et al., 1981), *N. torresi* (Hill), *N. triodiae* (Froggatt) and an undescribed species called here *N.* sp.F (as in Braekman et al., 1984, 1986, and in Pasteels et al., 1988). The samples of *N. gracilirostris* (nine nests), and of *N.* sp.F (nine nests) were collected in the Bogia district, on the northern coast of Papua New Guinea. Samples of *N. novarumhebridarum* were collected either in the Bogia

district (18 nests), or in the Bismarck Archipelago (five nests from New Britain and three from New Irland). Samples of *N. torresi* (three nests) and *N. triodiae* (three nests) were collected in the southern savannas of the Western Province of Papua New Guinea.

Preparation of Extracts. Various extraction procedures were tested with *N. novarumhebridarum* soldiers, using whole termites, termite heads, and natural secretion for extraction. Natural secretions were obtained by squeezing soldier heads with forceps and collecting the secretion droplet oozing at the nasus tip on a small square of filter paper which was extracted. There were no differences between the chromatograms of the extracts obtained by these methods, and we used whole soldiers for the following extractions. For comparative studies, a sample of 200 soldiers from each nest was extracted with twice-distilled pentane, and stored at −20°C. For the elucidation of the structures of two uncommon compounds, a sample of 25,000 soldiers from a *N. novarumhebridarum* nest was extracted with twice-distilled pentane.

Gas Chromatography (GC) and Gas Chromatography–Mass Spectrometry (GC-MS). Crude extracts were analyzed by GC and GC-MS in order to determine and quantify the compositions of the sesquiterpenoid fractions in the studied secretions.

GC analyses were carried out with a Packard 437A gas chromatograph fitted with a flame-ionization detector (FID). Injector and detector temperatures were, respectively, 240°C and 270°C. The column was a DB5 silica capillary column (30 m × 0.32 mm ID, J&W Scientific). Helium was used as carrier gas at a velocity of 26 cm/sec at 220°C, and oven temperature was programmed from 50 to 250°C at 4°C/min. The signal was recorded and integrated on a Shimadzu CR4-A computer. Kovats indices of the sesquiterpenes were calculated according to Van den Dool and Kratz (1963).

GC-MS analyses were carried out on a Nermag R 10-10 C quadrupole mass spectrometer coupled with a Girdel 31 gas chromatograph fitted with a split–splitless injector and a DB5 fused silica capillary column (60 m × 0.32 mm ID, J&W Scientific). The carrier gas (He) velocity was 35 cm/sec at 220°C. The column was directly connected to the ion source of the spectrometer through a heated transfer line maintained at 280°C. The injection port was maintained at 240°C, and oven temperature was programmed from 40 to 220°C at 3°C/min. Electron impact (EI) mass spectra were obtained at 70 eV on a 0.8 sec cycle, the instrument scanning from 25 to 300 amu with the ion source maintained at 150°C. Chemical ionization (CI) spectra, using either methane or ammonia with a source pressure of, respectively, 27 Pa and 40 Pa, were obtained at 90 eV with a source temperature of 90°C and the instrument scanning from 60 to 300 amu in 0.7 sec.

Gas Chromatography–Fourier Transform Infrared Spectrometry (GC-FTIR). The extract of 25,000 *N. novarumhebridarum* soldiers was analyzed by

GC-FTIR. The FTIR spectra were taken in the gas phase on a Bruker IFS 85 FTIR spectrometer connected via a Bruker gold-coated light-pipe (200 mm × 0.8 mm ID) to a Carlo-Erba 5160 gas chromatograph, equipped with an on-column injector and a flame ionization detector. A DB5 fused silica capillary column (60 m × 0.32 mm ID, J&W Scientific) was used with a helium flow rate of about 2 ml/min (velocity 35 cm/sec at 220°C). Oven temperature was programmed from 40 to 220°C at 3°C/min. Nitrogen make-up gas was introduced into the transfer line to reduce peak broadening with the light-pipe (maintained at 200°C), resulting in a total gas flow rate of 5 ml/min. The spectral resolution was 8 cm^{-1}, and time resolution reduced from 12 collected interferograms/sec to four effective ones by coadding three interferograms in real time. A narrow-band (4800–600 cm^{-1}) mercury–cadmium–telluride (MCT) detector was used.

Nuclear Magnetic Resonance (NMR). Previous to NMR analysis, preparative GC of the extract of *N. novarumhebridarum* was achieved with a Girdel 30 chromatograph, fitted with a Pyrex column (3 m × 3.2 mm ID) packed with 5% SE 30 on 100–120 mesh Chromosorb AW-DCMS, and a FID, with helium as carrier gas (flow rate: 15 ml/min). Injector and detector temperatures were, respectively, 240°C and 270°C. Oven temperature was programmed from 100 to 250°C at 2°C/min. An effluent splitter was used which allowed 50% of the effluent flow to the detector; fractions were collected in cooled glass tubes (1 mm ID). The trapped component was rinsed from the tube into a Teflon-lined capped vial with twice-distilled pentane, evaporated to dryness, and redissolved in deuterochloroform ($CDCl_3$, 99.96%d, CEN-CE, Gif-sur-Yvette) and benzene-D_6 (C_6D_6, 99.93%d, CEN-CE, Gif-sur-Yvette). The purity of collected fractions was monitored by capillary GC.

^1H NMR spectra were recorded on a Bruker WM400 instrument (400.13 MHz) in 5 mm ID tubes. The signal due to the residual protons of the deuterated solvents was used a as internal reference (δ = 7.24 ppm for $CDCl_3$; δ = 7.15 ppm for C_6D_6).

Identification. Kovats indices of the sesquiterpenes were compared with those of available commercial chemicals.

Kovats indices and MS data were compared with literature data and mass spectral data bases (Cornu and Massot, 1975; Heller and Milne, 1980–1983; Biougne et al., 1987; ten Noever de Brauw et al., 1987; Lias and Stein, 1990) and the data-base of the Laboratoire de Recherches sur les Arômes de l'INRA (Dijon, France).

Vapor-phase infrared spectra were also compared to the spectra of two compilations (Nyquist, 1984; Pouchert, 1989).

RESULTS

Identification of Sesquiterpenic Compounds. Fifteen compounds were detected in the sesquiterpenic fraction of the analyzed species; 10 of these were tentatively or positively identified (Figure 1). Table 1 summarizes the analytical

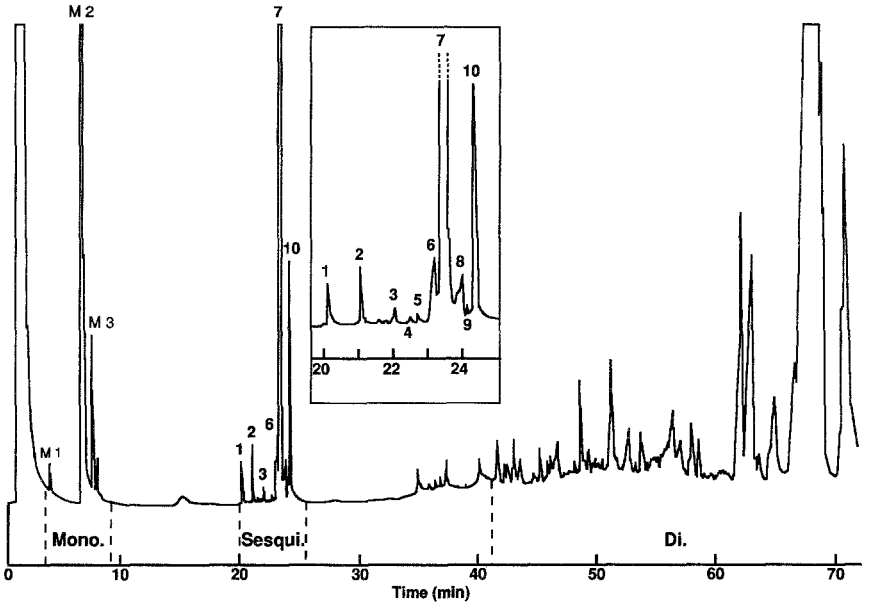

FIG. 1. Chromatogram of the soldier frontal gland secretion of *Nasutitermes novarum-hebridarum* (Packard 437A; injector temperature 240°C, FID temperature 270°C; column DB5 30 m × 0.32 mm ID, J&W Scientific gas vector: He at 26 cm/sec at 220°C; 50–250°C at 4°C/min). The three terpenic fractions are identified as follows: Mono. = monoterpenic fraction; Sesqui. = sesquiterpenic fraction (magnified in the box); Di. = diterpenic fraction. The identities of monoterpenes are indicated as follows: M 1, α-pinene; M 2, myrcene; M 3, limonene. The identities of sesquiterpenes are indicated numerically as follows: 1, β-elemene; 2, β-caryophyllene; 3, α-humulene; 4, α-muurolene; 5, γ-selinene; 6, β-selinene; 7, S1; 8, germacrene-A; 9, γ_2-cadinene; 10, S2.

techniques used for identification and the Kovats indices of the sesquiterpenes that were characterized.

GC and GC-MS analysis allowed us to identify most of the sesquiterpenes by comparison with their published analytical properties (see Methods and Materials). The following compounds were identified: β-elemene, β-caryophyllene, α-humulene, α-muurolene, γ-selinene, β-selinene, germancrene-A, and γ_2-cadinene.

The mass spectra of the two remaining compounds (Figure 2A and B) both contained a prominent ion at *m/z* 204, suggesting a molecular formula of $C_{15}H_{24}$. These parent ions were confirmed by chemical ionization GC-MS, with methane and ammonia as reagent gases. The mass spectrum of compound S1 (Figure 2A) is usual for a sesquiterpene hydrocarbon and a spectral search in data bases yielded essentially one candidate: (5*R**, 7*R**, 10*S**)-selina-4(14),11-diene (5,10-

TABLE 1. KOVATS INDICES CALCULATED ACCORDING TO VAN DEN DOOL AND KRATZ (1963)[a]

Compound	Kovats indices		Identification
	DB 5	DB Wax	
β-Elemene	1400	1582	GC, GC-MS, ref. sample
β-Caryophyllene	1429	1595	GC, GC-MS, ref. sample
α-Humulene	1464	1665	GC, GC-MS
α-Muurolene (tentative)	1480		GC, GC-MS
γ-Selinene (tentative)	1484	1682	GC, GC-MS
β-Selinene	1500	1703	GC, GC-MS, ref. sample
S1	1509	1714	GC, GC-MS, GC-FTIR, NMR
Germacrene-A	1517	1725	GC, GC-MS, ref. sample
γ_2-Cadinene (tentative)	1528	1737	GC, GC-MS
S2	1534	1749	GC, GC-MS, GC-FTIR, NMR

[a]Packard 437A; injector temperatures 240°C, FID temperature 270°C; DB5 silica capillary column 30 m × 0.32 mm ID, J&W Scientific; gas vector: He at 26 cm/sec at 220°C; 50°C–250°C at 4°C/min) and techniques used for the identification of sesquiterpenes found in the frontal gland secretion of the five *Nasutitermes* species studied (ref. sample: conparison with commercial chemicals). *S1* = (5*R**, 7*R**, 10*S**)-selina-4(14),11-diene,*S2* = (5*R**,7*R**,10*S**)-selina-3,11-diene.

epi-β-selinene). The mass spectrum of S2 (Figure 2B) is characterized by a major fragment ion at *m/z* 122, a rather uncommon feature for a sesquiterpene hydrocarbon. Spectral search in data bases gave only one candidate: (5*R**, 7*R**, 10*S**)-selina-3,11-diene (5,10-epi-α-selinene).

GC-FTIR spectra of both S1 and S2 displayed characteristic features for unsaturated hydrocarbons: important absorption bands near 3000 cm^{-1} (ν_{C-H}) and a weak absorption band near 1640 cm^{-1} ($\nu_{C=C}$). In both, a medium-sized absorption band near 890 cm^{-1} was attributed to the CH out-of-plane deformation (δ_{C-H}) of a terminal methylene group (=CH_2).

By preparative GC, we collected ca. 380 μg of S1 and 205 μg of S2, the respective purities of which were 93% and 88%.

S1: ^{1}H NMR (C_6D_6, δ/TMS): 4.98 (br dd, 1H), 4.94 (br s, 1H), 4.84 (br dd, 1H), 4.60 (br dd, 1H), 2.27 (m, 1H), 2.24 (m, 1H), 2.02 (dd, 1H), 1.94 (ddd, 1H), 1.87–1.76 (m, 2H), 1.65 (s, 3H), 1.70–1.00 (8H), 0.78 (s, 3H). ^{13}C NMR (C_6D_6, δ/TMS): 151.16 (C_{11}), 146.84 (C_4), 111.12 (C_{12}), 105.63 (C_{14}), 53.28 (C_5), 44.48, 42.49, 39.31, 37.32, 36.93, 26.32, 23.87, 23.63, 22.94, 16.28 (C_{15}).

The ^{1}H NMR of S1 revealed two independent vinylic groups at, respectively, δ 4.98, 4.94 and 4.84, 4.60, and two methyl singlets at δ 0.78 and 1.65, respectively, due to Me-15 and Me-13 protons. The results of the 2D ^{1}H NMR experiments (COSY-90 and COSY-45) fully agreed with the proposed structure

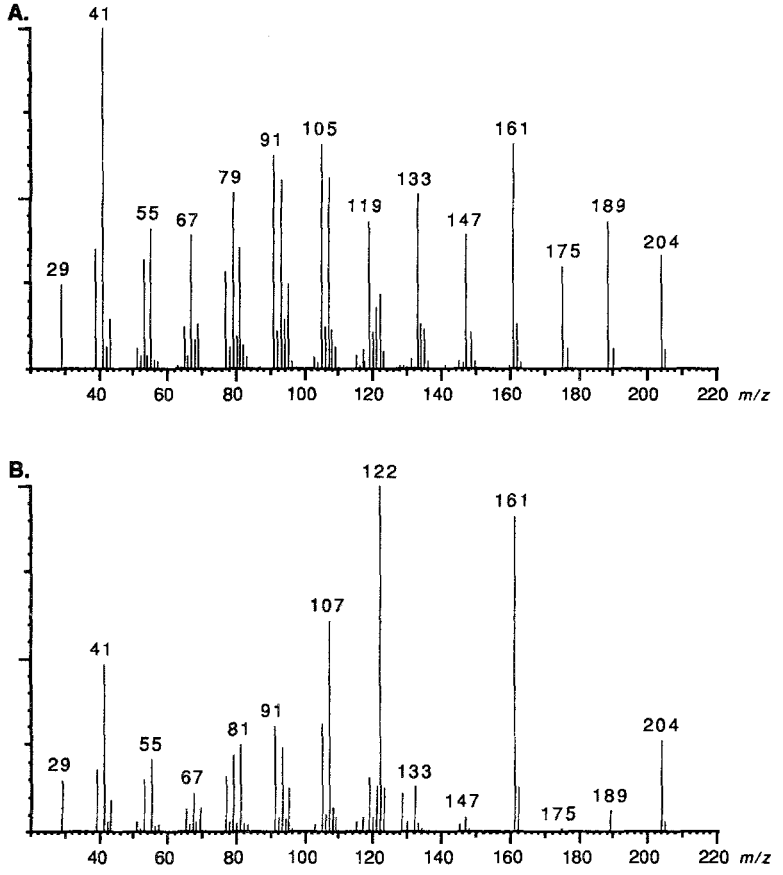

FIG. 2. EI-mass spectral scans of S1 and S2 (Nermag R 10-10 C quadrupole mass spectrometer coupled with Girdel 31 chromatograph; injector temperature 240°C; column DB5 60 m × 0.32 mm ID, J&W Scientific; gas vector: He at 35 cm/sec at 220°C; 40°–220°C at 3°C/min; EI mass spectra obtained at 70 eV on a 0.8 sec cycle, the instrument scanning from 25 to 300 amu with the ion source maintained at 150°C).

(Figure 3). Cross-peaks showed coupling between the deshielded methyl group and the vinylic protons at δ 4.98 and 4.94, revealing an isopropenyl group. These vinylic protons were coupled to the methine proton H-7 at δ 2.27. The other vinylic protons, at δ 4.84 and 4.60, were coupled to the methine proton H-5 at δ 1.94. A pair of cross-peaks revealed coupling interactions of H-5 with the C-6 protons at δ 2.24 and 1.47, and another pair of cross-peaks showed vicinal coupling of these C-6 protons with H-7. The C-1 protons at δ 1.76 and 1.15 are vicinal to the C-2 protons at δ 1.45, and the latter showed vicinal coupling with C-3 protons at δ 2.02 and 1.87.

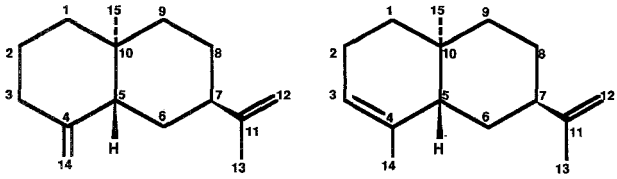

FIG. 3. Proposed structure for S1 [(5*R**, 7*R**, 10*S**)-selina-4(14), 11-diene] and S2 [(5*R**, 7*R**, 10*S**)-selina-3,11-diene].

The ^{13}C NMR spectrum of S1 exhibited 15 carbon resonances, of which six were attributed unambiguously by comparing with literature data of similar compounds (San Feliciano et al., 1990). Unfortunately the small amount available of S1 (ca. 380 μg) did not allow a ^{13}C–^{1}H heterocorrelation experiment to be performed.

For comparison, a ^{1}H NMR spectrum of S1 was obtained in $CDCl_3$. The signals for the methyl groups found at δ 0.75 and δ 1.74, and for the two terminal methylene groups found at δ 4.45, 4.70, 4.82, and 4.91 are comparable with reported NMR data for 5βH, 7β, 10α-selina-4(14), 11-diene (Sulser et al., 1971).

S2: ^{1}H NMR (C_6D_6, δ/TMS): 5.37 (br m, 1H), 4.98 (br m, 1H), 4.95 (br m, 1H), 2.27 (br s, 1H), 2.07 (br dd, 1H), 1.96–1.68 (3H), 1.65 (s, 3H), 1.60 (br s, 3H), 1.45–1.22 (6H), 1.13 (m, 1H), 0.88 (s, 3H). ^{13}C NMR (C_6D_6, δ/TMS): 121.63 (C_3), 111.32 (C_{12}), 53.28 (C_5), 41.56, 39.84, 38.68, 36.52, 25.68, 23.79, 23.26, 22.96, 21.38 (C_{14}), 15.76 (C_{15}), olefinic quaternary carbons not detected.

The ^{1}H NMR of S2 revealed an olefinic proton attached to a trisubstituted bond at δ 5.37 and a terminal methylene group at δ 4.98 and 4.95. A tertiary methyl group was found at δ 0.88 (Me-15) and two vinylic methyl groups gave signals at δ 1.60 (Me-14) and 1.65 (Me-13). The results of the 2D ^{1}H NMR experiments (COSY-90 and COSY-45) were fully compatible with the proposed structure (Figure 3). The isopropenyl group was demonstrated by the observation of coupling interactions between the methyl group at δ 1.65 and the vinylic protons at δ 4.98 and 4.95. These vinylic protons were coupled to the methine proton H-7 at δ 2.27. The latter was coupled to the methylene C-6 protons at δ ca. 1.3, and cross-peaks revealed coupling interactions between these C-6 protons and the methine proton H-5 at δ 1.92. A pair of cross-peaks revealed vinylic couplings between the latter and the olefinic proton H-3 at δ 5.37, and the methyl group Me-14 at δ 1.60.

The methine proton H-7 was coupled to a C-8 proton at δ 1.75, and the latter showed geminal coupling with a proton at δ 1.13. Cross-peaks showed

coupling interactions between these C-8 protons and the C-9 protons at δ 1.82 and 1.42, the latter showing geminal coupling. Finally, one of the methylene C-9 protons, at δ 1.42, displayed a small coupling interaction with the methyl Me-15 group at δ 0.88.

The ^{13}C NMR spectrum of S2 exhibited only 13 carbon resonances, the olefinic quaternary carbons not being detected due to the small amount available (205 μg). However, five resonances were unambiguously attributed by comparing with literature data of similar compounds (Van Beek et al., 1989; San Feliciano et al., 1990).

Again for the purpose of comparison, a ^1H NMR spectrum of S2 was obtained in $CDCl_3$. This spectrum agreed well with the reported NMR data for 5βH, 7β, 10α-selina-3,11-diene (Sulser et al., 1971) and of a compound identified as 7-epi-α-selinene, i.e., the enantiomer 5αH, 7α, 10β-selina-3,11-diene by Van Beek et al. (1989). The Kovats indices on a DB Wax column were found to be rather different [1775 in the report of Van Beek et al. (1989) vs 1749 in the present work], but this is probably due to differences in experimental conditions.

Interspecific Comparisons. The proportions of each terpenoid class in the secretions are given in Table 2, which also summarizes the composition of the sesquiterpenic fractions in each of the five species and the two populations of *N. novarumhebridarum*.

1. *Nasutitermes gracilirostris*: The sesquiterpene fraction represents almost 1% of the total secretion, and the major sesquiterpene of this species is β-elemene. Five other sesquiterpenes are also present, but in lower amounts: β-caryophyllene, β-selinene, germacrene-A, α_2-cadinene, and 5,10-epi-α-selinene.

2. *Nasutitermes novarumhebridarum*: The defensive secretion of this species possesses an important sesquiterpenic fraction (ca 5% of the total secretion), which is as large as the monoterpene fraction and consisted of all the cited compounds. 5,10-Epi-β-selinene (40% of the sesquiterpenic content) and 5,10-epi-α-selinene (27%) are the major constituents of this fraction. β-Elemene (15%), β-caryophyllene (3%), β-selinene (5%), germacrene-A (7%), and γ-selinene (1%) are present in lower quantities but always in appreciable amounts, while α-humulene, α-muurolene, and γ_2-cadinene are only present in trace quantities (lower than 1%). Comparison of the two analyzed *N. novarumhebridarum* populations demonstrated that there are no appreciable quantitative variations in their sesquiterpenic compositions.

3. *Nasutitermes torresi*, *N. triodiae*, and *N.* sp.F: In the secretions of these species, the sesquiterpenes are only present in trace amounts (lower than 1%). They consist of β-elemene and germacrene-A in *N. torresi*, traces of β-caryophyllene in *N. triodiae*, and β-elemene and traces of γ_2-cadinene in *N.* sp.F.

TABLE 2. RELATIVE QUANTITIES OF THE TERPENIC FRACTIONS AND SESQUITERPENES IN FRONTAL GLAND SECRETION OF *Nasutitermes spp.*[a]

	N.g. (9)	*N.n. 1* (18)	*N.n. 2* (7)	*N.to.* (3)	*N.tr.* (3)	*N.sp.F.* (9)
Fraction						
Monoterpenes (%)	5	4	6	7	6	5
Sesquiterpenes (%)	1	4	5	Trace	Trace	Traces
Diterpenes (%)	94	92	89	93	94	95
Compound						
β-Elemene (%)	0.7	0.6	0.7	Trace		Traces
β-Caryophyllene (%)	Trace	0.1	Trace		Trace	
α-Humulene (%)		Trace	Trace			
α-Muurolene (%)		Trace	Trace			
γ-Selinene (%)		0.1	0.2			
β-Selinene (%)	Trace	0.2	0.2			
S1 (%)	Trace	1	1.2			
Germacrene-A (%)	Trace	0.3	0.4	Trace		Trace
γ_2-Cadinene (%)	Trace	Trace	Trace			Trace
S2 (%)		1.6	1.8			

[a](Expressed as mean percentages; percentages were calculated from the peak area of the extracts; FID response factors were not determined). S1 = (5*R**,7*R**,10*S**)-selina-4(14),11-diene, S2 = (5*R**,7*R**,10*S**)-selina-3,11-diene; *N.g.* = *N.gracilirostris; N.n. 1* = *N. novarumhebridarum* from Bogia population; *N.n. 2* = *N. novarumhebridarum* from Bismark Archipelago population; *N.to.* = *N. torresi; N.tr.* = *N. triodiae*. Numbers in parentheses are numbers of colonies.

DISCUSSION

There are two reports of 5βH, 7β, 10α-selina-3,11-diene in termite defensive secretions. Naya et al. (1982) reported the existence of 5βH, 7β, 10α-eudesma-3,11-diene, i.e., 5βH, 7β, 10α-selina-3,11-diene, in the soldier cephalic secretion of *Noditermes wasambaricus*, but did not provide any analytical data. The same compound was also described as a minor constituent of the frontal gland secretion from soldiers of five *Synthermes* species (Baker et al., 1981a). The few ^{1}H NMR data available for this compound (Baker et al., 1981a) agreed with those for compound S2, but its completely different mass spectrum with a major peak at *m/z* 55 and no *m/z* 122 and 161 major fragment ions (characteristic of compound S2) suggests different structures. Besides these reports, neither 5βH, 7β, 10α-selina-4(14),11-diene nor 5βH, 7β, 10α-selina-3,11-diene have been positively identified as animal natural products, although they were previously characterized as dehydration products of a sesquiterpene alcohol, paradisiol, isolated from grapefruit oil (Sulser et al., 1971) and from *Carthamus lanatus* (San Feliciano et al., 1990). 7-Epi-β-selinene was also iden-

tified as a major sesquiterpene hydrocarbon in *Aristolochia indica* (Govindachari et al., 1973), while 7-epi-α-selinene was recently characterized in the essential oil of *Amyris balsamifera* (Van Beek et al., 1989). The reported ^{1}H NMR and MS data for the 7-epi-α-selinene (Van Beek et al., 1989) agreed well with the data for S2. However, Van Beek et al. (1989) have not determined the absolute configuration of the alleged 7-epi-α-selinene. Moreover, other original data available for epi-α-selinene isolated from plants (Klein and Rojahn, 1970) or characterized as a dehydration product of an alcohol isolated from plants (Sulser et al., 1971, San Feliciano et al., 1990), always displayed a 5βH, 7β, 10α-stereochemistry. As higher plants generally biosynthesize sesquiterpenoids with unique absolute configuration (Asakawa et al., 1981), it is probable that the compound isolated by Van Beek et al. (1989) was, in fact, 5βH, 7β, 10α-selina-3,11-diene, i.e., 5,10-epi-α-selinene. The corresponding isomer of the β-selinene isolated from various higher plants was also found to possess the same configuration, i.e., 5βH, 7β, 10α-selina-4(14), 11-diene or 5,10-epi-β-selinene (Sulser et al., 1971; Govindachari et al., 1973).

The absolute configurations of S1 and S2 were not determined. However, owing to their related structural analogy, since the two positional isomers are found together in large amounts in *Nasutitermes novarumhebridarum*, and since they likely arise from the same precursors by the same biosynthetic pathway, we assume that they have the same configuration.

In termites, sesquiterpenes are present in the defensive secretions of several species of Rhinotermitidae and Termitidae. In the Rhinotermitidae, while α-farnesene is described as a minor compound in the secretion of *Prorhinotermes simplex* (Rhinotermitinae) (Naya et al., 1982), sesquiterpenic compounds are always present in large proportions in the secretions of soldier of various *Reticulitermes* species (Heterotermitinae) (Zalkow et al., 1981; Baker et al., 1982; Clément et al., 1988). In the family Termitidae, sesquiterpenes were found in the soldier frontal gland secretions of Macrotermitinae (Evans et al., 1977, 1979), Termitinae (Wadhams et al., 1974; Baker et al., 1978; Naya et al., 1982; Scheffrahn et al., 1983, 1984, 1987, 1988), and Nasutitermitinae (Baker et al., 1981a,b; Prestwich and Chen, 1981; Prestwich and Collins, 1981; Valterovà et al., 1987).

It was reported that the genus *Amitermes* was the only termite genus known to possess characteristic eudesmane sesquiterpenes in soldier frontal secretion (Deligne et al., 1981; Prestwich, 1983, 1984). This seems true for epoxy-eudesmane, but not for eudesmane hydrocarbons, i.e., selinane, which were described both in *Amitermes* (Scheffrahn et al., 1983, 1984, 1987, 1988), *Syntermes* (Baker et al., 1981a), *Noditermes* (Naya et al., 1982), and now in *Nasutitermes*.

β-Caryophyllene, α-hymulene, α-muurolene, γ-selinene, β-selinene, and

γ_2-selinene have never been reported to be present in termite defensive secretion, whereas they have been detected in other insects and in plants.

Since they exist in many termite genera belonging to several families, the presence of sesquiterpenes in frontal gland secretions is probably of very little phylogenetic value. However, some particular classes of sesquiterpenic compounds might constitute reliable taxonomic characters such as selinanes (eudesmanes), which all result from cyclization of the germacrenium cation.

In the genus *Nasutitermes*, sesquiterpenes were characterized in only two species (Prestwich and Collins, 1981; Valterovà et al., 1987). In *N. octopilis*, the sesquiterpenic fraction consists of only one unidentified sesquiterpene, in small amounts in comparison with the mono- and diterpenic fractions (Prestwich and Collins, 1981). In *N. nigriceps*, this fraction consists of *cis*-caryophyllene (isocaryophyllene) and γ-gurjunene (Valterovà et al., 1987). In these two *Nasutitermes* species, as in four of the five species studied in the present report, sesquiterpenes are not quantitatively important, whereas in *N. novarumhebridarum*, the sesquiterpenes are almost equally abundant as the monoterpenes.

Except for *N. triodiae*, we found β-elemene in the frontal secretions studied. This compound was previously identified in the secretions of *Syntermes dirus*, *S. molestus* (Baker et al., 1981a), and *Noditermes wasambaricus* (Naya et al., 1982). However, even at low temperatures β-elemene is readily formed from germacrene-A by a Cope rearrangement (Weinheimer et al., 1970), so it is impossible to know if β-elemene actually exists in natural secretions.

Defensive secretions of Nasutitermitinae are often species-specific and sometimes characterize a given population or even a particular colony (Prestwich, 1979b; Gush et al., 1985; Roisin et al., 1987; Pasteels et al., 1988; Everaerts et al., 1988b; Chuah et al., 1989). The existence of sesquiterpenic components in the secretions of *N. novarumhebridarum*, *N. gracilirostris* and *N.* sp.F increases the species-specificity of these secretions. In addition to the species-specific characteristics of the secretions, the monoterpenic and diterpenic compositions allow allopatric populations of *N.*sp.F and *N. novarumhebridarum* to be distinguished, while they do not for *N. gracilirostris* (Pasteels et al., 1988). Sesquiterpene content does not display such differences between allopatric populations of *N. novarumhebridarum*.

Various biological activities of sesquiterpenes have been described in other insects and plants. In termites, some sesquiterpenes are involved in chemical defense as repellents against ants (Evans et al., 1977, 1979; Scheffrahn et al., 1983, 1984, 1987), as toxins (Wadhams et al., 1974), and perhaps as antihealants when added to wounds made with mandibles (Naya et al., 1982), but at present the precise role of sesquiterpenes from *Nasutitermes* frontal gland secretions is unknown.

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SURVIVAL OF PINE SAWFLIES IN COCOON STAGE IN RELATION TO RESIN ACID CONTENT OF LARVAL FOOD

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Abstract—Several experiments were conducted to determine whether the ingestion of diterpenoids (resin acids) by pine sawfly larvae influences the survival of postlarval stages. Larvae of two diprionid sawfly species were reared on shoots of two Scots pine clones, one with a low (1.5% dry wt) concentration of resin acids and the other with a high (5.2% dry wt) concentration. No significant treatment-related differences were found in any of the experiments with respect to (1) resistance against parasitoids, (2) preference of predatory shrews and carabids, and (3) apparency of cocoons in the field to predators. A preference of sawfly prepupae to spin cocoon in feces from larvae reared on high resin acid needles was found. Possible explanations for these results are discussed. Detection of an unknown compound, possibly a breakdown product of the major resin acid in pine needles (pinifolic acid), in prepupae indicate that resin acids may be metabolized by the sawflies.

Key Words—Three-trophic-level interaction, *Pinus sylvestris*, diterpenoid resin acids, *Neodiprion sertifer*, Hymenoptera, Diprionidae, predators, *Sorex araneus*, carabid beetle, Coleoptera, Carabidae, parasitoids.

INTRODUCTION

Many species of herbivorous insects sequester host plant allelochemicals for use in defending against natural enemies (Eisner, 1970; Blum, 1979; Duffey et al.,

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1986). In addition to deterring enemies before they attack, sequestered allelochemicals can be used against parasitoids after an attack; for example, some insects can "intoxicate" parasitoid eggs and larvae after these have entered their body (Campbell and Duffey, 1979; Vinson and Iwantsch, 1980; Duffey et al., 1986; Thorpe and Barbosa, 1986). This internal defense can operate in both the larval and the pupal stage, but requires that the compound or its derivatives be present in the body fluid of the insect. Only a few insect species have been studied from this more subtle aspect of interactions among three trophic levels.

Resin acids (diterpenoids), a major group of allelochemicals in Scots pine, are sequestered, without structural alteration, in the foregut of pine sawfly (Hymenoptera: Diprionidae) larvae (Eisner et al., 1974). The larvae store the resin acids in a pair of pouches connected to the foregut. When attacked, the gregarious larvae regurgitate this obnoxious fluid. The defense capability of larvae depends on the concentration of resin acids in their food (Björkman and Larsson, 1991). Thus, larvae feeding on high resin acid needles are better protected against predators (i.e., they regurgitate larger defense droplets) than are larvae on low resin acid needles (Björkman and Larsson, 1991).

According to Eisner et al. (1974), sequestration in the foregut is extremely effective, leaving no traces of resin acids in the midgut or feces. This suggests that resin acids cannot enter the body fluid. However, more recent data imply that resin acids can be sequestered in the midgut; i.e., fairly high concentrations of resin acids (i.e., more than 15% of those found in needles) were found in the feces of pine sawfly larvae (Larsson et al., 1986).

In this study we attempted to determine whether there is a relationship between resin acid concentration in the food ingested by sawfly larvae and the degree of protection against natural enemies shown by later stages (prepupal and cocoon). We also present data suggesting that resin acids can be metabolized by the sawflies. To our knowledge there are no data published on metabolism or de novo rearrangement of diterpenoids by pine sawflies.

METHODS AND MATERIALS

Host Insects. Two species of pine sawflies (Hymenoptera: Diprionidae) were used in the experiments. The reason for using two species was that *Neodiprion sertifer* (Fourcroy), with which most experiments were performed, was not available at the time of certain tests and *Diprion pini* (L.) was considered to be a good alternative. The two species have a similar biology (Prop, 1960), and their populations appear to be regulated by similar factors (Hanski, 1987; Larsson et al., 1993).

Plant Material and Rearing of Larvae. In all experiments, we used two clones (E4006 and W1040) of Scots pine (*Pinus sylvestris* L.) (cf. Larsson et

al., 1986). These two clones were selected out of a group of 36, growing in a seed orchard 40 km SW of Uppsala, to represent a "high" (W1040) and a "low" (E4006) resin acid clones (Table 1). Sawfly larvae were reared to the cocoon stage on the two Scots pine clones under constant environmental conditions (18:6 hr light–dark photoperiod, 18°C, 70% relative humidity) in the laboratory. Needles for resin acid analysis were sampled from the shoots prior to feeding. The two clones did not differ substantially in other chemical properties [see Larsson et al. (1986) for detailed information]. Samples of feces from the larvae in these laboratory rearings were collected for resin acid analysis.

Parasitoid Experiment. A laboratory experiment was conducted to determine whether the ability of sawfly prepupae to survive a parasitoid attack is influenced by the concentration of resin acids in the larval food. One *N. sertifer* prepupa, raised on either low or high resin acid needles, and one field-collected *Exenterus abruptorius* Thunbg. (Hymenoptera: Ichneumonidae) female were placed together in a clear plastic jar. The insects were observed continuously until the parasitoid had laid one egg. The parasitoid was then removed, and the prepupa was left to spin its cocoon. In each trial a new *E. abruptorius* individual

TABLE 1. SUMMARY OF COCOON CHARACTERISTICS AND RESULTS FROM EXPERIMENTS ON SURVIVAL OF PINE SAWFLY PREPUPAE AND COCOONS IN RELATION TO RESIN ACID CONCENTRATION IN LARVAL FOOD

Trait	Resin acid concentration in larval food: Low (1.5%)	Resin acid concentration in larval food: High (5.2%)	Statistical analysis
Proportion of parasitized prepupae emerging as sawfly imagines (%)	21.7	11.5	$\chi^2 = 0.33$, $P > 0.50$, $df = 1$
Resin acid concentration (% dry wt) in			
Cocoon shell	0	0	[a]
Prepupae	0.84	1.25	[a]
Cocoon shell weight relative to total cocoon weight (%)[b]	9.6	10.0	$t = 1.95$, $P > 0.05$, $df = 8$
Proportion of cocoons taken by			
Carabids	47.2	52.8	$\chi^2 = 0.06$, $P > 0.50$, $df = 1$
Shrews	56.4	43.6	$\chi^2 = 3.07$, $P > 0.05$, $df = 1$
in preference experiments (%)			
Proportion of cocoons preyed upon in the field predation experiment (%)	6.1	8.3	$\chi^2 = 0.37$, $P > 0.50$, $df = 1$

[a] No statistical test could be performed because of pooled samples.
[b] SD = 0.51 and 0.44 for low and high resin acid cocoons, respectively.

was used. Later, the success of the parasitoid was recorded. A total of 32 and 35 prepupae originating from larvae fed low and high terpene needles, respectively, were used in this experiment. *Exenterus* spp. are the principal parasitoids of pine sawflies (Griffiths, 1960; Pschorn-Walcher, 1965; Larsson and Tenow, 1984).

Behavior of Prepupae. This experiment was performed to see whether prepupae, which normally attach some litter material (e.g., pieces of needles, feces) to their cocoon, differentiate between material based on its resin acid content. Prepupae that had fed as larvae on low ($N = 22$) and high ($N = 23$) resin acid needles, respectively, were individually offered a choice of media in which to spin their cocoon. Thus, each prepupa could choose from feces from larvae raised on low resin acid needles and feces from larvae raised on needles rich in resin acids, placed in two separate heaps in a large Petri dish.

Chemical and Mechanical Properties of Cocoons. Nine cocoons of each type were cut open, and the prepupa and cocoon shell were weighed separately. The relationship between these two measurements was used to estimate the degree to which prepupae raised as larvae on the two clones invested in what could be considered "mechanical protection." In this regard, it was assumed that a larger investment in cocoon shell would render cocoons more difficult for predators to penetrate. Prepupae and cocoon shells were then analyzed with respect to resin acid concentrations (chemical protection).

Cocoon Predation. Prey preferences of shrews (*Sorex araneus* L.) and carabid beetles (*Pterostichus niger* Schall.) were investigated in laboratory experiments. In both experiments, individual predators were presented with one low and one high resin acid cocoon at the time. After the predator had taken one cocoon, it was offered two new cocoons. Three shrew individuals were used, and they were offered 110 cocoons (55 of each type). A total of 20 carabid beetles were used, and they successfully preyed upon 72 cocoons.

A field experiment was conducted to compare the "apparency" of low and high terpene cocoons. Along a 5-m stretch of ground two cocoons, one of each type, were placed 10 cm apart at half-meter intervals. With this design, it was assumed that the two cocoons in each pair ($N = 181$ pairs) had an equal probability of being detected by chance. The cocoons were placed just below the litter layer, which is where most cocoons are normally found. Each cocoon position was marked with a headless match stuck into the ground. Cocoons were left in the field for one month. Both *N. sertifer* and *D. pini* cocoons were used in this experiment. Experiments with cocoons of the former species were performed in late summer or early autumn, whereas the experiments with *D. pini* cocoons were performed in September to October. The habitat in both cases was pine forest interspersed with mixed-species stands, with lichens and mosses dominating on the ground. The sites used in this experiment had very low natural

densities of pine sawflies; thus the natural occurrence of cocoons should not have influenced the results.

Chemical Analysis. Resin acids were analyzed using a Varian 3700 gas chromatograph after being extracted and treated as described in Gref and Ericsson (1985). The chromatograph was equipped with a fused silica capillary column (15 m × 0.25 mm ID) with a 0.25 μm film of DB-1 (J&W Scientific), a split injector, and a flame ionization detector. Hydrogen was used as carrier gas at a flow rate of 1.60 ml/min. The chromatograph was operated isothermally at 210°C or temperature programmed to increase from 160° to 240°C at 4°/min. Peak areas, relative to the internal standard, and retention times were measured with an electronic integrator.

RESULTS

No difference between treatments was found in the ability of prepupae to survive parasitoid attack (Table 1).

Irrespective of which type of food prepupae had fed upon as larvae, they preferred to spin their cocoon in feces from larvae raised on high resin acid needles (Figure 1; $\chi^2 = 5.77$, $P < 0.05$).

The weight of the cocoon shell relative to the total cocoon weight was similar for the two treatments (Table 1), indicating that the cocoons did not differ with respect to mechanical properties. Cocoon shells did not contain any traces of resin acids (Table 1).

The total concentration of resin acids was lower in prepupae than in needles (Table 2), but the relative difference between needles of the two types was much

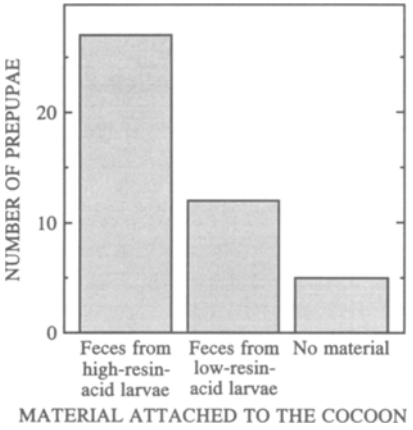

FIG. 1. Choice of cocoon-spinning medium by *Neodiprion sertifer* prepupae.

TABLE 2. CONCENTRATIONS OF RESIN ACIDS (% DRY WT) IN LARVAL FECES AND PREPUPAE OF *Neodiprion sertifer* RAISED ON TWO SCOTS PINE CLONES DIFFERING IN NEEDLE RESIN ACID CONCENTRATIONS

	Clone E4006			Clone W1040		
Resin acid	Needles	Feces	Prepupae	Needles	Feces	Prepupae
Isopimaric	0	0	0.25	0	0	0.27
Levopimaric + palustric	0.12	0.05	0.29	0.19	0.09	0.41
Dehydroabietic	0	0	0.16	0	0	0.23
Abietic	0.07	0.01	0.08	0.14	0.12	0.20
Neoabietic	0	0	0.06	0	0	0.06
4-epiimbricatolic	0.30	0.05	0	0.37	0.12	0
Pinifolic	1.02	0.13	0	4.53	0.72	0.08
Total	1.51	0.24	0.84	5.23	1.05	1.25
Unknown	0	0	1.95	0	0	1.88

larger than between prepupae, which had fed on them (3.5- vs. 1.5-fold). Prepupae of both kinds contained high concentrations of an unknown substance with diterpenoid-like retention characteristics on GLC and TLC (Table 2). The total concentration of resin acids was lower in feces than in prepupae, but for two resin acids (pinifolic and 4-epiimbricatolic) the opposite relationship was evident. Because the data from feces and prepupae were estimates from pooled samples, no statistical analysis could be made.

Both shrews and carabids took equal proportions of low resin acid and high resin acid cocoons in the preference experiments (Table 1). Among the 20 carabids used, only 10 were successful in preying upon cocoons. The unsuccessful beetles were slightly smaller than the successful ones ($T = 1.38$, $P > 0.10$), which might explain their problem; mean length ($\pm$SD) of successful and unsuccessful carabids was 17.85 mm ($\pm$1.37) and 16.95 mm ($\pm$1.54), respectively.

A low, but about equal, proportion of cocoons of each type disappeared in the field predation experiment (Table 1).

DISCUSSION

None of the data presented here indicate that there is a direct relationship between the resin acid concentration in larval food and the survival of pine sawflies in the cocoon stage.

Similarities in chemical and mechanical properties between low and high

resin acid cocoons may explain why polyphagous cocoon predators did not prefer one type over the other in the laboratory and field experiments.

There are at least two possible reasons why resin acid content did not influence the ability of sawfly larvae to survive a parasitoid attack: (1) resin acids and their derivatives may not be toxic to eggs and larvae of parasitoids, and (2) the resin acids may have never reached the body fluid of the sawfly larvae. The latter explanation is supported by the work of Eisner et al. (1974), who found that sequestration in the foregut is extremely effective. However, the relatively high concentrations of resin acids in larval feces found in our study conflict with their results. One explanation to this discrepancy may be that the methods for analyzing diterpenoids have become more sensitive and thus increased the detectability of resin acids in low concentrations.

Total resin acid concentrations differed considerably more between needles of the two pine clones than between prepupae that had fed on them. The reason for this difference could be that larvae raised on low resin acid needles ate more bark, which is richer in resin acids compared with needles (Gref, 1982) than larvae fed needles rich in resin acids (cf. Larsson et al., 1986). Similarly, bark feeding by larvae may explain the qualitative differences in resin acid composition between needles and prepupae (Table 2). However, the presence of high concentrations of an unknown diterpenoid-like compound in the prepupae, but not in the needles or in the feces, cannot be explained by larval feeding behavior.

The extremely low concentrations of pinifolic acid in prepupae, as compared with needles (and feces), indicate that the unknown compound found in prepupae may be a breakdown product or metabolite of pinifolic acid (cf. Kutney et al., 1981; Ohmart and Larsson, 1989). It is interesting to note that the concentration of this unknown compound was about the same in prepupae regardless of whether they had been raised as larvae on the high- or low-resin acid needles. One possible reason for this similarity may be that the metabolism of pinifolic acid is limited. If sawflies seldom or never experience the high concentrations of the high resin acid clone used here, it seems likely that the insects have not evolved a metabolic pathway with such a capacity. In fact, the needles of trees in natural stands of *P. sylvestris* often have much lower concentrations of resin acids (C. Björkman and R. Gref, unpublished data). Furthermore, the high intake of resin acids by pine sawfly larvae relative to the size of the pouches connected to the foregut where resin acids are stored (Larsson et al., 1986) implies that there could be selection for a metabolic pathway of these compounds. If the unknown compound is a metabolite of pinifolic acid, it must have entered the midgut, and also probably the body fluid, in order to get chemically rearranged because it is not likely that this could be accomplished in the foregut.

The lack of any relationship between resin acids in larval food and resistance to parasitoid eggs and larvae thus favors the hypothesis that neither resin acids nor their derivatives are affecting parasitoids within the sawfly body.

Life-table data suggest that the ability to intoxicate parasitoid eggs and larvae is weak in *N. sertifer* because mortality from parasitoids may be very high (Pschorn-Walcher, 1987). The fact that parasitoids generally account for most of the mortality due to natural enemies in the larval stage (e.g., Lyons, 1977) infers that some parasitoids have evolved behaviors to avoid the defense droplets of these larvae. Indeed, some ichneumonid parasitoids place their eggs just behind the head of the larvae or prepupae (personal observations), which is one of the few places on the body that larvae cannot reach with their defense droplets (also cf. Prop, 1960). Interestingly, defenses that may be highly effective against insect predators often seem to be quite ineffective against specialized parasitoids (Gross, 1993).

The only significant difference found in any of the experiments was the preference of prepupae to spin their cocoons in feces from larvae fed high resin acid needles. Resin acid concentrations in this type of feces were more than four times higher compared to feces from larvae fed low resin acid needles. We did not measure any other properties of the feces, and thus the possibility cannot be excluded that the preference for high resin acid feces was due to some other property (e.g., texture) that made it different from low resin acid feces. Nevertheless, by attaching resinous material to the cocoon, the insect might reduce the risk of predation in two ways: First, resinous feces on the cocoon surface could deter predators from biting. Second, resinous feces attached to the cocoon could make it difficult for shrews, which use olfactory stimuli for detecting their prey (Holling, 1958), to sense the smell of the insect. In fact, shrews do not respond to the resinous compounds that pine sawflies store in the pouches connected to the foregut (Holling, 1958). Holling (1958) suggested that predators responding to resinous compounds would be at a selective disadvantage because they would have to waste their time investigating such inedible and common objects as pine needles. The behavior of prepupae may represent an example of a subtle type of interaction among three trophic levels mediated by variation in plant chemistry (cf. Price et al., 1980). The role of such interactions in the population dynamics of diprionid sawflies have only recently been appreciated (Larsson et al., 1993).

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PHYSIOLOGICAL EFFECTS OF ALARM CHEMOSIGNAL EMITTED DURING THE FORCED SWIM TEST

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Abstract—Two experiments were conducted. In the first, male rats were immersed for 25 min in fresh water or water previously swum in by another rat. Control rats were not immersed in water. Rats tested in water previously swum in by another rat were significantly less immobile than rats tested in fresh water. Water immersion resulted in significant increases in serum corticosterone, glucose, and phosphorus levels, a decrease in potassium levels, and a higher phosphorus/potassium ratio, compared to nonimmersed controls regardless of water condition. When the two water-immersed groups were compared, rats tested in previously swum water had significantly higher glucose and significantly lower potassium levels and a higher phosphorus/potassium ratio than rats tested in fresh water. Immobility times were significantly correlated with the phosphorus/potassium ratio. In the second experiment, blood gases were measured prior to testing and at 25 min after immersion in rats tested in fresh and previously swum water. Rats in soiled water hypoventilated to a significantly greater extent than rats in fresh water but did not differ significantly in blood oxygenation. These two studies demonstrate that alarm chemosignals can produce physiological effects in conspecifics.

Key Words—Alarm chemosignals, forced swim test, depression, stress, rat, *Ratus ratus*, electrolytes, blood gases.

INTRODUCTION

We have previously reported that, in contrast to the long immobility times exhibited by rats tested in fresh water in the forced swim test, rats tested in

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soiled water, i.e., water previously swum in by another rat, are unable to maintain an immobility response (Abel and Bilitzke, 1990, 1992; Abel and Subramanian, 1991; Abel, 1991a, 1992, 1993a,b; Abel and Hannigan, 1992). We have interpreted this inhibitory effect on immobility as evidence that rats secrete an alarm chemosignal in response to the stress of immersion, which affects the behavior of conspecifics subsequently exposed to it (Abel, 1991a,c, 1992, 1993a,b; Abel and Bilitzke, 1990; Abel and Subramanian, 1991). However, despite a more than 10-fold difference in immobility times between rats tested in these two water conditions, we have not been able to differentiate between rats tested in fresh and soiled water in terms of their physiological responses to testing. For example, while rats tested in fresh water or water containing alarm chemosignals have large increases in serum corticosterone and prolactin levels as a result of testing compared to nonimmersed controls (Abel, 1991b; Abel and Subramanian, 1991; Satoh et al., 1985; Manev and Pericic, 1988; Yamada et al., 1987), rats tested in fresh water do not differ significantly from those tested in the presence of alarm chemosignals on these two hormonal measures (Able and Subramanian, 1991). One reason for the failure to detect treatment-related differences in these two stress hormones is that while corticosterone and prolactin are both reliable stress markers (Axelrod and Reisine, 1984; Amario et al., 1986; Hennessey et al., 1979; Hennessey and Levine, 1978; Gala, 1990; Natelson et al., 1987; Natelson et al., 1981), they do not reflect the behavior of rats in the forced swim test since they are poorly correlated with immobility times (Abel, 1993a,b).

Increases in corticosterone and prolactin are not the only physiological changes occurring in conjunction with forced swim testing, however. In addition to these changes, rats in the forced swim test undergo a metabolic acidosis (Abel, 1993a,b). The acidemia provokes mobilization of serum bicarbonate to buffer the test-induced acidosis along with a significant increase in alkaline phosphatase activity, which promotes the release of phosphorus from bone (Biltz et al., 1981) so that it, too, can buffer the testing-induced acidosis (Biltz et al., 1981). Under normal circumstances, acidemia also induces hyperventilation to relieve the build-up of carbon dioxide, but under conditions of water immersion, hyperventilation is attenuated so that a respiratory acidosis likewise occurs, and the increase in bicarbonate is attenuated (Harrington et al., 1986). The metabolic and respiratory acidoses have an additive effect such that plasma acidity rises dramatically and there is a significant increase in partial carbon dioxide pressure (P_{CO_2}). Whereas the correlation between corticosterone and immobility just reaches statistical significance ($r = -0.38$), the correlations between immobility times and carbon dioxide, potassium, phosphorus, and the phosphorus/potassium ratio are very high, e.g., $r = 0.59, 0.67, -0.73, -0.82$, respectively). The phosphorus/potassium ratio, in fact, accounts for as much as 67% of the variance in immobility (Abel, 1993a,b). Since these latter measures reflect

immobility to a much greater extent than corticosterone, we hypothesized they should also be capable of differentiating between rats tested in fresh water and water previously swum in by another rat. An alternative way of stating this hypothesis in terms of alarm chemosignals is that alarm chemosignals, if present in the test condition, should evoke not only behavioral but also physiological changes in rats that differ significantly in degree from those evoked by testing in their absence. The results of experiment 1 corroborated this hypothesis.

In a second study, we explored the changes in blood gases associated with testing rats in the presence of alarm chemosignals. Our previous study (Abel, 1993a,b) was unable to do this except for total carbon dioxide because samples were collected aerobically. For this study, we cannulated animals and sampled blood under anaerobic conditions.

METHODS AND MATERIALS

Experiment 1

Male Sprague-Dawley rats (Charles River, Portage, Michigan) 70–90 days of age were housed in polycarbonate cages in a vivarium at a constant room temperature of 21° ± 1°C, and humidity (40 ± 5%), with a 12-h light–dark cycle (lights on at 7:00 AM). Food and water were available ad libitum. Animals were tested in a Plexiglas cylinder (Corning Glass, Corning, New York) (45.7 cm high, 22.2 cm inside diameter) filled to a height of 38 cm containing either fresh tap water (30° ± 1°C) ($N = 14$) or water previously swum in for 25 min by another rat ($N = 14$). Rats were tested for 25 min. All sampling was done at the same time between 8:00 and 11:00 AM to minimize circadian rhythms in hormonal levels. Immobility was recorded on video tape and was scored at a later time. Behavioral scoring was performed by only one "blinded" experimenter. A nonimmersed control group ($N = 12$) was also sampled to evaluate the effects of water immersion per se.

Immediately after removal from the water, each animal was blotted dry and sacrificed by decapitation. Decapitation occurred in a room adjacent to and separate from the room in which testing took place. Less than 15 sec elapsed from removal of an animal from water. This time interval produces no detectable increases in plasma corticosterone response following maximal ACTH stimulation (Urquhart and Li, 1969). Trunk blood was collected in tubes and stored on ice for 20 min prior to centrifugation (300*g* for 10 min). The serum was then separated and stored at −70°C until subsequent assay.

Serum corticosterone levels were determined using a commercially available radioimmunoassay kit containing corticosterone antibody (ICN Biomedicals). All samples were analyzed in duplicate. The intraassay coefficient of

variation (CV) for corticosterone was 1.9%. All other measures were obtained directly using a Kodak Ektackem Analyzer (model No. 700).

Experiment 2

Catheterization. Polyethylene (PE 50; Clay Adams) silastic (0.02-in. ID × 0.037-in. OD; Dow Corning) catheters were prepared in advance and stored in 0.1% benzalkonium chloride until implanted. Rats were anesthetized with methoxyflurane (Metofane; Pitman-Moore, Inc.), secured to a surgical board, and the jugular vein exposed and cleared of connective tissue. The catheter was attached to a catheter extension that was connected to a 1-ml tuberculin syringe. The syringe, catheter extension, and the catheter were filled with heparin (50 IU/ml) diluted with sterile 0.9% saline. The silastic end of the catheter was inserted through the vein into the atria, secured in position, and the free end led over the shoulder and out under the skin through an incision in the back. Approximately 2 cm of the catheter was exteriorized and closed with an amphenol pin (No. 220-PO2-100-8049). Blood samples were taken following a recovery period of five days.

Blood Sampling. For sampling, an extension was attached to the exteriorized portion of the catheter. The extension, consisting of a three-way stopcock (K 75; Pharmaseal), polyethylene tubing (PI 50; Clay Adams), and a connector (22-gauge stainless steel tubing 1.5 cm long), was filled with heparinized 0.9% saline. A preimmersion 0.3-ml blood sample was withdrawn and was replaced by an equal infusion of 0.9% saline via the catheter. Animals were then immersed in either fresh ($N = 9$) or soiled ($N = 8$) water as described above. After 25 min of immersion, a second blood sample was obtained. Blood was analyzed within 60 sec of removal using a Nova Biomedical analyzer (Waltham, Massachusetts).

RESULTS

Experiment 1

Compared to nonimmersed controls, water immersion resulted in significant increases in corticosterone, glucose, and phosphorus ($F = 157.8, 73.9, 8.52$; $df = 2, 37$, $P < 0.0001$, respectively) whereas potassium levels were reduced ($F = 14.1$, $df = 2, 37$, $P < 0.0001$) and the phosphorus/potassium ratio was significantly increased ($F = 33.5$, $df = 2, 37$, $P < 0.0001$). The data are shown in Figure 1.

Rats in soiled water were much less immobile than those tested in fresh water ($t = 12.5$, $df = 21$, $P < 0.0001$). Rats in soiled water did not differ from those in fresh water in serum corticosterone or phosphorus levels, but had

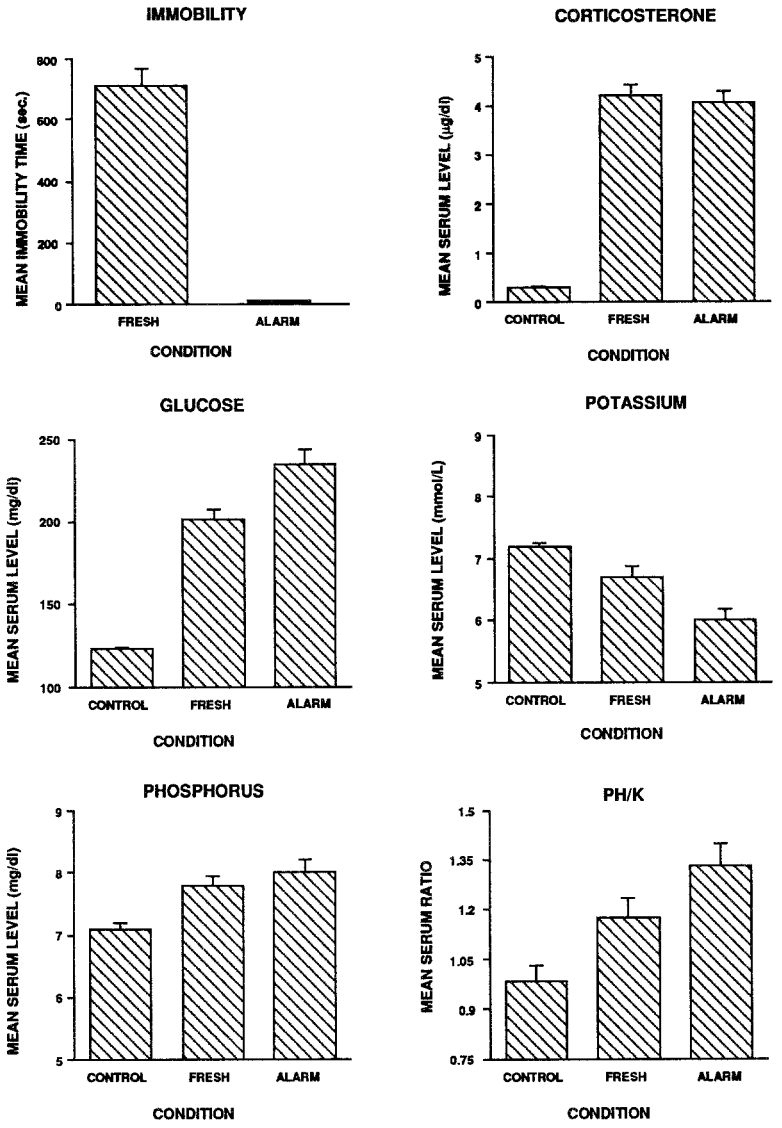

FIG. 1. Mean (±SEM) immobility times and physiological responses of rats tested for 25 min in fresh water ($N = 14$) or water containing alarm chemosignals ($N = 14$) compared to controls ($N = 12$).

higher serum glucose ($t = 3.11$, $df = 26$, $P < 0.005$) and lower potassium levels ($t = 2.73$, $df = 26$, $P < 0.02$) and a higher phosphorus/potassium ratio ($t = 3.61$, $df = 26$, $P < 0.002$). The correlation between immobility time and corticosterone levels was not significant, whereas the correlations between immobility time and serum glucose and phosphorus levels ($r = -0.56$, $r = 0.53$) and the phosphorus/potassium ratio ($r = 0.63$) were all highly significant ($P < 0.006$, $P < 0.009$, $P < 0.001$, respectively).

Multiple regression analysis indicates that the phosphorus/potassium ratio was the only variable significantly related to immobility time and accounted for 63% of the variance in immobility time.

Experiment 2

Sampling via cannulation allowed us to obtain preimmersion baseline measures as well as responses to testing. As a result, animals were able to serve as their own controls.

Using baseline levels as a covariate, testing rates in the presence of alarm chemosignals resulted in a significant decrease in pH and bicarbonate (HCO_2) and total carbon dioxide (TCO_2) ($F = 4.64$; 5.11, $df = 1, 14$, $P < 0.01$), coupled with an increase in PCO_2 ($F = 8.19$, $df = 1, 14$, $P < 0.01$) indicative of a mixed metabolic and respiratory acidosis (Harrington et al., 1986) (Figure 2), whereas differences in PO_2, O_2 saturation, O_2 concentration, and hematocrit were not significant.

DISCUSSION

As previously reported in all our studies (Abel, 1991a,c, 1992, 1993a,b; Abel and Bilitzke, 1990, 1992; Abel and Hannigan, 1992; Abel and Subramanian, 1991), rats tested in water previously swum in by another rat were much less immobile than those tested in fresh water.

The forced swim test is obviously stressful, as reflected in the frantic paddling of rats when they are first placed into the test, and by the increases in corticosterone, prolactin, and glucose (Abel, 1993a,b; Abel and Subramanian, 1991; Satoh et al., 1985; Manev and Pericic, 1988; Yamada et al., 1987) and hypoventilation (Abel, 1993a,b, and current study) that occur in response to testing. Although corticosterone, prolactin, and glucose are reliably increased in response to stressors of all kinds (Axelrod and Reisine, 1984; Amario et al., 1986; Hennessey et al., 1979; Hennessey and Levine, 1978; Natelson et al., 1987; Natelson et al., 1981), they are not highly correlated with immobility, whereas carbon dioxide levels, phosphorus, and acid–base status, and especially the phosphorus/potassium ratio, are highly correlated with immobility (Abel, 1993a,b). The present study demonstrates that rats tested in the presence of

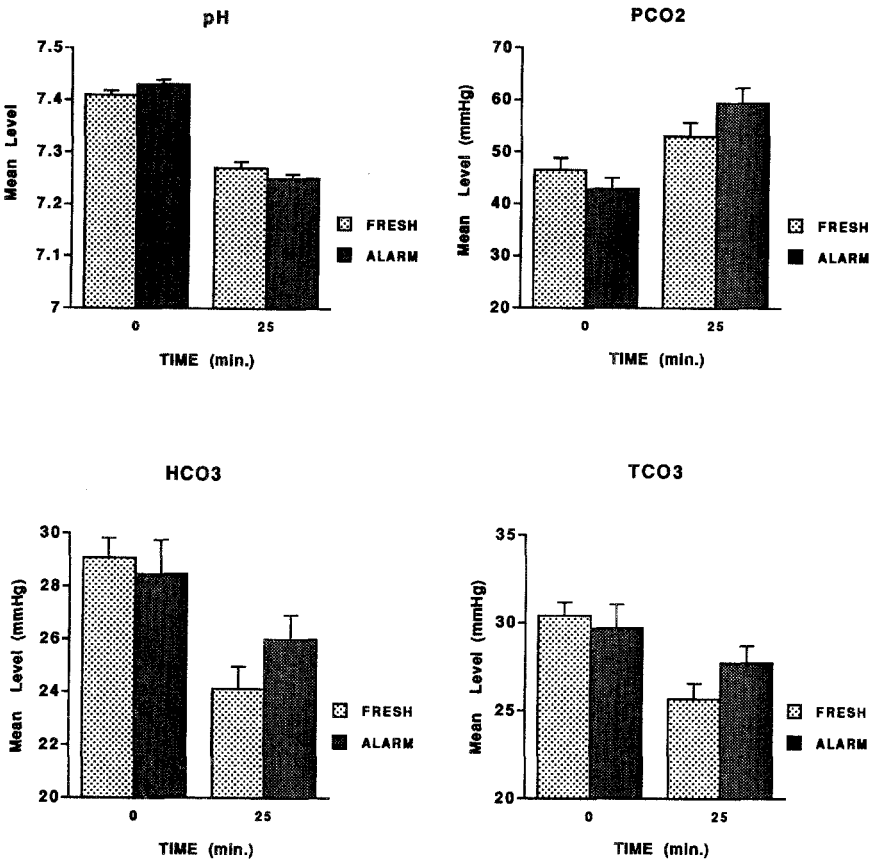

FIG. 2. Mean (±SEM) changes in blood gases for rats tested for 25 min in fresh water ($N = 9$) or water containing alarm chemosignals ($N = 8$).

alarm chemosignals experience a mixed metabolic–respiratory acidosis (decreased pH and bicarbonate and increased P_{CO_2}) (Harrington et al., 1986) but do not become significantly more hypoxic than rats in fresh water. Within minutes of water immersion, the rat enters a state of anaerobic glycolysis and produces lactic acid instead of pyruvic acid. The lactic acid enters the blood and dissociates into hydrogen and lactate, causing blood acidity and lactate levels to rise (Abel, 1993a,b). The ensuing acidemia is potentially life-threatening if not corrected (Cogan, 1991) and is neutralized by serum bicarbonate stores and by mobilization of other neutralizing anions such as phosphorus from bone (Biltz et al., 1981). Ordinarily, an acidemia would provoke hyperventilation as a way of releasing the ensuing build-up of carbon dioxide, but because the rat is

immersed in water, its breathing is suppressed and the partial pressure of carbon dioxide continues to increase, resulting in a respiratory acidosis. Acidosis should have the effect of increasing serum potassium levels (Cogan, 1991); however, other factors (e.g., insulin, catecholamines) have the opposite effect (Williams and Epstein, 1989). The net impact of these and possibly other factors) (e.g., hyperosmolarity) is an ultimate hypokalemia.

We have previously found that phosphorus and potassium levels are very closely linked in the forced swim test (Abel, 1993 a,b), and, as in the present study, the phosphorus/potassium ratio can account for over 60% of the variance in immobility. The present study indicates that soiled water, which we propose contains an alarm chemosignal, induces a greater stress response in terms of acidemia and attendant changes in certain cations (potassium) and anions (phosphorus) during the forced swim test, than occurs when rats are tested in fresh water.

Alarm chemosignals are released by a wide variety of plants, insects, and animals and communicate danger to conspecifics (Pfeiffer, 1963). These chemicals elicit freezing, defensive behavior, dispersal, or aggregation, depending on the species and test conditions. Alarm chemosignals can be inherently aversive to rats (Hornbuckle and Beall, 1974; Stevens and Saplikowski, 1973; Mackay-Sim and Laing, 1980), and aversion increases if test rats are themselves stressed (Williams and Groux, 1993). The chemosignals can suppress ongoing behavior and interfere with learning in a shock-avoidance task (Dua and Dobson, 1974; Valenta and Rigby, 1968) and prod-burying behavior in response to shock (Williams, 1987). Mice, likewise, will generally avoid area previously traversed by stressed conspecifics (Carr et al., 1970; Rottman and Snowdon, 1972), and these chemosignals cause mice to become more vigilant (Zalaquett and Thiessen, 1991) and move to areas of higher ambient temperature (Thiessen et al., 1991).

Recent studies have also found that in some species alarm chemosignals may also suppress immune function (Cocke and Thiessen, 1990), induce analgesia (Fanselow, 1985), and precipitate convulsions in conspecifics (Abel et al., 1992a; Cocke and Thiessen, 1990). The present study elucidates some of the physiological changes that occur in response to alarm chemosignals encountered during the forced swim test which may underlie some of these other effects.

Although alarm chemosignals in mammals have not been isolated, we have found that ACTH but not adrenal steroids are involved in secretion of alarm chemosignals in the forced swim test, since removal of the pituitary eliminates production/secretion of chemosignals and ACTH replacement restores it, whereas adrenalectomy has no effect on production/secretion (Abel, 1993b; Abel and Bilitzke, 1992). Although alarm chemosignals have not been isolated, we have characterized those released during water immersion as being very stable (Abel, 1991a), not readily depleted from the body (Abel, 1991a), and nonvolatile (Abel, 1991c). They also produce different levels of responsiveness in different rat

strains (Abel et al., 1992a,b). Studies of the ontogeny of responsiveness to alarm chemosignals in the forced swim test indicate a rapid onset of response occurs around day 24, shortly after ontogeny of the immobility response itself (Abel, 1993b).

Although we have found that the pituitary, and at least ACTH, is involved in production of alarm chemosignals, we are unable to identify the area of the body from which they are eliminated or whether their production/secretion is mediated by another organ. We have, however, shown that urine or feces excreted in response to foot shock do not affect the immobility response when rats are exposed to them during the forced swim test (Abel and Bilitzke, 1990). Mackay-Sim and Laing (1980) likewise reported that electric shock did not cause rats to emit alarm chemosignals. This indicates either the alarm chemosignal is not produced in response to all stressors or that the chemosignal is not carried in urine or feces. Related studies in tadpoles have shown that alarm chemosignals in this species are produced by mechanical injury but not by electrical shock (Pfeiffer, 1963). Several studies in fish and reptiles have shown that skin is an important source of alarm chemosignals for these species (Pfeiffer, 1963). Skin actively secrets these chemosignals; skin from dead fish is ineffective (Pfeiffer, 1963).

No studies have reported the chemical nature of any alarm chemosignal. Studies in fish have shown that skin containing alarm substance remains active for many days and boiling reduces its potency (Pfeiffer, 1963). A study from our laboratory also found alarm chemosignals emitted during the forced swim test remain active for many days (Abel, 1991a) and boiling likewise destroys their effectiveness (unpublished observation). We have also found that the alarm chemosignal in water taken from cylinders previously swum in, fluoresces at a wavelength of 300 nm (unpublished observation).

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INDUCTION OF CYTOCHROME P-450 ACTIVITIES BY NICOTINE IN THE TOBACCO HORNWORM, *Manduca sexta*

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Abstract—The induction by dietary nicotine of a series of cytochrome P-450 enzyme activities was investigated in early fifth-instar *Manduca sexta* larvae. At a low nicotine concentration in the diet (0.1%), three of 12 midgut microsomal enzyme activities were significantly increased. At a higher concentration (0.75%) commonly found in plants of the genus *Nicotiana*, nine of 12 activities were induced by 1.4- to 10.0-fold. Total cytochrome P-450, P-450 reductase activity, and midgut microsomal metabolism of nicotine were also increased by feeding 0.75% nicotine. Nicotine was metabolized by midgut microsomes to nicotine-1-*N*-oxide and cotinine-*N*-oxide. Fat body microsomal nicotine metabolism was low and unaffected by dietary nicotine. Isolated nerve cords were able to metabolize nicotine in vitro but this metabolism was not inducible by dietary nicotine. Nicotine-fed fifth-instar *M. sexta* larvae showed an increased tolerance to subsequent nicotine injection when compared to larvae fed a control diet. These results support the idea that induction of midgut cytochrome P-450-related metabolism is an adaptation of *Manduca sexta* to dietary nicotine.

Key Words—Cytochrome P-450, induction, nicotine, *Manduca sexta*, Lepidoptera, Sphingidae.

INTRODUCTION

Microsomal cytochrome P-450 monooxygenases are involved in the metabolism of endogenous and exogenous chemicals in all organisms thus far examined.

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Since the work of Krieger et al. (1971), the involvement of insect cytochrome P-450 enzymes in the detoxification of plant secondary compounds has received considerable attention (Hodgson, 1985, for review). In Lepidoptera, the midgut is generally recognized as a major site of microsomal P-450 activities induced in response to plant chemicals, although the fat body, Malpighian tubules, and other tissues also show induced activities in certain species (Hodgson, 1985).

Induction of microsomal cytochrome P-450 activities is thought to protect insects against subsequent plant allelochemical exposures (Hodgson, 1985, Yu, 1986). Midgut P-450 activities are low around the time of ecdysis and increase when larvae are actively feeding (Tate et al., 1982; Yu and Ing, 1984; Feyereisen and Farnsworth, 1985). Induction of P-450 enzyme activities is dependent on either the host plant or the inducer added to an artificial diet (Brattsten et al., 1977; Yu, 1982, 1984, 1987; Yu and Ing, 1984; Brattsten, 1987; Rose et al., 1991). Little is presently known about the levels and number of different cytochrome P-450 enzymes induced by endogenous and exogenous chemicals in insects. Cohen et al. (1990) showed that at least two different P-450 proteins were induced by xanthotoxin in the black swallowtail, *Papilio polyxenes*. Xanthotoxin tolerance in this species (Ivie et al., 1983) is linked to the induction of at least one identified P-450, CYP6B1 (Cohen et al., 1992).

Interest in nicotine as a possible inducer of insect P-450 enzymes arose from the observation that this chemical is tolerated by tobacco-feeding insects such as *Manduca sexta* at concentrations that are toxic to non-tobacco feeders (Hodgson, 1985). Nicotine tolerance by *M. sexta* was initially thought to result from rapid excretion of large amounts of unmetabolized nicotine (Self et al., 1964). Brattsten et al. (1977) showed that feeding α-pinene (a known P-450 inducer) to the southern armyworm increased its tolerance to subsequent dietary nicotine exposure. Dietary piperonyl butoxide resulted in greater nicotine toxicity, thus implicating P-450 in nicotine metabolism in this insect. Recently, Rose et al. (1991) showed that nicotine was a good inducer of midgut P-450 activities in the tobacco budworm, *Heliothis virescens*.

In this paper, we describe the induction of midgut cytochrome P-450 enzyme activities by dietary nicotine in fifth instar larvae of *M. sexta*. Metabolism of nicotine by midgut and fat body microsomes was also examined. Induction of P-450 activities was correlated with an increased capacity to cope with a subsequent nicotine challenge.

METHODS AND MATERIALS

Insects. Fourth-instar *Manduca sexta* larvae, in the latter stages of head capsule slippage, were selected from a colony reared by established protocols (Prasad et al., 1986). They were maintained (26°C with a 16:8 hr light–dark

photoperiod) on a control diet (Bioserv) for 24 hr after molting to the fifth instar and then kept on that diet for an additional 72 hr or placed on the same diet with either 0.1% or 0.75% nicotine by weight.

Chemicals. Nicotine and cotinine were obtained from Sigma. Authentic metabolite standards were obtained from the following: nicotine-1′-*N*-oxide was supplied by Dr. N. Castagnoli (VPI&SU), and cotinine-*N*-oxide was from Dr. G.D. Byrd (R.J. Reynolds). Radiolabeled nicotine, [*pyrrolidine*-2-^{14}C]nicotine (specific activity 59.9 mCi/mmol) was obtained from NEN. The cytochrome P-450 inhibitors piperonyl butoxide and SKF 525A were obtained from Aldrich and Smith Kline & French, respectively.

Preparation of Microsomes. Midguts from 4-day-old fifth-instar larvae were dissected free of peritrophic membrane, associated gut contents, and Malpighian tubules. Pooled groups of three to four midguts were homogenized in 0.1 M $NaPO_4$ buffer (pH 7.5) containing 10% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, and fresh 1 mM PMSF. The homogenates were centrifuged at 10,000*g* for 10 min and the resulting low-speed supernatants were filtered through glass wool and then centrifuged at 100,000*g* for 60 min. The microsomal pellets were individually resuspended in the same buffer prior to immediate freezing in liquid nitrogen. Microsomes were stored at −80°C until used in enzyme assays. Total microsomal protein was measured using the Bicinchoninic acid procedure (Sigma) for microtiter plates using bovine albumin as the standard.

P-450 Enzyme Assays. Cytochrome P-450 assays using synthetic substrates were conducted using 7–24 different microsome preparations (each from three to four midguts) collected from each diet treatment (control, 0.1%, or 0.75% nicotine), and the data were analyzed by ANOVA and by the Student-Newman Keuls procedure.

Benzo[*a*]pyrene metabolism was assayed according to Yang and Kicha (1978). Microsomal protein (100 μg) was preincubated in buffer for 3 min followed by the addition of 0.2 μmol of NADPH and 0.2 nmol of substrate. The continuous disappearance of benzo[*a*]pyrene fluorescence, i.e., hydroxylation, was monitored at wavelengths of 387 nm excitation and 407 nm emission on a Perking-Elmer 650-10S fluorescence spectrophotometer.

Biphenyl hydroxylation was measured according to the procedure of Burke and Prough (1978) using 1 mg of microsomal protein per assay.

Aminopyrine *N*-demethylation and 6-methylthiopurine *S*-demethylation were measured according to Tate et al. (1982) and Yu (1988), respectively. Modifications to these procedures were that 1 mg of microsomal protein was used per assay and final deproteinization was accomplished by the addition of 20% trichloroacetic acid in both assays. Samples of the final Nash reagent-treated supernantants were aliquoted in triplicate to 96-well microplate and the absorbance read immediately at 405 nm in a Thermomax microplate reader (Molecular Devices).

Aldrin and heptachlor epoxidation assays were performed according to Feyereisen and Vincent (1984) using 0.5 mg of microsomal protein per assay. The products dieldrin and heptachlor epoxide were measured by electron capture on a Shimadzu GC-8A gas chromatograph.

O-Demethylation of *p*-nitroanisole, 7-methoxycoumarin, and 7-methoxy-4-methylcoumarin were assayed according to Feyereisen and Vincent (1984) using 0.5 mg of microsomal protein per assay. Products were measured by fluorometric detection as indicated above.

Methoxyresorufin *O*-demethylation was measured in a kinetic assay according to Feyereisen and Farnsworth (1985) using 0.25 mg of microsomal protein per assay.

N-demethylation of *p*-chloro-*N*-methylaniline was assayed as follows. Microsomes (200 μg protein) were preincubated for 5 min at 34°C in 0.1 M MOPS buffer, pH 7.2, containing a NADPH regenerating system. Incubations with *p*-chloro-*N*-methylaniline were started by the addition of 0.6 mM substrate and stopped after 20 min by the addition of 6% *p*-dimethyl-amino-benzaldehyde. The formation of *p*-chloroaniline was compared against a standard curve at 450 nm using 96-well microplates in the Thermomax plate reader.

Phorate sulfoxidation was measured according to the procedure of Yu (1985) using 1.0 mg of microsomal protein per assay.

Total P-450 was measured according to the procedure of Omura and Sato (1964). Spectra were measured at 12°C on a Perkin-Elmer Lambda 19 spectrometer.

NADPH-cytochrome *c* reductase activity was assayed by a modification of the procedure of Halliday et al. (1986). Assays were conducted at 30°C in 96-well plates in the Thermomax plate reader in 250 μl total volumes. Duplicate assay wells contained 10 μg of microsomal protein preincubated for 5 min prior to the addition of the NADPH regenerating system (corresponding controls run in parallel received an additional volume of buffer instead of the regenerating system). The assay was run in kinetic mode for 5 min at 550 nm absorbance and the results expressed using an extinction coefficient of 2.52 OD/mM (calculated from absorbance values of known concentrations of dithionite-reduced cytochrome *c*).

Nicotine Metabolism. The metabolism of [^{14}C]nicotine by microsomes was studied as follows. One milligram of microsomal protein in 0.1 M MOPS buffer, pH 7.2, an NADPH regenerating system, 1 μM nicotine, and 0.5 μCi [^{14}C]nicotine in a final volume of 1 ml were incubated for 1 hr at 30°C. The reactions were terminated by the addition of 3 ml of 100% methanol, centrifuged, and the pellets reextracted three times with 100% methanol. The pooled supernatants for each reaction were concentrated by evaporation under reduced pressure, redissolved in a small volume of buffer, centrifuged, and the super-

natants injected directly onto a reverse-phase high-performance liquid chromatography system (Perkin-Elmer, Series 400) with Zorbax R_x C_8 column (4.6 mm ID × 25 cm). The elution conditions were 10–40% acetonitrile in 50 mM acetate buffer (pH 5.0) linear for 10 min changing to 40–100% acetonitrile in a linear gradient from 10 to 20 min followed by 100% acetonitrile for an additional 10 min. The flow rate was 0.75 ml/min with 0.5-min fractions collected directly into vials for scintillation counting. The elution times of ^{14}C metabolites were compared with the retention times of authentic nicotine-1′-*N*-oxide, cotinine-*N*-oxide, nicotine, and cotinine standards monitored at 254 nm using a Perkin-Elmer LC-95 detector.

The inhibition of microsomal [^{14}C]nicotine metabolism was also studied. The cytochrome P-450 inhibitors SKF525A or piperonyl butoxide (both at 10^{-4} M) were preincubated with microsomes for 10 min before the addition of substrate. In another set of incubations, the NADPH regenerating system was deleted. The effect of added house fly anti-cytochrome P-450 reductase antibodies (Feyereisen and Vincent, 1984) was studied as follows. Trial studies determined that 5× concentration of antisera versus microsomal protein levels gave 80% inhibition of cytochrome *c* reductase activity. Equivalent levels of control (nonimmune) rabbit sera protein served as controls for inhibition of nicotine metabolism by anticytochrome reductase antisera.

The in vitro nicotine metabolism by isolated midgut, Malpighian tubules, fat body, and nerve cords was also studied. Incubation conditions were 1 μM [^{14}C]nicotine in insect saline at 25°C for either 15 min (nerve cords) or 1 hr (midgut, Malpighian tubules, and fat body).

Behavioral Assays. One-day-old fifth-instar *M. sexta* larvae were fed on diets containing 0, 0.1%, or 0.75% nicotine. After three days of feeding on the diets, individual larvae were injected with 0.29 mg/g wet weight of nicotine dissolved in insect saline. The following parameters were monitored immediately after the injections: (1) the time interval following injection required for the insect to right itself three times in succession after placement on its dorsal surface each time, (2) the time until the larva resumed normal locomotory movement and feeding, and (3) any abnormalities in normal movement patterns. Statistical comparisons between groups were made using ANOVA and multiple comparison tests.

RESULTS

P-450 Enzyme Activities. One-day-old fifth-instar *M. sexta* larvae were placed on 0, 0.1%, or 0.75% nicotine diets for three days. Microsomes were then prepared and assayed for 12 enzyme activities representing structurally diverse substrates and different types of reactions catalyzed by P-450 enzymes

(Table 1). In addition, the metabolism of nicotine, NADPH cytochrome P-450 reductase activity (as cytochrome *c* reductase), and the total P-450 level were measured. The latter represents the sum of all P-450 proteins present in microsomes regardless of their activity, and significant changes in minor forms of P-450 are difficult to observe if major P-450 forms remain relatively unaffected. At the low concentration of dietary nicotine (0.1%), only three enzyme activities were significantly increased: *p*-chloro-*N*-methylaniline *N*-demethylation (1.4-fold), methoxyresorufin *O*-demethylation (1.4-fold), and benzo[*a*]pyrene hydroxylation (2.5-fold). At the higher nicotine concentration (0.75%), nine enzyme activities were induced. Two (*p*-chloro-*N*-methylaniline *N*-demethylation and methoxyresorufin *O*-demethylation) were induced to levels significantly higher than those reached at 0.1%. Nicotine metabolism was also induced at 0.75% dietary nicotine, and the total P-450 level and NADPH-cytochrome *c* reductase activity were increased 1.5-fold. Only aminopyrine *N*-demethylation, 6-methylthiopurine *S*-demethylation, and phorate sulfoxidation activities were unchanged by the addition of nicotine (either 0.1% or 0.75%) to the diet. Whereas aldrin epoxidation was increased the most (10-fold when compared to controls), the epoxidation of heptachlor was decreased significantly.

Nicotine Metabolism. We have previously shown that cotinine-*N*-oxide and one other metabolite (identified tentatively as nicotine-1-*N*-oxide) are the major in vivo metabolites produced by *M. sexta* larvae fed a diet containing nicotine (Snyder et al., 1993) (Figure 1). Study of the midgut microsomal metabolism by RP-HPLC indicated three different metabolites, two eluting prior to and one after nicotine (Figure 2). The metabolite eluting just before nicotine (8–8.5 min, Figure 2) coeluted with an authentic cotinine-*N*-oxide standard. Metabolite 1 is probably nicotine-1-*N*-oxide, but this has not been unambiguously demonstrated (Snyder et al., 1993). The metabolism of nicotine by midgut microsomes was doubled in 0.75% nicotine but was unaffected in 0.1% nicotine-fed larvae (Table 1). Cotinine-*N*-oxide accounted for most of the induced nicotine metabolism in microsomes from 0.75% nicotine-fed larvae. Metabolism of nicotine by fat body microsomes was barely detectable (less than 10% of the midgut values) and was not inducible by 0.75% nicotine in the diet (data not shown).

Similar RP-HPLC metabolite profiles were found with in vitro incubations of midgut or Malpighian tubules, although the amount of nicotine metabolism was very low in the tubules (data not shown). Nerve cords (target for the neurotoxic action of nicotine) were assayed for their ability to metabolize nicotine because Morris (1983) had shown conversion of nicotine by isolated nerve cords to polar products. We wanted to confirm Morris' results and determine whether this metabolism was inducible by dietary nicotine. Nerve cords from both control and 0.75% nicotine diet-fed larvae metabolized 12.3 and 13.9 nmol/min/cord, respectively (no significant difference), indicating a lack of nicotine induction. A single high polarity metabolite with an elution time similar to

TABLE 1. MIDGUT CYTOCHROME P-450 ACTIVITIES AFTER 72 hr OF DIETARY NICOTINE EXPOSURE

P-450 activity	Units[a]	Control	0.1% nicotine	0.75% nicotine
p-Cl-*N*-Me-aniline *N*-demethylation	1	12.6 ± 0.6[a]	17.9 ± 2.2[b]	23.7 ± 3.4[c]
p-Nitroanisole *O*-demethylation	2	0.9 ± 0.7[a]	1.5 ± 1.4[a]	4.5 ± 2.2[b]
Aminopyrine *N*-demethylation	1	2.5 ± 0.3[a]	2.4 ± 0.3[a]	2.4 ± 0.6[a]
Methoxyresorufin *O*-demethylation	2	6.3 + 0.2[a]	8.9 ± 1.0[b]	18.7 ± 2.3[c]
6-Methyl-thiopurine *S*-demethylation	1	0.7 ± 0.1[a]	n.d.	0.6 ± 0.1[a]
Benzo[a]pyrene hydroxylation	1	54.6 ± 20.1[a]	119.3 ± 52.8[b]	139.9 ± 52.3[b]
Biphenyl hydroxylation	1	0.3 ± 0.1[a]	0.5 ± 0.1[a]	1.4 ± 0.8[b]
7-Me O-coumarin *O*-demethylation	1	111.91 ± 38.57[a]	183.08 ± 41.42[a]	460.71 ± 154.91[b]
7-MeO-4-Me-coumarin *O*-demethylation	1	3.98 ± 1.41[a]	6.31 ± 2.17[a]	13.03 ± 4.19[b]
Aldrin epoxidation	2	31.5 ± 15.6[a]	49.6 ± 21.1[a]	295.8 ± 220.5[b]
Heptachlor epoxidation	2	19.7 ± 4.3[a]	n.d.	9.7 ± 3.5[b]
Phorate sulfoxidation	1	22.2 ± 5.6[a]	27.6 ± 11.1[a]	34.5 ± 13.7[a]
Nicotine metabolism	1	56.3 ± 11.5[a]	58.2 ± 11.9[a]	110.3 ± 29.5[b]
Total cytochrome P-450	3	58 ± 55[a]	72 ± 16[a]	137 ± 52[b]
NADPH-cytochrome *c* reductase	1	70.8 ± 4.5[a]	81.2 ± 7.4[a]	91.6 ± 9.0[b]

[a]The units of activity reported are 1: nmol of product formed × mg midgut microsomal protein^{-1} × min^{-1}; 2: pmol product formed × mg midgut microsomal protein^{-1} × min^{-1}; and 3: pmol × mg midgut microsomal protein^{-1} all with an n = 7–24 samples for each enzyme determination (means ±1 standard deviation). Statistically significant differences between treatment groups for each P450 activity are denoted by different superscript Roman letters.

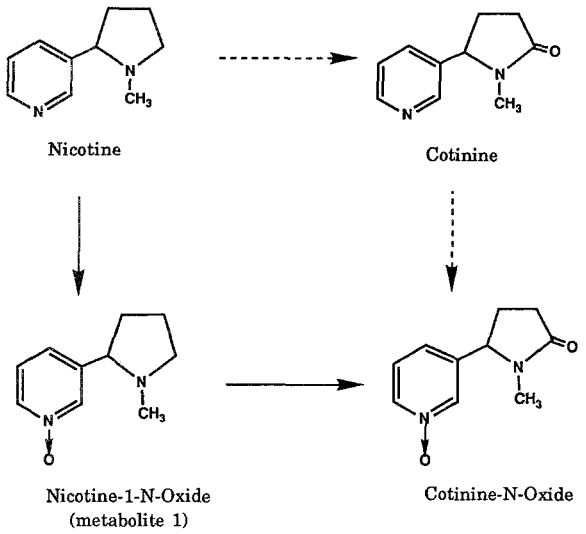

FIG. 1. Structures of nicotine, nicotine-1-*N*-oxide, cotinine, and cotinine-*N*-oxide.

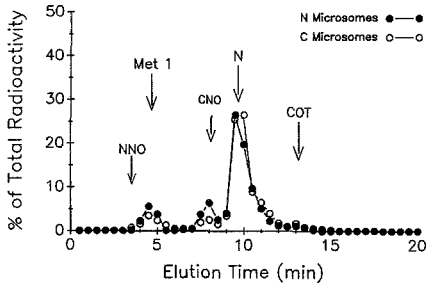

FIG. 2. RP-HPLC profile of [^{14}C]nicotine metabolites produced by control (open circles) and 0.75%-nicotine diet-fed (filled circles) *M. sexta* midgut microsomes. The elution times for nicotine metabolites are given by the following abbreviations: NNO, nicotine-1′-*N*-oxide; CNO, cotinine-*N*-oxide; N, nicotine; C, cotinine.

nicotine-1′-*N*-oxide, believed to be nicotine-1-*N*-oxide (Snyder et al., 1993) was the major product formed by nerve cords in vitro (Figure 3). Cotinine-*N*-oxide, the major microsomal nicotine metabolite in the midgut, was not produced in vitro by nerve cords from either control or nicotine-fed larvae.

Evidence for the role of cytochrome P-450 in the metabolism of nicotine by midgut microsomes was obtained with the help of inhibitors (Table 2). First, nicotine metabolism was shown to be NADPH-dependent because a significant reduction was observed in the absence of NADPH regenerating system. The

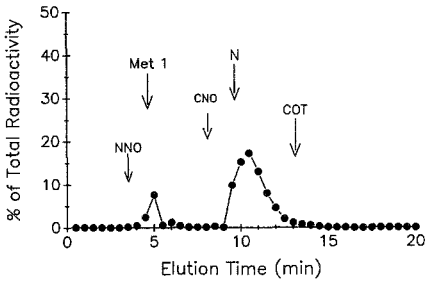

FIG. 3. RP-HPLC profile of [^{14}C]nicotine metabolites produced by 0.75%-nicotine diet-fed *M. sexta* nerve cords in vitro. See Figure 2 for abbreviations of the different nicotine metabolites.

TABLE 2. INHIBITION OF NICOTINE METABOLISM IN MIDGUT MICROSOMES FROM *M. sexta* FED CONTROL DIET OR DIET SUPPLEMENTED WITH 0.75% NICOTINE

Treatment[a]	Inhibition (%)[b]	
	Control Diet	Nicotine Diet
No NADPH	55.5 ± 36.1*	76.8 ± 10.8*
SKF525A	74.0 ± 4.4*	81.0 ± 6.1*
Piperonyl butoxide	49.9 ± 24.8*	67.1 ± 17.1*
Anti-reductase Ab	59.2 ± 10.1*	40.1 ± 25.0

[a] Cytochrome P-450 inhibitors SKF525A or piperonyl butoxide were added at 10^{-4} M in methyl-cellosolve (MC). Paired control microsomes received MC only. Anti-reductase antibody was incubated at 5× the microsomal protein concentration. Respective controls received 5× microsomal protein concentration of control rabbit sera.

[b] Data are reported as mean ±1 standard deviation of the percentage inhibition of [^{14}C]nicotine metabolism of the treated versus control portion of the same sample of microsomes (N = 3 for all samples). Numbers followed by the asterisk indicate a significant reduction by the treatment versus its respective paired control group. Microsomes were isolated from *M. sexta* fed on either control or 0.75% nicotine diets.

addition of typical P-450 inhibitors, SKF525A and piperonyl butoxide, also resulted in a marked inhibition of metabolism in microsomes from both control and nicotine-fed larvae. SKF525A was the most effective inhibitor at the concentration tested (0.1 mM). All these inhibitory treatments resulted in a lowered overall nicotine metabolism and did not specifically decreased the formation of cotinine-*N*-oxide or metabolite 1. When rabbit antibodies raised against house fly cytochrome P-450 reductase were added to control microsomes, nicotine metabolism was significantly reduced by 59%. Although addition of reductase

TABLE 3. EFFECTS OF NICOTINE FEEDING ON FIFTH-INSTAR LARVAE OF *M. sexta* CHALLENGED BY SUBSEQUENT NICOTINE INJECTION

Nicotine Conc. in diet (%)	*N*	Righting time (min)[a]	Time to normal (min)[b]	Twitching bouts[c]
0	10	35.1 ± 17.7	95.0 ± 46.5	6/10
0.1	10	47.4 ± 33.6	82.9 ± 32.0	3/10
0.75	10	13.5 ± 8.1*	43.5 ± 24.9*	1/10

[a] Righting time was defined as the period from injection of nicotine (0.29 mg/g) until each larvae was able to turn itself over upon being placed on its dorsal surface three times in succession. Data are the mean ±1 standard deviation, and the asterisk denotes significant difference from controls (0% dietary nicotine prior to injection).

[b] Time to normal was defined as the period from nicotine injection until larvae resumed normal searching and feeding. Data are the means ±1 standard deviation, and the asterisk denoted significant difference from controls.

[c] Refers to the number of larvae in each treatment group that displayed multiple bouts of constant twitching following nicotine injection.

antisera to nicotine-fed larval microsomes also appeared to inhibit subsequent nicotine metabolism, the reduction was not significant.

Behavioral Tests. The results of prior nicotine exposure fifth-instar *Manduca sexta* larvae in protecting against subsequent nicotine injection are shown in Table 3. Larvae fed with a 0.75% dosage of nicotine recovered more quickly from subsequent nicotine exposure than did the 0- or 0.1%-fed groups. Only feeding on the higher nicotine concentration significantly shortened both the time until larvae were able to right themselves and the return to normal behavior, as compared with controls. The lower dietary nicotine level (0.1%) had no effect on these variables. In addition, fewer nicotine-exposed larvae exhibited bouts of constant twitching (Table 3).

DISCUSSION

Ingestion of nicotine by *Manduca sexta* larvae resulted in an induction of midgut cytochrome P-450 enzyme activities against a variety of substrates. There were significant increases in *N*- and *O*-demethylations, hydroxylations, epoxidations, total cytochrome P-450 levels, and cytochrome P-450 reductase activities in midgut microsomes from larvae fed 0.75% nicotine. Many P-450 activities towards artificial substrates are induced by treatment of insects with single chemicals either topically or mixed into an artificial diet (Hodgson, 1985; Yu, 1986, for reviews). Nicotine also induces a variety of cytochrome P-450 activities in the tobacco budworm, *Heliothis virescens* (Rose et al., 1991).

The induction of detoxifying activity by nicotine is therefore expected.

Levels used in this study are within the normal range of nicotine concentrations in tobacco plants and are known to result in diminished efficiency of growth and survival of *M. sexta* (Parr and Thurston, 1972; Schoonhoven and Meerman, 1978; Bentz and Barbosa, 1990; Appel and Martin, 1992; Snyder et al., unpublished observations). With such an array of induced activities (11 of 13 different assays), it is probable that more than one P-450 gene is induced by nicotine. Additionally, only three of 13 activities were significantly induced by 0.1% dietary nicotine, suggesting that a subset of P-450 genes is differentially expressed at low doses. Thus, there seems to be a complex regulatory mechanism triggered by dietary nicotine, where some genes are expressed in response to low nicotine levels, more are expressed at higher levels, and some are repressed as well, e.g., the gene encoding the P-450 that catalyzes heptachlor epoxidation. It is not possible to determine how many P-450 genes are involved in this apparently pleiotropic effect of nicotine, but recent progress in cloning P-450 genes and mapping the catalytic capacity of their products should soon provide some answers. Densitometric scans of polyacrylamide gels indicated that microsomes from isosafrole-treated *Spodoptera litura* showed increased expression of two P-450 proteins (Reidy et al., 1987). Likewise, Cohen et al. (1990) showed, via immunochemical means, that at least two P-450 proteins were induced by xanthotoxin in *Papilio polyxenes*. Recently, it was also shown that mRNA levels for a specific P-450 (CYP6B1) increase in the midguts of several *Papilio* species in response to feeding on xanthotoxin-containing diets (Cohen et al., 1992). Relative staining intensities of seven different presumed P-450 bands (on SDS-PAGE) increased in susceptible house fly microsomes after feeding phenobarbital (Lee and Scott, 1989).

The levels of induction of enzymatic activity towards the different substrates were quite variable (1.4- to 10.0-fold, Table 1). At first glance, nicotine does not appear to be as effective as other inducers in other insects (Hodgson, 1985). Tate et al. (1982) have suggested that *M. sexta* midgut microsomal P-450 activities are not as readily inducible by foreign chemicals as in other insects. A smaller percentage change in specific activities may underestimate the true magnitude in the appearance and/or disappearance of specific P-450 proteins or in alterations of P-450 turnover rates (Yu, 1987; Lee and Scott, 1989). The percentage changes in P-450 activity in response to inducers are also difficult to ascertain since no artificial diet can be considered an absolute control. Most diets are based on plant components and therefore can be considered to contain inducers of xenobiotic metabolizing enzymes prior to the addition of any other compounds of interest. This can be shown in some adult cockroaches where short-term starvation causes a reversible depression in P-450 levels without an effect on reproduction (Feyereisen and Farnsworth, 1985). In rodents, it is well known that apparently innocuous components of the environment (e.g., cage bedding material) can influence drug-metabolizing activities (Vesell, 1967).

Therefore, care must be taken in the interpretation of the magnitude of inducibility of particular enzyme activities in response to an inducer chemical.

Three lines of evidence support the involvement of microsomal cytochrome P-450 in nicotine metabolism leading to the production of cotinine-*N*-oxide by *M. sexta*. The first is that NADPH was required for nicotine metabolism by microsomes (Table 2). Secondly, cytochrome P-450 inhibitors significantly reduced microsomal nicotine metabolism. The requirement for cytochrome P-450 reductase activity for microsomal nicotine metabolism was also demonstrated by the addition of anti-reductase antiserum. Although we cannot exclude the potential involvement of flavin-containing monooxygenases (FMO) in nicotine metabolism by *M. sexta*, these enzymes have not been detected in insects. In studies of mammalian nicotine metabolism, oxidation of either the pyridyl or pyrrolidine nitrogens to form nicotine-1-*N*-oxide or nicotine-1′-*N*-oxide can involve P-450 or FMO depending on the species. However, the oxidation of the pyrrolidine ring to form cotinine is specific to cytochrome P-450s (Kyerematen and Vesell, 1991).

Metabolism of nicotine was also induced by 0.75% dietary nicotine in midgut but not in fat body microsomes. Brattsten et al. (1977) have reported that prior feeding with α-pinene confers resistance to subsequent dietary nicotine in southern armyworm larvae. Feeding piperonyl butoxide significantly increased mortality to subsequent nicotine exposure, and this evidence supported the involvement of cytochrome P-450 enzymes in nicotine detoxification in this species. These results support the idea that the lepidopteran midgut is a primary detoxification organ (Hodgson, 1985). However, Tate et al. (1982) showed that total P-450 and several monooxygenase activities were significantly induced in fat body by phenobarbital and 3-methylcholanthrene.

The seeming failure of *M. sexta* to metabolize nicotine (Self et al., 1964) and the lack of evidence for nicotine induction of P-450 activities were some of Gould's (1984) major arguments against a role of P-450 metabolism in the survival of polyphagous herbivores. In insects, α-pinene, xanthotoxin, and indole-3-carbinol are known to induce their own metabolism by midgut P-450 (Brattsten, 1983; Ivie et al., 1983; Yu, 1987; Cohen et al., 1989; Nitao, 1989). In addition, xenobiotics such as phorate can induce their own metabolism by midgut microsomal preparations (Yu, 1986). It is likely that other plant secondary compounds induce their own metabolism in herbivores that consume them. Our in vivo data (Snyder et al., 1993) and the in vitro data presented here place nicotine and the oligophagous *M. sexta* in a teleologically pleasing pattern where the potential toxicant from the host plant is an inducer of its own metabolism in the herbivore.

The potential protective effects of cytochrome P-450 induction to the individual insect was also demonstrated. Larvae fed a 0.75% nicotine-containing diet recovered significantly faster from subsequent nicotine injection (Table 3).

It was previously demonstrated that *M. sexta* larvae fed nicotine exhibited significantly induced metabolism of an injected dose of nicotine to cotinine-*N*-oxide (Snyder et al., 1993). Induction of midgut detoxification mechanisms by allelochemical exposure may be an important mechanism of adaptation by herbivores such as *M. sexta* and *Papilio polyxenes* (Cohen et al., 1992).

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DEVELOPMENTAL INHIBITION OF *Spodoptera litura* (FAB.) LARVAE BY A NOVEL CAFFEOYLQUINIC ACID FROM THE WILD GROUNDNUT, *Arachis paraguariensis* (CHOD et HASSL.)

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Abstract—A novel compound, 1-caffeoyl-4-deoxyquinic acid (1-CdQA) has been identified along with 3-caffeoylquinic acid (3-CQA) and 5-caffeoylquinic acid (5-CQA) (syn. chlorogenic acid) in the foliage of *Arachis paraguariensis*, a wild species of groundnut that is highly resistant to attack by the larvae of *Spodoptera litura*. When neonate larvae were fed on diets treated with 3-CQA or 1-CdQA, their development was severely inhibited compared to larvae on untreated diets, and the effects were similar in nature and magnitude to those observed for larvae feeding on diets treated with 5-CQA, rutin, and quercetin. The effects of all the compounds were dose related, and their optimal concentration was approximately 3 mM, which corresponds to the total foliar concentration of both caffeoylquinic acids and quercetin diglycosides in *A. paraguariensis*. After 24 h, the development of third stadium larvae feeding on diets treated with 5-CQA, rutin and 1-CdQA was promoted compared to larvae on control diets, but after 96 hr larvae feeding on treated diets had gained significantly less weight than those on the control diets. 1-CdQA, 3-CQA, and quercetin dyglycosides (previously identified in the foliage of *A. paraguariensis*) are considered to be valuable components in resistance of

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groundnuts to *S. litura* and may provide useful genetic markers in future breeding for resistance to this pest.

Key Words—*Spodoptera litura*, Lepidoptera, Noctuidae, *Arachis paraguariensis*, wild groundnuts, caffeoylquinic acids, resistance, rutin, quercetin, phenolics, growth inhibition, *Arachis hypogaea*.

INTRODUCTION

Spodoptera litura Fabricius (Lepidoptera: Noctuidae), the tobacco armyworm, is a swarming, polyphagous, foliage-feeding insect that is distributed throughout South and East Asia and eastern Australia (Feakin, 1973). Since 1980 *S. litura* has become an increasingly important pest of groundnuts (*Arachis hypogaea* L.) in India (Amin and Mohammad, 1980; Wightman and Amin, 1988).

High yield losses in groundnuts have been directly associated with high larval density (Panchabhavi and Nethradani Raj, 1987) and consequent high levels of defoliation by *S. litura* (Wightman et al., 1990). The development of varieties of groundnut with resistance to *S. litura* has been identified as an essential component of current and future integrated pest management programmes in India (Wightman and Amin, 1988). Subsequently, low levels of resistance to the larvae of *S. litura* have been reported in some genotypes of *A. hypogaea* (Wightman et al., 1990) but some wild species of *Arachis* have been reported to be highly resistant to attack (Stevenson, 1992, Stevenson et al., 1993)

The identification of factors in the foliage of wild species of *Arachis* that affect the feeding behavior or development of *S. litura* may provide plant breeders and biotechnologists with valuable information for the development of cultivars of *Arachis* with resistance to *S. litura*. A previous study reported that the methanol extract of the foliage of three wild species of *Arachis* (*A. paraguariensis*, *A. appresipila*, and *A. chacoensis*) significantly inhibited larval development (Stevenson et al., 1993). Phytochemicals in the methanol extract were concluded as being responsible, at least in part, for the observed field resistance of these species to the larvae of *S. litura*.

Chlorogenic acid [synonymous with 5-caffeoylquinic acid after IUPAC (1976)] (5-CQA) and rutin have been shown by many authors to inhibit the development of neonate lepidopterous larvae and their potential role in the natural resistance of crops has been proposed (Elliger et al., 1980, 1981; Isman and Duffey, 1982; Wiseman, et al., 1990).

Foliar phenolic acid esters isolated from one of these resistant species of groundnut, *Arachis paraguariensis* (Chod et Hassl.), were shown to be absent from or at very much lower concentrations in susceptible cultivated and wild species of groundnuts (*Arachis hypogaea* (Stevenson, 1992, 1993). In the work

described here, these phenolic acid esters were isolated, identified, and subsequently bioassayed on the larvae of *S. litura*. The observed effects are discussed, and their potential role in the resistance of cultivated groundnuts to this species of Lepidoptera is considered.

METHODS AND MATERIALS

Extraction of Plant Material. Foliage of field plants of *Arachis paraguariensis* was collected as previously described (Stevenson et al., 1993). Dried, milled leaves (100 g) were extracted twice into 500 ml of 80% methanol for 12 hr. The extract was filtered under vacuum through Whatman grade 1 paper and the residue was reextracted in 500 ml of water and filtered. The filtrates were combined and evaporated to approximately 100 ml with a rotary evaporator under water pump vacuum. The concentrated aqueous extract was then filtered to remove components that were soluble in 80% methanol but that had subsequently precipitated out of the extract. The remaining water-soluble extract was evaporated on a rotary evaporator to 2 g of dry plant material extracted per 1 ml of water.

Paper Chromatography of Foliar Extract. A 3-μl aliquot of the aqueous extract was applied to a two-dimensional paper chromatogram (20 × 20 cm) and was developed initially in butanol–acetic acid–water (3:1:1) (BAW) and then in 15% acetic acid (aq. HOAc). A spot, previously shown to be absent from the foliage of *A. hypogaea* (Stevenson, 1992, 1993), was cut out and eluted from the paper in methanol. The UV spectrum of the eluted spot was recorded in absolute methanol on a Shimadzu 2000 spectrophotometer.

HPLC Analysis of Isolate from Paper Chromatogram. The eluent from the paper chromatogram was analyzed by HPLC using a Merck-Hitachi Intelligent Pump and Waters 991 photodiode array detector by injection directly onto an analytical HPLC column (4.6 mm ID × 25 cm) packed with Spherisorb 5 reverse-phase C8 silica. Compounds were eluted with an isocratic solvent delivery of 15% methanol in 5% aqueous acetic acid at 2 ml/min adapted from the system employed by Lattanzio (1982).

Quantification of Peaks. The concentration of each compound in the foliage was calculated by comparison of their peak area from the HPLC trace with that of a chlorogenic acid standard (5-CQA) (5-caffeoylquinic acid after IUPAC, 1976).

Isolation of Compounds. A flash column (0.7 cm ID × 20 cm) (BioRad) was packed with 2 g of dry Bondesil Reverse Phase C_8 silica (40–60μm) (Analytichem International) such that the packed column had a solvent volume of approximately 3 ml. The column was washed with three column volumes of methanol and three column volumes of water. The whole extract was fraction-

ated in 1-ml aliquots by flash chromatography eluting with: 3 × 3 ml water, 1 × 3 ml of 5% methanol, 4 × 3 ml 10% methanol, and 3 × 3 ml 15% methanol. The eluent was collected in 3-ml fractions, and the composition of each fraction was determined by HPLC analysis as described above. By this method it was possible to isolate crudely two compounds, S1 and S2. Fractions containing compounds S1 and S2 were further purified by HPLC using a semipreparative column (10 mm ID × 25 cm) packed with LiChrosorb RP 8 using gradient elution at 7 ml/min as follows: 0–10 min, 5–10% methanol (MeOH) in 5% acetic acid (HOAc); 10–13 min, 10–13% MeOH in 5% HOAc; 13–15 min, 13–5% MeOH in 5% HOAc. Peaks were collected on a Gilson 203 Micro Fraction collector.

NMR Spectral Determination of Isolated Components. A 10-mg aliquot of each of two unknown components from the methanol extract of *A. paraguariensis* was freeze-dried for spectral analysis. Sample were dissolved in deuterated methanol and ^{1}H NMR spectra were recorded on a Bruker AM-500 spectrometer at 500 MHz. The spectra of the unknown compounds were compared to that of 5-CQA (Sigma Chemical Company).

Fast Atom Bombardment Mass Spectroscopy. Mass spectra were recorded on VG Autospec Mass Spectrometer with static fast atom bombardment from a cesium ion gun at 25 kV. The sample was dissolved in a minimum of methanol and applied to a 3-nitrobenzylalcohol matrix prior to analysis.

Insects. Field-collected pupae were incubated at 26 ± 2°C. Adults were allowed to emerge in wire cages (50 × 50 × 50 cm) containing groundnuts (*A. hypogaea* cv. TMV2) on which the moths oviposited. Egg masses were collected and the larvae were allowed to emerge onto an artificial diet based on chickpea flour and sorghum leaf (Taneja and Leuschner, 1985) and were maintained under a 12:12 hr light–dark regime.

Selection of Larvae for Bioassays. Bioassays were conducted on larvae of known age and known feeding history. Neonate (prefeeding) larvae were selected when less than 6 hr old and third stadium larvae were selected between 6 and 12 hr after ecdysis.

Effect of 5-CQA, Rutin, and Quercetin on Development of Neonate Larvae: Diets: 5-CQA and rutin (3,3′,4′,5,7-pentahydroxy-flavone-3-rhamnogalactoside) were incorporated into artificial diets (Taneja and Leuschner, 1985) at 0.025, 0.05, 0.10, and 0.20% (5-CQA only) and 0.25% (rutin only). Quercetin (3,3′,4′,5,7-pentahydroxy-flavone) was incorporated at the same concentration as rutin but with one extra test diet containing 0.0125% quercetin. The highest concentrations of 5-CQA and rutin used in diets represented the natural foliar concentration of caffeoylquinic acids in the leaves *A. paraguariensis*. Because of comparatively poor solubility in water, quercetin and rutin were dissolved in approximately 10 ml of methanol, which was then poured on to and mixed into the dry ingredients of the diet to ensure an even distribution of the compounds

in the diets. The methanol was allowed to evaporate for 24 h before incorporation into the test diet. 5-CQA was added to the diet dissolved in the water–agar component. Two control diets were used: an untreated artificial diet and a solvent control (treated with 10 ml of methanol only).

Two more diets were made up in which rutin and 5-CQA were incorporated together at 0.1% and 0.2% of each compound. The concentration of the second diet represents the approximate total concentration of quercetin glycoside (Stevenson, 1993) and caffeic acid ester (Table 1) in the foliage of *A. paraguariensis*. The new compounds isolated in this study, S1 and S2, were incorporated individually into diets at a concentration equivalent to that observed for these compounds in the foliage by the method described above for 5-CQA.

Bioassays with Neonate Larvae. Ten neonate larvae were placed onto 15 ml of treated or control diet in 25-ml plastic cups and each treatment was replicated 10 times so that a total of 100 insects were fed on each diet. The weights of larvae in each of the 10 replicates were pooled. In the first bioassay, testing the effects of quercetin, rutin, and 5-CQA on larval development, larvae were weighted after seven days. This bioassay was performed to establish the likely effect at specified concentrations of the caffeoylquinic acids and quercetin glycosides found in the foliage of *A. paraguariensis* on the feeding and development of the larvae.

In the second bioassay on neonate larvae, the effects of S1 and S2 in comparison to the effects of 5-CQA and rutin were studied in more detail. larval weight, larval stage attained and larval mortality were evaluated as a mean value of the larvae on each replicated diet for each treatment or control on each of days 3, 6, 9, and 14 after starting the experiment. Thus, the results table shows the mean values of the pooled means from each of the ten individual replications. Mean larval stage attained was evaluated according to the system of Lynch et al. (1981) where stages 1–6 were equivalent to instars 1–6, stage 7 was equivalent to prepupal stage, and stage 8 equivalent to pupal stage.

Bioassays with Third Stadium Larvae. Third stadium larvae were weighed

TABLE 1. HPLC ANALYSIS OF THREE COMPONENTS OF AQUEOUS FOLIAR EXTRACT OF *A. paraguariensis* RELATIVE TO 5-CAFFEOYLQUINIC ACID STANDARD

Peak No./compound	R_t (min)	% wt[a]
Peak 1 (S1)	3.31	0.094
Peak 2 (S2)	4.98	0.108
Peak 3 (5-CQA)	6.70	0.018
5-CQA (standard)	6.63	0.100

[a] % wt = percentage weight of an extract of 1 g of leaf/ml solvent.

and placed singly into 25-ml plastic cups with cylinders of treated diet made up as described above. Pieces of diet were cut with a 2.1-cm-diameter cork borer. Larval weight gain was recorded after 24, 48, and 96 hr. Each treatment was replicated 10 times; thus the values for weight gain on each treatment or control are the means of 10 larvae.

Bioassays with Sixth Stadium Larvae. The effect of isolated compounds on the food choice by larvae was tested on sixth stadium larvae. Glass fiber disks (GFDs) (Whatman GF/A 2.1 cm) were treated with 100 μl of 0.1 M sucrose to make them palatable (Blaney et al., 1990). Control disks were impregnated with sucrose only, whereas the test disks were impregnated with an additional 100 μl of the compound being tested.

After the disks had been treated, they were oven dried at 100°C and weighed. The larvae were placed individually in Petri dishes with a control disk and a treatment disk, either for 8 hr or until the larva had consumed at least 50% of one of the disks, whichever was the sooner; each bioassay was replicated 10 times for each treatment as described for third stadium larvae. After the bioassay was complete, the disks were dried again and reweighed, and the data were used to calculate a feeding index: $[(C - T)/(C + T)\%$ where C represented the weight of the control disk eaten and T represented the weight of the treatment disk eaten. The feeding index distinguished a phagostimulant, which had a negative value, from a feeding deterrent, which had a positive value (Simmonds et al., 1990). Compounds were tested at their approximate foliar concentration.

RESULTS

Paper Chromatography. The spot that had been isolated from paper chromatograms appeared bright blue under long-wave UV light (354 nm) and changed to yellow–green after fuming in ammonia. The spot had R_f value of 0.5 and 0.67 in BAW and 15% acetic acid, respectively, and the UV spectrum of the eluted spot gave an absorbance maximum of 325 nm with a shoulder at 300 nm. These color characteristics and spectral values were very similar, although not identical, to those of the 5-CQA standard.

HPLC Analysis of Aqueous Foliar Extract. HPLC analysis showed that the spot that had been eluted from the paper chromatogram consisted of two major peaks and one minor peak. The minor peak was shown to be 5-CQA by comparison of retention time (6.70 min) (Table 1) and UV spectral data (λ_{max} 325 nm with shoulder at 300 nm) with the standard. The two major peaks were described as S1 with retention time 3.31 min and S2 with retention time 4.98 min. The spectral and chromatographic characteristics of S1 and S2 were similar to those of 5-CQA, which indicated that they might have related structures.

Structural Determination of Unknown Compounds in Foliar Extract by ^1H

NMR Spectroscopy and Fast Atom Bombardment Mass Spectroscopy. All of the spectral data for S1, S2, and 5-CQA are presented in Table 2. These data exclude signals for carboxy and hydroxyl groups, which were observed with single peaks and characteristic low field resonance at δ10.6 and 9.0–9.7, respectively. Although the structure of 5-CQA is well known and has been fully characterized (Waiss et al., 1964; Morishita et al., 1984) the proton spectral data recorded for this compound are included to make the interpretation of the data from the spectra of the other compounds more clear. In addition, previous interpretations of data (e.g., Butt and Lamb, 1981; Barz and Koster, 1981) have been based on the structural configuration used by Waiss et al. (1964), in which the esterified carbon of the quinic moiety of chlorogenic acid is designated as C-3′, i.e., 3-caffeoylquinic acid. Under current IUPAC recommendations (IUPAC, 1976), however, chlorogenic acid is 5-caffeoylquinic acid (Figure 1A).

From a direct comparison with the spectra of 5-CQA, it is clear that both S1 and S2 contain the caffeoyl moiety (Table 2).

In the case of 5-CQA, the low field H-5′ proton (δ5.32) α to the ester is a triple doublet (J = 9.0,4.3 Hz). The large triplet coupling constant (9.0 Hz) is indicative of two vicinal protons in an axial–axial configuration. For S1, however, the multiplicity of the proton α to the ester (δ5.39) was a double double doublet ($J \approx 3 \times 4$ Hz), which is characteristic of three equatorial–equatorial and/or axial–equatorial configurations. There were also two other protons α to oxygen atoms (δ3.71–3.80 and 3.97), which were presumably α to hydroxyls by virtue of their chemical shifts and by comparison with the NMR spectrum of 5-CQA. If the absolute and relative configurations of the stereocenters in S1 are assumed to be the same as those in 5-CQA, then the multiplicity of H-3′ in S1 can be interpreted from the structure in Figure 1B. In this rep-

TABLE 2. ^{1}H NMR CHEMICAL SHIFTS AND MULTIPLICITIES OF S1, S2, AND 5-CQA (δPPM; 500Mhz; CD_3OD)

S1	1.88–2.08(m,4H,2 × H-2′,2 × H-6′), 3.71–3.80(m,1H,H-5′), 3.97(dd,J = 10.4,5.5 Hz,1H,H-4′), 5.39(br ddd, J = 3 × 4Hz,1H,H-3′), 6.30(d,J = 15.9Hz,1H,H-8), 6.76(d,J = 8.2Hz,1H,H-5), 6.94(dd,J = 8.2,2.0Hz,1H,H-6), 7.04(d,J = 2.0Hz,1H,H-2), 7.57(d,J = 15.9Hz, 1H,H-7)
S2	1.97–2.15(m,6H,2 × H-2′, 2 × H-4′,2 × H-6′), 4.19–4.20(m,1H,H-3′ or 5′), 4.28(br s,1H,H-3′ or H-5′), 6.36(d,J = 16.0Hz,1H,H-8), 6.77(d,J = 8.2Hz,1H,H-5), 6.96(1H,dd,J = 8.2,2.1 Hz, H-6), 7.06(1H,d,J = 2.0 Hz,H-2), 7.62(1H,d,J = 15.9Hz, H-7)
5-CQA	2.01–2.24 (4H,m,2 × H-2′,2 × H-6′), 3.72(1H,dd, J = 8.5,3.2,H-4′), 4.16(1H,dt,J = 5.3,3.2,H-3′), 5.32(1H,td,J = 9.0,4.3,H-5′), 6.25(1H,d,J = 15.9Hz,H-8), 6.77(1H,d,J = 8.2Hz,H-5), 6.95(1H,dd,J = 8.2,2.1,H-6), 7.04(1H,d,J = 2.1Hz,H-2), 7.55(1H,d,J = 15.9Hz,H-7)

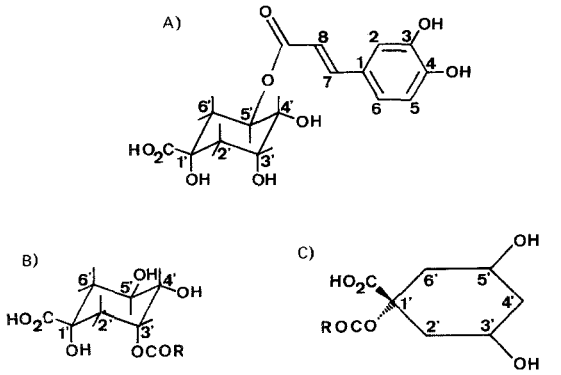

FIG. 1. Structural features of the quinic moeity of caffeoylquinic acids illustrating ^1H NMR interpretations. (see text for explanations of A, B, and C).

resentation H-3′ should have three similar coupling constants of 3–6 Hz (Haasnoot et al., 1980). The signals produced by H-5′ and H-4′ in S1 appear to be broadened compared to H-5′ and H-4′ in 5-CQA. This could be due to some conformational fluxionality of the cyclitol due to the bulky ester function not wanting to adopt a sterically congested axial configuration. Thus, we tentatively assign S1 to 3-caffeoylquinic acid (3-CQA).

In the case of S2, the ester is present but there is no characteristic low field resonance indicative of a methine proton next to an ester. This indicates that the ester is attached to a quaternary center, i.e., at the C-1′ of the quinic acid moiety. There were only two signals from protons α to hydroxyl groups (δ4.19–4.20 and δ4.28) and, furthermore, the integral of the high field methylene protons (δ1.97–2.15) revealed six signals, so presumably S2 is a deoxy derivative of 1-caffeoylquinic acid. In ^1H decoupling experiments, irradiation of the protons α to hydroxyls affected the multiplicities of four of the methylene proton signals but not each other. Thus, the two hydroxyl groups are not adjacent and S2 is therefore proposed to be 1-caffeoyl-4-deoxyquinic acid (1-CdQA) with the part structure shown in Figure 1C. The configuration of the hydroxy groups at C-3′ and C-5′, however, remains unknown.

The largest ion recorded from the fast atom bombardment of 3-CQA and 5-CQA gave major signals at *m/z* 355 (M+H) with daughter ions at *m/z* 307 and *m/z* 289. The value for the major signal corresponded to the molecular weight of both 3-CQA and 5-CQA. The largest ion of S2, however, gave a major signal at *m/z* 339 (M+H) supporting the NMR interpretation that this compound is a deoxy derivative of 5-CQA.

Effect of Rutin, Quercetin, and 5-CQA on Weight Gain of Neonate Larvae. Figure 2 shows that the inhibition of larval growth by rutin, quercetin, and

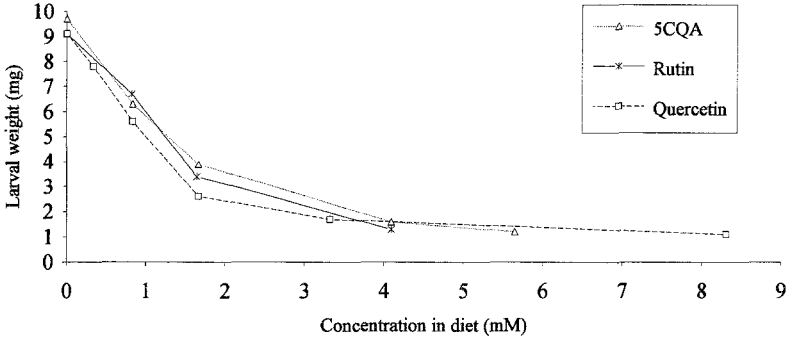

FIG. 2. *S. litura* larval weight after 7 days on diets incorporating 5-CQA, rutin, and quercetin.

5-CQA was dose-related. At concentrations up to 3 mM, the larval response to 5-CQA, rutin, and quercetin resembled that of lepidopteran larvae reported by other workers (Isman and Duffey, 1982; Duffey and Bloem, 1986). However, the response curve leveled off after approximately 3 mM for each compound, which suggested that they had an optimal effect at the natural foliar concentration of 5-CQA, 1-CdQA, and quercetin diglycosides.

Figure 2 also shows that when larvae consume diets containing rutin and quercetin at up to 2.5 mM the inhibitory effect of these compounds was greater than that of 5-CQA, although above this concentration there was no significant (Mann-Whitney; $P < 0.05$) difference in effect of any of the compounds. Quercetin did not inhibit larval development significantly more than rutin.

Effect of 5-CQA, 3-CQA, and 1-CdQA on Development and Mortality of Neonate Larvae. The development of neonate larvae that had fed on diets treated with 3-CQA (S1) and 1-CdQA (S2) was severely impeded over a 14-day period compared to that of larvae feeding on control diets (Table 3). Whereas larvae on control diets were entering the prepupal stage and had a mean weight of almost 1 g, the majority of larvae on diets treated with 3-CQA (S1) and 1-CdQA (S2) were still second instars and their mean weight was less than 5 mg.

The effect of both 5-CQA and rutin on larval development was similar to that observed with 3-CQA and 1-CdQA (Table 3). The potency of 3-CQA and 1-CdQA to inhibit larval development, however, appeared to be slightly greater than that of 5-CQA and rutin because values for larval weight and mean stadia attained after 14 days were significantly [Duncan's multiple range test (*Dmrt*); $P < 0.05$)] greater on 5-CQA and rutin than on either 1-CdQA or 3-CQA when the compounds were presented to larvae at similar concentrations. Furthermore, the inhibitory effect of 5-CQA at 0.2% was not significantly different to that of either 3-CQA or 1-CdQA at 0.091% and 0.11%, respectively.

With the exception of larvae on diet treated with 1-CdQA, the mortality of test larvae was not significantly different than that of larvae on the control diet. It is not known why the mortality of larvae feeding on 1-CdQA treated diets was so low.

Figure 3 shows the growth rates over 14 days of larvae on 3-CQA, 1-CdQA, and different concentrations of 5-CQA. From day 0 to day 3 growth rates were similar. The subsequent rate of growth was much greater for larvae

TABLE 3. EFFECT OF 1-CdQA, 3-CQA, 5-CQA, AND RUTIN ON DEVELOPMENT AND MORTALITY OF NEONATE LARVAE AFTER 14 DAYS ON TREATED ARTIFICIAL DIETS[a]

Treatment, Conc. in diet (% wt) (mM)	Larval weight (mg)	Mean larval instar	Mortality (% ± SEM)
Control	912.7a	6.3a	22 ± 3.8
3-CQA 0.09% 2.54	4.1f	2.2e	14 ± 4.3
1-CdQA 0.11% 2.95	2.9f	2.1e	4 ± 2.7
5-CQA			
0.05% 1.41	84.7c	3.0bc	20 ± 5.2
0.10% 2.82	24.7d	2.6cd	24 ± 8.1
0.20% 5.65	2.9f	2.2e	21 ± 8.5
Rutin			
0.05% 0.82	185.3b	3.5b	18 ± 3.1
0.10% 1.64	19.4d	2.6d	26 ± 6.7
0.25% 4.09	8.7e	2.2d	24 ± 7.3

[a] Values in the same column with different letters differ significantly ($P < 0.05$; Duncan's multiple range test).

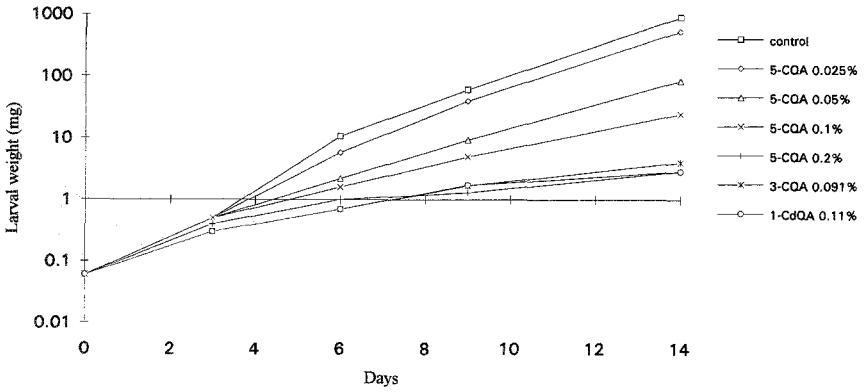

FIG. 3. Growth curves of larvae on diets treated with 3-CQA 1-CdQA, and different concentrations of 5-CQA from days 0–14.

on control diets compared with those on diets treated with 5-CQA, 3-CQA, and 1-CdQA.

Weight Gain of Third Stadium Larvae on Artificial Diets Incorporating 1-CdQA, 5-CQA, and Rutin. After 24 hr the weight gain of larvae feeding on diets containing 5-CQA and 1-CdQA was significantly [Mann-Whitney (M-W), $P < 0.05$] higher than that of larvae on control diets. The weight of larvae on diets incorporating rutin after 24 hr was also greater than that of larvae on control diets, but this difference was not significant (M-W; $P > 0.05$). These results indicate that the compounds tested were feeding stimulants or growth promoters for third stadium larvae. The stimulant effect was short lived, however, and after 96 hr the weights of larvae feeding on all of the treated diets were significantly (M-W; $P < 0.05$) lower than on the control diets. Furthermore, the inhibitory effect of these compounds on third stadium larvae was associated with the concentration of the compounds in the diets and thus reflected the effects of the same compounds on neonate larvae. For example, the weight gain of larvae on diets treated with 0.1% of both rutin and 5-CQA was significantly greater than that recorded for larvae on diets treated with the same compounds at 0.2% of each (Table 4).

Although the previous bioassays (Table 3) showed that the susceptibility of neonate larvae to 1-CdQA at 0.11% and 5-CQA at 0.2% were not significantly different, when the same compounds were presented to third stadium larvae at the same concentrations (0.1%), 5-CQA inhibited larval growth significantly more than 1-CdQA. The most severe inhibition of third stadium larval

TABLE 4. WEIGHT GAIN OF THIRD STADIUM LARVAE FEEDING ON ARTIFICIAL DIETS INCORPORATING 5-CQA, 1-CdQA, AND RUTIN AFTER 24, 48 AND 96 HOURS

Treatment	Weight gain (% ± SEM)		
	24 hr	48 hr	96 hr
Control	64 (10.5)	159 (22.3)	573 (66.1)
1-CdQA 0.11%	90 (10.5)[a]	140 (16.3)	435 (57.7)[b]
5-CQA 0.20%	91 (11.4)[a]	155 (19.4)	336 (36.9)[b]
Rutin 0.20%	78 (12.9)	146 (15.7)	400 (37.1)[b]
5-CQA and rutin			
Each at 0.1%	84 (13.5)	153 (16.4)	357 (55.8)[b]
Each at 0.2%	78 (9.7)	143 (14.3)	275 (30.5)[b]

[a] Larval weight gain is significantly greater on treated diets than on the control diet (M − W; $P < 0.05$).

[b] Larval weight gain is significantly lower on corresponding diets than on the control diet (M − W; $P < 0.05$).

growth was recorded for larvae feeding on diets incorporating both 5-CQA and rutin, each at 0.2% (Table 4).

Effect of 5-Caffeoylquinic Acid and Rutin on Feeding Behavior of Sixth Stadium Larvae. The feeding indices in Table 5 show that sixth stadium larvae preferred the control disks to those disks treated with 5-CQA, suggesting that this compound is a mild feeding deterrent for final stadium larvae. Disks treated with rutin, however, were preferred to the control disks, suggesting that rutin is a feeding stimulant for the larvae in the sixth stadium. This stimulant effect was eliminated when rutin was combined with 5-CQA at both 0.1% and 0.3% of each compound.

DISCUSSION

Chemical Analysis. The spectral shifts of 5-CQA compared well with those of Morishita et al. (1984). By comparison we tentatively assign the structures of 3-CQA and 1-CdQA. In addition, the FAB mass spectrum showed these compounds to have their predicted molecular weight, and the UV spectra of both 5-CQA and 3-CQA compared well with those reported by Bailey et al. (1990).

This is the first report of 1-caffeoyl-4-deoxyquinic acid either from nature or as a synthesized product and the first report of 5-CQA and 3-CQA together in *Arachis* species. Although esterification at C-5 (e.g., chlorogenic acid) is the most common in caffeoylquinic acid esters and glycosides, 3-*O* and 4-*O* substitutions are not uncommon in the plant kingdom and are known to occur together in many plant groups (Molgaard and Ravn, 1988). In contrast, 1-*O* substitution is less common, and there are few phytochemical surveys of caffeoylquinic acids that report the presence of 1-*O*-substituted derivatives. Litvinenko et al. (1975) published a survey of cinnamic acid derivatives in the Labiatae and found 3-CQA, 4-CQA, and 5-CQA in all but a few of the 55 genera analyzed, but comparatively few of the plants studied were observed to

TABLE 5. EFFECT OF 5-CAFFEOYLQUINIC ACID AND RUTIN ON FEEDING BEHAVIOR OF SIXTH STADIUM LARVAE

Test compounds	Feeding index (± SEM)
5-CQA, 0.2%	23.8 (13.36)
Rutin, 0.2%	−49.2 (20.32)
5-CQA and rutin:	
Each at 0.1%	9.7 (24.41)
Each at 0.3%	18.2 (26.96)

contain 1-*O* caffeoylquinic acid. Thus, the presence of 1-*O* substitution in *A. paraguariensis* may be useful as a taxonomic marker for this species of *Arachis*.

Effect of Caffeoylquinic Acids and Rutin on Development of Neonate Larvae. The development of neonate larvae was severely impaired when they were allowed to feed on artificial diets incorporating 5-CQA, 3-CQA, and 1-CdQA at their natural foliar concentrations. Thus, it is likely that these compounds are, at least in part, responsible for the field and laboratory resistance of *A. paraguariensis* to *S. litura* reported previously (Stevenson et al., 1993).

The opinion that phenolic compounds such as 5-CQA and rutin are important contributors to the natural resistance of plants to different species of insect is well established (Elliger et al., 1980; Isman and Duffey, 1982; Wiseman et al., 1990), although this is not always the case. For example, Cole (1985) showed that the incidence of carrot root fly larval damage increased with increasing concentrations of 5-CQA in carrot roots. There are few studies, however, that investigate the biological effects of caffeoylquinic acids other than 5-CQA on lepidopterous larvae. An example of one of these studies (Kato and Yamada, 1963) showed that all the known caffeoylquinic acids (1-*O*, 3-*O*, 4-*O*, and 5-*O*) are essential ingredients of the artificial diet of the silk worm (*Bombyx mori*). In the present study the nature and magnitude of the biological effects of 3-CQA and 1-CdQA were similar to 5-CQA, thus, it is appropriate to discuss the results of this study in light of the numerous previous studies that have only investigated the effects of 5-CQA. The similarity in effect with 5-CQA, 3-CQA, and 1-CdQA also indicates that the inhibitory properties of caffeoylquinic acids towards the larvae of *S. litura* may not depend upon the position of substitution on the quinic moiety, but on another common structural feature, e.g., the dihydroxy group on the caffeic acid moiety. This would agree with the conclusions drawn by Duffey and Felton (1991) about the actual mechanism of developmental inhibition of caffeoylquinic acids and rutin.

The level of developmental inhibition by 5-CQA, 3-CQA, and 1-CdQA was dependent upon the dose, and up to a concentration of 2 mM the increase in effect was linear. This linear dose effect began to level out when the concentration of the compounds in the diet was greater than 2 mM, and the effect of each compound appeared to be optimal above a concentration of approximately 3 mM. This concentration corresponds, approximately, to the total caffeoylquinic acids (Table 1) and quercetin diglycosides (Stevenson, 1993) in the foliage of *A. paraguariensis* A similar response was also reported by Isman and Duffey (1982) and Duffey and Bloem (1986) when 5-CQA and rutin were fed to two other lepidopterous larvae, *Heliothis zea* and *Spodoptera exigua*. Thus caffeoylquinic acids may provide a useful broad-spectrum mechanism of insect resistance.

The dose of 5-CQA required to inhibit the development of *H. zea* in the study of Isman and Duffey (1982) was greater than that required in the present

study for the larvae of *S. litura*. Furthermore, Duffey and Bloem (1986) reported that *H. zea* required a higher concentration of rutin in the diet than *S. exigua* to produce the same level of developmental inhibition. The reason for these differences is not clear, although variations in the protective effects of midgut catalase activity (Felton and Duffey, 1991a) or gut alkalinity (Felton and Duffey, 1991b) between different species of lepidopterans could be important factors. At these concentrations, however, the possibility of insect tolerance building up to the inhibitory effects may be high (Duffey and Felton, 1991). It may therefore be appropriate to use caffeoylquinic acids in resistance breeding programs but with at least one other mechanism.

Farrar and Kennedy (1990) also observed the inhibition of larval development of *H. zea* by 5-CQA, but they reported the effect to be slight and of little if any value in plant protection. This reflected the previously reported lower susceptibility of *H. zea* to rutin as well as chlorogenic acid (Isman and Duffey, 1982) compared to *S. litura*. Farrar and Kennedy (1990) also disputed the value of weighing insects after a specified time rather than allowing larvae to complete their life cycle because the growth curves of larvae of *H. zea* were sigmoidal in their study. Thus, the ED_{50} value (Effective dosage required to reduce larval growth by 50%) after a specified time may not necessarily imply that the time taken to pupate will be twice as long as that on the control. In the present study, the experiment was terminated after 14 days, which coincided with the pupation of larvae on the control diets. The experiment was terminated because larval growth was so poor on treated diets that it was not considered necessary to continue in order to show that the compounds were toxic. Furthermore, to maintain individual larvae in culture for long periods of time may have influenced the results. For instance, if the diet was changed regularly, the persistent handling of the insects would have increased the stress, adding to that already induced by feeding on "toxic" diets. To avoid this additional stress factor, the larvae could have been left on the same diet to complete the life cycle. The possibility of the degradation of dietary components, however, might have had an additional influence on larval development. Additionally, the $\log_{10}$ curves of larval growth do illustrate the considerable differences in growth rate between larvae on treated and control diets, and the crude extrapolation of the curves gives an indication of the likely differences in development time for larvae to pupate.

Although the biochemical mechanism by which these compounds inhibit development of lepidopteran larvae has not been unequivocally determined, some recent studies have shown that the compounds reduce the nutritional quality of the food (Duffey and Bloem, 1986). Furthermore, foliar enzymes such as polyphenoloxidases and perioxidases increase the inhibitory effect of 5-CQA (Felton et al., 1989) by oxidizing dihydroxy groups (e.g., on caffeoylquinic acid) to *o*-quinones (Matheis and Whitaker, 1984), which covalently bind

nucleophillic -SH and -NH_2 groups of proteins, peptides, and amino acids, thus reducing the available nutrients. It is possible, therefore, that the inhibitory effects of 3-CQA and 1-CdQA on larvae observed in the present study may only be a part of the effect that would result if a whole leaf were eaten. This may explain why the effects on the larvae feeding on whole plants (Stevenson et al., 1993) were more severe than those observed for larvae feeding on the isolated compounds. Rutin can be oxidized by foliar peroxidases and can also behave as an electrophile and thus can also covalently bind to protein (Duffey and Felton, 1991).

Effect of Caffeoylquinic Acid Esters and Rutin on Development of Third Stadium Larvae. Lindroth and Peterson (1988) evaluated the effect of rutin and chlorogenic acid on the development of *Spodoptera eridania* and concluded that chlorogenic acid had no deleterious effects. Their experiments, however, only investigated the effects of 5-CQA on fifth stadium larvae. Isman and Duffey (1982) also showed that the development of older larvae was not affected by the consumption of 5-CQA. Our experiments, however, demonstrated that the individual inclusion of both 5-CQA and rutin in diets mildly stimulated development after 24 hr and, after an additional three days, the development was significantly retarded compared to larvae on control diets, especially when rutin and 5-CQA were present at concentrations equivalent to those found in the foliage of *A. paraguariensis* When third stadium larvae feed on whole leaves of *A. paraguariensis*, their weight gain is significantly lower than that of larvae feeding on susceptible varieties of groundnut (Stevenson et al., 1993). It is possible, therefore, that the presence of orthos dihydroxy phenolics combined with the oxidative activity of PPO and peroxidases in the whole leaf may be responsible for the resistance of *A. paraguariensis* to third stadium larvae observed in the field.

Effect of Rutin and 5-CQA on Feeding Preferences of Sixth Stadium Larvae. Rutin was shown to stimulate feeding of sixth stadium larvae, and 5-CQA was shown to significantly deter feeding. These results are in accord with those of Boer and Hanson (1987), who worked with *Manduca sexta*. Although rutin does stimulate feeding, it is unlikely to influence the choice of food by the larvae of *S. litura* in favor of *A. paraguariensis* because the presence of rutin in the foliage was concurrent with 5-CQA, and our experiments showed that rutin was ineffectual as a stimulant in the presence of 5-CQA.

Overall, the results from this study indicate that both the flavonoid glycosides (Stevenson, 1993) and caffeoylquinic acids identified in the foliage of *A. paraguariensis* play a crucial role in the defense of the plant to *S. litura* and thus may well provide breeders or biotechnologists with an easily screened marker for resistance of groundnuts to *S. litura* and other Lepidoptera.

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SYNTHESIS OF ATTRACTANTS FOR ORIENTAL FRUIT FLY *Dacus dorsalis* HENDEL USING A CATALYTIC ORGANOCOPPER COUPLING REACTION[1]

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Abstract—A general synthetic approach to various catechol derivatives was developed using a copper-catalyzed cross-coupling reaction of 1,2-dimethoxy-4-bromomethyl, 1-ethoxy-2-methoxy-4-bromomethyl- and 2-ethoxy-1-methoxy-4-bromomethylbenzenes with Grignard reagents. Dilithium tetrachlorocuprate was an acceptable catalyst in the dimethoxy series, whereas copper(I) iodide in THF-HMPA was a superior catalyst in all cases due to decreased side reactions, i.e., reduction and reductive coupling. Methyl-substituted analogs of methyl eugenol, a potent attractant of Oriental fruit fly, *Dacus dorsalis* Hendel, were synthesized by this method and evaluated for attractancy in field tests.

Key Words—Oriental fruit fly, *Dacus dorsalis* Hendel, Diptera, Tephritidae, methyl eugenol analogs, synthesis, coupling reaction, benzylic bromides, attractants, lures.

INTRODUCTION

Methyl eugenol (ME), 1,2-dimethoxy-4-(2-propenyl)benzene, is an extremely potent natural attractant for the male Oriental fruit fly, *Dacus dorsalis* Hendel.

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It has been used as the standard survey and detection lure for 40 years (Steiner, 1952) and was used in conjunction with a toxicant to eradicate *D. dorsalis* from the Islands of Rota (Steiner et al., 1965). In a similar fashion, it was used to annihilate incipient infestations of the Oriental fruit fly in California (Chambers et al., 1976; Cunningham et al., 1975) and Okinawa (Koyama et al., 1984).

Reports have indicated that ME causes hepatic tumors in mice (Miller et al., 1983), induces intrachromosomal recombination in a yeast assay (Schiestl et al., 1989), and elicits a positive response in a bacterial DNA repair test (Sekizawa and Shibamoto, 1982). Further, naturally occurring phenylpropanoids, such as safrole and eugenol gave positive responses in the yeast assay and both, including structurally related estragole (Drinkwater et al., 1976), were carcinogenic in mice (Miller et al., 1983; Mitzutani et al., 1991; Borchert et al., 1973). However, ME is on the "Generally Regarded as Safe" list and is used as a food flavoring agent. Moreover, ME gave a negative response in the Ames mutagenicity test (Schiestl et al., 1989). Presently, ME is being evaluated by the National Toxicology Program to determine its safety. Should regulatory and/or health agencies ultimately declare ME as being unsafe, there is no current alternative lure.

From the large number of compounds evaluated as attractants for *D. dorsalis* (Beroza and Green, 1963; Metcalf et al., 1975, 1981, 1983), Mitchell et al. (1985) selected three isosteres of methyl eugenol as promising replacements. Two ethoxy analogs of methyl eugenol, 1-ethoxy-2-methoxy-4-(2-propenyl)benzene and 2-ethoxy-1-methoxy-4-(2-propenyl)benzene, as well as 1,2-dimethoxy-4-ethylbenzene (DeMilo et al., 1993; Metcalf et al., 1990) have also been identified as possible substitutes. However, as yet no compound has been found to equal the attractiveness of methyl eugenol, nor are toxicity data available for these analogs. Furthermore, reports on structure–activity relationship studies on attractancy (Metcalf et al., 1975, 1981) or carcinogenicity (Miller et al., 1983) were not comprehensive. One reason to explain this might be the inherent difficulty in obtaining the necessary compounds to conduct this type of research. Consequently, we sought to develop a simple and general method for the synthesis of a large variety of 4-substituted catechol derivatives. The introduction of a methyl group to the 4-allyl moiety of methyl eugenol was of particular interest to us because of strong evidence that enzymatic oxidation of the methylene group or epoxidation of the double bond might generate potentially carcinogenic metabolites (Mitchell et al., 1985; Miller et al., 1983; Mitzutani et al., 1991). Aside from potential activity, methyl-branched analogs would provide valuable information concerning the compatibility of the receptor site of the Oriental fruit fly with this part of methyl eugenol molecule (Jönsson et al., 1991, 1992). Additionally, information derived from toxicity studies of the analogs containing a modified allylic moiety would allow predictions on how

other substituents might affect biotransformation pathways leading to potentially carcinogenic metabolites.

In this study we describe the general utility of a catalytic organocopper coupling reaction of benzylic substrates with Grignard reagents as a means of synthesizing various analogs of methyl eugenol.

METHODS AND MATERIALS

All reagents were obtained from commercial suppliers and used without further purification. Diethyl ether and tetrahydrofuran (THF) were distilled from solutions of sodium benzophenone ketyl. Hexamethylphosphoramide (HMPA) was distilled from calcium hydride prior to use. Caution must be taken when handling HMPA since it is a carcinogen. Dilithium tetrachlorocuprate was prepared according to known procedures (Tamura and Kochi, 1971). Isopropenylmagnesium bromide and 1-propenylmagnesium bromide (*Z*/*E* 2.5:1) were made from the corresponding bromides in THF. GC-MS analyses were carried out on a Hewlett-Packard 5890 (series II) GC fitted with a HP-5 fused-silica capillary column (25 m × 0.22 mm ID, film thickness 0.11 μm) and combined with a 5971 mass selective detector (EI 70 eV) and Vectra 386/25 computer system. Methane was used in the chemical ionization method.

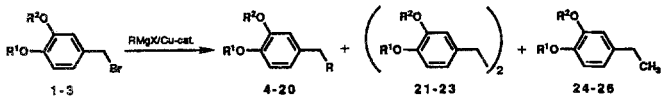
TLC analyses were performed on Baker-flex sheets with silica gel 1B-F. Flash chromatography was done with Merck Silica gel (230–400 mesh). Purity of the synthesized compounds was determined on a Shimadzu GC-14A fitted with a SPB-1 Supelco fused-silica capillary column (60 m × 0.25 mm ID, film thickness 0.25 μm). Helium was used as a carrier gas with a linear velocity of 19.5 cm/sec. Argentation chromatography was performed on silica gel (200 mesh) with 10% $AgNO_3$ (Aldrich).

NMR spectra were recorded on a GE-Brucker QE Plus spectrometer. Unless otherwise specified, $CDCl_3$ was used as solvent. Chemical shifts are given downfield to TMS. The $CHCl_3$ signal was used as an internal standard.

Syntheses of methyl eugenol analogs are outlined in Schemes 1 and 2.

1,2-Dimethoxy-4-bromomethylbenzene ***(1).*** Compound **1** was prepared according to Haworth et al. (1925) and used without purification.

1-Ethoxy-2-methoxy-4-bromomethylbenzene ***(2).*** 4-Ethoxy-3-methoxybenzyl alcohol (12.75 g, 0.07 mol) was dissolved in anhydrous benzene (120 ml) and dry hydrogen bromide was passed through this solution at 5–10°C until saturation. The mixture was then treated with anhydrous sodium carbonate (50 g) at 5–10°C and then filtered in vacuo. The resulting solution was dried (Na_2SO_4) and concentrated using a rotary evaporator (40°C) to give bromide **2** (15.6 g, 91%), which was used without further purification. NMR: 1.46 (t, 3H, $\mathbf{CH_3}CH_2$), 3.89 (s, 3H, CH_3) 4.10 (q, 2H, $CH_3\mathbf{CH_2}$), 4.50 (s, CH_2Br), 6.81 (d, 1H, 6-H), 6.91 (s, br., 1H, 3-H), 6.93 (dd, 1H, J = 7.8 and 2.1 Hz, 5-H).



	1,21,24	2,22,25	3,23,26	4	5	6	7
R^1	CH_3	C_2H_5	CH_3	CH_3	CH_3	CH_3	CH_3
R^2	CH_3	CH_3	C_2H_5	CH_3	CH_3	CH_3	CH_3
R	-	-	-	CH_2=CH	C_2H_5	CH_3	$(CH_3)_2CH$

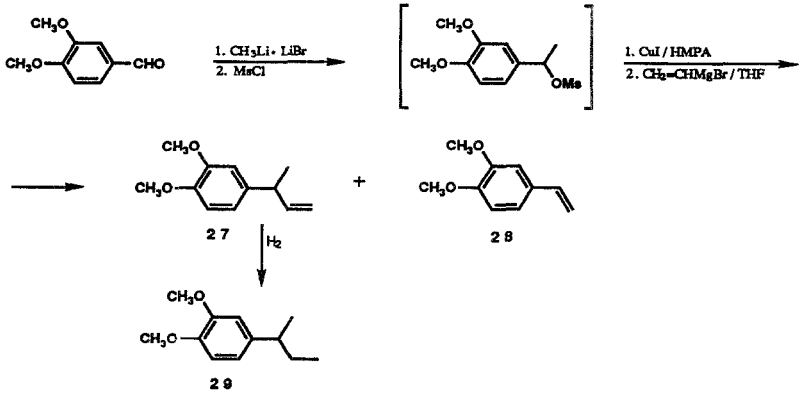
	8	9	10	11	12	13
R^1	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
R^2	CH_3	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5
R	CH_2=C(CH_3)	CH_2=CH	C_2H_5	CH_3	$(CH_3)_2CH$	CH_2=C(CH_3)

	14	15	16	17	18	19	20
R^1	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	CH_3	CH_3
R^2	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
R	CH_2=CH	C_2H_5	CH_3	$(CH_3)_2CH$	CH_2=C(CH_3)	$CHCH_3$=CH (*Z*)	CH_3CH=CH (*E*)

SCHEME. 1.

2-Ethoxy-1-methoxy-4-bromomethylbenzene ***(3).*** This was prepared in 90% yield from 3-ethoxy-4-methoxybenzyl alcohol using the same method used for **2.** NMR (acetone-d_6): 1.37 (t, 3H, $\mathbf{CH_3}CH_2$), 3.81 (s, 3H, CH_2), 4.06 (q, 2H, $\mathbf{CH_2}CH_3$), 4.61 (s, CH_2Br), 6.92 (d, 1H, 3-H), 7.01 (dd, 1H, J = 8.1 and 2.1 Hz, 4-H) 7.05 (d, 1H, 2-H).

Dilithium Tetrachlorocuprate-Catalyzed Coupling Reaction of Benzylic Bromides ***1–3*** *with Grignard reagents (Method A).* To the appropriate Grignard reagent in THF (13 mmol, Table 1), under an inert atmosphere, dilithium tetrachlorocuprate (0.3 mmol; 0.5 ml of 0.6 M) was added at −5 to 0°C. Subsequently, a solution of bromide **1–3** (10 mmol) in THF (20 ml) was added while maintaining the temperature at 5–10°C. The reaction mixture was



SCHEME. 2.

stirred at room temperature 2 hr, then poured into ice-cooled aq. NH_4Cl (200 ml) and extracted with ether–hexane mixture (1:1). The organic layer was washed with brine, dried (Na_2SO_4), and concentrated in vacuo. Distillation of the residue in vacuo afforded cross-coupling products **4–9**, and **14** in 97–99% purity, contaminated with small amounts of reduction products **24–26**. The yields of compounds **4–9**, **14**, and **24–26** are presented in Table 1. Structures were confirmed by NMR and GC-MS, the data of which are presented in Table 2. Three new analogs have also been synthesized by this coupling reaction: 1,2-dimethoxy-4-(2-methylpropyl)benzene **(7)**; 1,2-dimethoxy-4-(2-methyl-2-propenyl)benzene **(8)**; and 2-ethoxy-1-methoxy-4-(2-propenyl)benzene **(9)**. In selected experiments (Table 1, entries 4, 6, and 8) pot residues after distillation were subsequently chromatographed on silica gel. Elution with hexane–ethyl acetate (7:3) afforded dimers **21–23**: 1,2-bis(3,4-dimethoxyphenyl)ethane **(21)**, 33% yield, mp 104–105°C (hexane–acetone, 10:1), NMR: 2.85 (s, 4H, CH_2CH_2), 3.84 (s, 6H, OCH_3), 3.86 (s, 6H, OCH_3), 6.66 (d, 2H, 2′-H, J = 1.8 Hz), 6.71 (dd, 2H, J = 8.1 and 1.8 Hz, 6′-H), 6.79 (d, 2H, 5′-H). [NMR data are consistent with those described in the literature (Iida et al., 1977)]; 1,2-bis(4-ethoxy-3-methoxyphenyl)ethane, **(22)**, 57% yield, mp 129–130°C (hexane); NMR: 1.45 (t, 6H, $CH_2\mathbf{CH_3}$), 2.84 (s, 4H, CH_2CH_2), 3.82 (s, 6H, OCH_3), 4.07 (q, 4H, $\mathbf{CH_2}CH_3$), 6.66 (s, br., 2H, 2′-H), 6.68 (dd, 2H, 6′-H, J = 7.8 and 2.1Hz), 6.79 (d, 2H, 5′-H); 1,2-bis(3-ethoxy-4-methoxyphenyl)ethane **(23)**, 30% yield, mp 84–85°C (hexane); NMR: 1.44 (t, 6H, $CH_2\mathbf{CH_3}$). 2.82 (s, 4H, CH_2CH_2), 3.85 (s, 6H, OCH_3), 4.05 (q, 4H, $\mathbf{CH_2}CH_3$), 6.66 (d, 2H, 2′-H), 6.69 (dd, 2H, 6′-H, J = 8.1 and 1.8 Hz), 6.79 (d, 2H, 5′-H). GC-MS (CI): 331($M+1^+$).

Copper(I) Iodide-Catalyzed Cross-Coupling of Benzylic Bromides (1–3) with Grignard Reagents (Method B). A solution of CuI (190 mg, 1 mmol) in

TABLE 1. COPPER-CATALYZED REACTION OF BENZYLIC BROMIDES 1–3 WITH GRIGNARD REAGENTS

Entry	Benzylic bromide No.	Grignard reagent, (R)[a]	Method[b]	Reaction products: Cross-coupling No.	Cross-coupling Yield[c] (%)	Cross-coupling bp (°C/mm)	Dimer 21–23 (% yield)	Reduction 24–26 (% yield)[d]
1	1	CH_2=CH	A	4	73	65–67/0.25	17	0.5
2	1	C_2H_5	A	5	66	66–68/0.25	20	2.5
3	1	CH_3	A	6	80	58–60/0.25	10	0.9
4	1	$(CH_3)_2CH$	A	7	52	74–75/0.25	33	1.8
5	1	CH_2=C(CH_3)	A	8	84	84–85/0.3	7	0.5
6	3	CH_2=CH	A	9	54	78/0.3	30	0.6
7	3	CH_2=CH	B	9	74		5	
8	2	CH_2=CH	A	14	25	77–78/0.25	57	0.4
9	2	CH_2=CH	B	14	88		5	
10	2	CH_2=C(CH_3)	B	18	87	87–89/0.25		
11	2	CH_3	B	16	82	65–67/0.25	4	
12	2	C_2H_5	B	15	72	78–79/0.35	10	0.5
13	2	$(CH_3)_2CH$	B	17	56	74–75/0.25	20	3.5
14	3	CH_3	B	11	83	80–82/0.8	5	
15	3	C_2H_5	B	10	75	69/0.3	6	0.2
16	3	CH_2=C(CH_3)	B	13	80	85–86/0.2	5	
17	3	$(CH_3)_2CH$	B	12	57	75–76/0.25	18	2.5
18	1	CH_3CH=CH	B	19 + 20	84	82–92/0.25		

[a] 1 M CH_2=CHMgBr, 1 M C_2H_5MgBr, 3 M CH_3MgCl, 3 M i-C_3H_7MgCl, 0.3 M CH_2=C(CH_3)MgBr, 0.67 M CH_3CH=CHMgBr (*Z*/*E*, 2.5:1).
[b] A: Li_2CuCl_4/THF, 5–10°C. B: CuI/THF/HMPA, −20° – (−25)°C.
[c] Column represent isolated yields. Purities of cross-coupling products are 97–99%.
[d] Calculated from GC data.

HMPA (6.9 ml, 40 mmol) was added to a solution of benzylic bromide **1–3** (10 mmol) in THF (10 ml) at −10°C. The mixture was cooled to −20° to −25°C, and the Grignard reagent in THF (15 mmol) was added dropwise. Stirring was continued for an additional hour at this temperature, then the mixture was allowed to warm to 0°C and poured into aq. NH_4Cl (150 ml) containing 2 g of NaCN. The mixture was extracted with ether–hexane (1:1) and the extract was washed with brine, dried (Na_2SO_4), and concentrated in vacuo. Products **9–20** were isolated by distillation in vacuum. Yields, boiling points, as well as NMR and GC-MS data are presented in Tables 1 and 2. (*Z*)-1,2-Dimethoxy-4-(2-butenyl)benzene **(19)** and (*E*)-1,2-dimethoxy-4-(2-butenyl)benzene **(20)** were separated by argentation chromatography. Eluent: hexane–ethyl acetate, 99:1 to 9:1. β-Branched analogs of methyl eugenol: 2-ethoxy-1-methoxy-4-(2-meth-

ylpropyl)benzene **(12)**, 2-ethoxy-1-methoxy-4-(2-methyl-2-propenyl)benzene **(13)**, 1-ethoxy-2-methoxy-4-(2-methylpropyl)benzene **(17)**, and 1-ethoxy-2-methoxy-4-(2-methyl-2-propenyl)benzene **(18)** were also prepared by this method.

1,2-Dimethoxy-4-(1-methyl-2-propenyl)benzene (27). An ethereal solution of the complex of methylithium and lithium bromide (11 mmol, 7.3 ml of 1.5 M) was added dropwise to a solution of 3,4-dimethoxybenzaldehyde (2.66 g, 10 mmol) in THF (20 ml) at −10°C. After about 1 hr, methanesulfonyl chloride (0.85 ml, 11 mmol) was added at − 10°C, and the mixture was stirred for 1.5 hr at the same temperature. A solution of copper(I) iodide (190 mg, 1 mmol) in HMPA (20 ml) followed immediately by a solution of vinylmagnesium bromide in THF (13 mmol, 13 ml of 1M) were added via syringe at −25°C. The mixture was stirred at −20°C for 0.5 hr and then warmed gradually to room temperature during 2 hr. After stirring for an additional 0.5 hr at room temperature, the mixture was poured onto ice-cooled aq. NH_4Cl containing 3 g NaCN. The mixture was extracted with ether–hexane (1 : 1), and the extract was washed with brine and dried (Na_2SO_4). Evaporation of the solvent and distillation of the residue afforded 1.12 g of the mixture of 1,2-dimethoxy-4-methylbenzene (**24**, 1.0%), 1,2-dimethoxy-4-vinylbenzene (**28**, 16.0%), 1,2-dimethoxy-4-(1-methyl-2-propenyl)benzene (**27**, 79%), and 3,4-dimethoxy-α-methylbenzyl alcohol (4.0%). Flash chromatography of this mixture on silica gel (60 g) with hexane–ethyl acetate (5:1) afforded 768 mg (40%) of **27** of 98% purity, bp 67–68°C/0.25 mm. NMR: 1.35 (d, 3H, 1′-CH_3, J = 7.2 Hz), 3.42 (dq, 1H, 1′-H, J = 7.2 and 6.3 Hz) 3.86 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 5.05 (ddd, 1H, =CH**H**) 5.02 (ddd, 1H =C**H**H), 6.0 (ddd, 1H, C**H**=CH_2, J = 17.0, 10.5, 7.2 Hz), 6.74 (s, br, 1H, 3-H) 6.76 (dd, 1H, 5-H), 6.81 (d, 1H, 6-H). MS: 192 (M^+, 97), 177 (M^+-CH_3, 100), 165(7), 161(36.4), 146(44.1).

1,2-Dimethoxy-4-(2-methylpropyl)benzene (29). Compound **27** (1.0 g, 5.2 mmol) in ethanol (10 ml) was shaken with a catalytic amount of PtO_2 under a slight positive pressure of H_2 at room temperature. After H_2 uptake ceased, the catalyst was removed by filtration through Celite. The filtrate was concentrated in vacuo and distilled to give **29** (710 mg, 70.9%) of 97% purity, bp 72–73°C/0.25 mm. NMR: 0.83 (t, 3H), 1.22 (d, 3H, J = 6.9 Hz, 1′-CH_3) 1.57 (dq, 2H), 2.54 (tq, 1H), 3.86 and 3.88 (OCH_3), 6.7–6.82 (m, 3H, ar). MS: 194(M^+, 25.4) 165 (M^+-C_2H_5, 100), 150 (8.1).

RESULTS AND DISCUSSION

Cross-coupling reactions involving catalytic organocopper compounds are used routinely for carbon–carbon bond construction. Grignard reagents have been used most effectively to achieve good yields of coupling products with a

wide variety of organic halides (Posner, 1980; Normant, 1978). Despite their normally high reactivity toward nucleophilic displacement, benzylic halides have not been widely explored in coupling reactions. Dilithium tetrachlorocuprate, initially reported by Tamura and Kochi (1971) for preparation of aliphatic compounds, was shown to be an effective catalyst for some coupling reactions of benzylic halides (Nunomoto et al., 1983; Erdik, 1984; Seltzman et al., 1991). We also found it useful for the reaction of 3,4-dimethoxybenzyl bromide **1** and Grignard reagents (Table 1). Yields of cross-coupling products were good in most cases (entries 1–3, 5), but moderate with isopropylmagnesium bromide (entry 4). Reductive dimerization was a major side reaction, but the high molecular weights of the dibenzyl by-products **21–23** allowed easy purification of the desired product. However, the reverse mode of addition of the reagents (i.e., benzylic bromide added to the Grignard reagent and catalyst) seems important in decreasing reductive dimerization. For example, addition of vinylmagnesium bromide to the mixture of bromide **1** and Li_2CuCl_4 afforded only 51% yield of methyl eugenol (**4**), whereas the reverse addition increased the yield up to 73% (entry 1). It is also noteworthy that noncatalytic reaction of these reagents resulted mainly in dimerization of benzylic bromide **1** and that replacement of Grignard reagent by vinyllithium led to dibenzyl **21** almost exclusively. The last observation was in sharp contrast with the alkylation of vinylithium by alkyl halides in THF (Millon et al., 1975) and might possibly be attributed to the predisposition of benzylic bromides **1–3** to single electron transfer (Mojima and Kusabayashi, 1989). The relatively high degree of dimerization and reduction even under catalytic reaction conditions in the case of Grignard agents with high electron donating capability (Table 1, entry 4) supports this hypothesis. The tendency of benzylic bromides to reductive coupling was more profound in the ethoxy series. The yield of the Li_2CuCl_4-catalyzed cross-coupling reaction of the 3-ethoxybenzylic bromide **3** and vinylmagnesium bromide (entry 6) decreased to 54% and dropped even further (entry 8) when 4-ethoxybenzylic bromide **2** was studied. The yields of these coupling reactions were dramatically increased by substituting Li_2CuCl_4 for copper(I) iodide and adding 4 eq of HMPA to the solvent. Nicolaou et al. (1986) and Mori and Brevet (1991) utilized these conditions successfully for coupling reactions of haloepoxides. We found that CuI-catalyzed cross-coupling reaction of all three benzylic substrates with Grignard reagents proceeded smoothly at $-20°$ to $-25°C$ and afforded high yields of various methyl eugenol analogs (Table 2). Despite these improvements, the highly basic isopropylmagnesium bromide (entries 13 and 17) still provided substantial amounts of dimers.

The application of cross-coupling reactions to the synthesis of an α-methylated analog of methyl eugenol was also studied. Usually, substitution at the secondary carbon atom with organocopper reagents is sluggish and nonchemoselective (Lipshutz and Wilhelm, 1981; Nunomoto et al., 1983). For example,

TABLE 2. ^{1}H-NMR AND MS DATA OF CROSS-COUPLING PRODUCTS **4–20**

Compound	^{1}H NMR ($CDCl_3$)	MS m/z (%)
4[a]	3.33(d), 3.86(s), 3.87(s), 5.06 (dm), 5.08(ddt), 5.96 (ddt), 6.71(s), 6.73(dd) 6.81(d)	Cl 179 (M + 1^+)
5[b]	0.94(t), 1.62(tq), 2.53(dd), 3.86(s), 3.87(s), 6.7–6.81 (3H,ar)	180(M^+, 30.2), 151 (M^+-C_2H_5, 100)
6[c,d]	1.22(t), 2.58(g), 3.86(s), 6.71–6.82(3H ar)	166(M^+, 64.6), 151 (M^+-CH_3, 100)
7	0.90(d), 1.83(qqt), 2.41(d), 3.86(s), 3.87(s), 6.67–6.74 (3H,ar)	194(M^+, 19.8), 151 [M^+-$(CH_3)_2CH$, 100]
8	1.68(s,br), 3.26(s,br.), 3.87(s,br.6H,OCH_3), 4.73(s,br.1H), 4.88(s,br.1H), 6.71–6.81(3H,ar)	192(M^+, 100), 177(36.1), 161(37.8), 151(39.5), 91(21.9)
9	1.45(t,3H), 3.32(d,2H), 3.85(s,3H), 4.09(q,2H), 5.03–5.11(m,2H), 5.95(ddt,2H), 6.70–6.83(m, 3H, ar)	Cl 193(M + 1^+)
10[d]	0.93(t,3H), 1.45(t), 1.62(tq,2H), 2.52(t,2H), 3.85(s), 4.09(q), 6.69–6.81(ar)	194(M^+, 69.7), 165 (M^+-C_2H_5, 100), 137(92.9)
11[d]	1.22(t), 1.46(t), 2.58(q), 3.85(s), 4.10(q), 6.71–6.82(m)	180(M^+, 61.1), 165 (M^+-CH_3, 13.1), 152(22.8), 137(100)
12	0.89(d), 1.45(t), 1.82(qqt), 2.40(d), 3.85(s), 4.10(q), 6.65–6.81(m,ar)	208(M^+,28.3), 165 [M^+-$CH(CH_3)_2$, 100], 137(24.7)
13	1.45(t), 1.67(s.br), 3.25(s.br), 3.85(s), 4.09(q), 4.72(m,1H), 4.79(m,1H), 6.70–6.81(m,ar)	206(M^+,100), 177(8.6), 163(31.9), 137(19), 131(22.1), 91(14.6)
14[d]	1.45(t,3H), 3.33(d,2H), 3.86(s,3H), 4.08(q,2H), 5.02–5.12(m,2H), 5.96(ddt,1H), 6.68–6.83(m,3H,ar)	192(M^+,100), 164(21.6), 163(16.9), 149(33.3)
15[d]	0.94(t), 1.44(t), 1.63(tq,2H), 2.53(t,2H), 3.86(s,3H), 4.07(q,2H), 6.67–6.81(3H,ar)	194(M^+,36), 165 (M^+-C_2H_5, 31.8), 137(100)
16[d]	122(t,3H), 1.44(t,3H), 2.59(q), 3.87(s,3H), 4.07(q,2H), 6.69–6.82(m,3H,ar)	180(M^+,56.3), 165 (M-CH_3,4.3), 152 (M^+-C_2H_4,22.5), 151(M^+-C_2H_5, 13), 137(100)
17	0.90(d,6H), 1.45(t,3H), 1.82(qqt,1H), 2.41(d,2H), 3.86(s,3H), 4.08(q,2H), 6.63–6.81(m,3H)	208(M^+,32.8), 165(60.9), 137(100)
18	1.45(t,3H), 1.68(s,br.,3H), 3.26(s,br., 2H), 3.85(s,3H), 4.08(q,2H), 4.73(m,1H), 4.80(m,1H) 6.68–6.82(m,3H)	206(M^+,100), 178(7.3), 177(13.0), 163(24.2), 137(26.8), 131(30), 91(15.2)
19	1.70(dm,3H,$\mathbf{CH_3}$CH,J = 4.8Hz), 3.36(m,2H,CH_2), 3.86(s,3H,OCH_3), 3.87(s,3H,OCH_3), 5.57(m,CH_3**CH**=**CH**), 5.59(dq,CH_3**CH**=CH,J = 10.8 and 4.8 Hz), 6.72(s,br.1H,3-H, 6.73(dd,1H,5-H,J = 8.4 and 2.1Hz), 6.8(d,1H,6-H)	192(M^+100), 177(43.7), 161(35), 146(13.4)
20	1.69(dm,3H,CH**CH**$_3$,J = 6.0 Hz), 3.26(dm,2H,CH_2,J = 6.0 Hz), 3.85(s,3H,OCH_3), 3.88(s,3H,OCH_3), 5.51(dqm,1H,CH_3**CH**=CH,J = 15.0 and 6.0 Hz), 5.58(dtq, 1H, CH_3CH=**CH**$_2$,J = 15.0, 6.1 and 1.2 Hz), 6.70(s,br.,1H), 6.72(dd,1H), 6.8(dm,1H)	192(M^+,100), 177(46), 161(40.9), 146(16.5)

[a] Methyl eugenol.
[b] Metcalf et al. (1975).
[c] Metcalf (1990).
[d] DeMilo et al. (1992).

the Li_2CuCl_4-catalyzed reaction of 1,3-butadien-2-ylmagnesium chloride with 2-bromooctane afforded only a 5% yield of cross-coupled product (Nunomoto et al., 1983). While the main reaction pathways appear to follow a reduction and/or elimination mode, benzylic halides appeared to cause additional problems, such as reductive coupling. Since 1,2-dimethoxy-4-(1-bromoethyl) benzene (Redeuilh et al., 1973) is extremely unstable, the benzylic substrate was prepared and immediately used without isolation (Scheme 2). Accordingly, addition of $CH_3Li \cdot LiBr$ to 3,4-dimethoxybenzaldehyde followed by conversion of the lithium salt to a mesylate and subsequent alkylation of mesylate by vinylmagnesium bromide in the presence of CuI/THF/HMPA afforded a 40% yield of α-methylated analog **27**. Dimethoxystyrene **28** was removed from the desired product by flash chromatography. Despite its moderate yield, this catalytic cross-coupling procedure appears superior to the organocuprate approach (Lipshutz and Wilhelm, 1981) because of its simplicity.

Bioassay. Several field-plot experiments were conducted in Hawaii during January–March 1992 to evaluate the attractiveness of synthesized compounds against feral populations of the Oriental fruit fly. Candidate attractants were pipetted neat onto small cotton dental roll wicks that were secured inside tent-shaped Jackson traps (Harris et al., 1971). Fly catches were recorded at predetermined intervals over the course of the experiment. Similar to methyl eugenol, analogs were almost exclusively attractive to males. To assess the comparative effectiveness of methyl eugenol analogs, wicks were removed from the traps and analyzed by GC for nonvolatilized residues. These data were then used to calculate the mean catch per mass unit of volatilized compound. A brief description of the bioassay results is presented here; complete attractancy data, including a full range of dose–response experiments, will be published elsewhere.

The 2-ethoxy analog **9** exhibited an initial attractiveness close to that observed for methyl eugenol and was consistently more attractive than its corresponding 1-ethoxy analog **14**. The enhancing effect of an ethoxy group located meta to the allyl moiety was even more profound for β-methyl branched compounds **13** and **18**, i.e., **13** elicited 75% of the attractancy of methyl eugenol and was 3× more attractive than **18** (1-ethoxy). Methylation at the α position of the allyl moiety (i.e., **27**) substantially decreased attractancy, whereas methylation at the γ position (i.e., **19**, **20**) led to compounds with appreciable attractancy and high persistency. Effective trapping of males by β- and γ-methylated compounds was surprising in light of previous reports (Metcalf et al., 1975, 1983) that most promising compounds required a three-atom, nonbranched chain at the four-position of the benzene ring.

In summary, we have developed a general synthetic approach to conveniently prepare a variety of 4-substituted catechol ethers related to methyl eugenol. Several of these show moderate to high attractancy against the male

Oriental fruit fly under field conditions and are therefore considered worthy of further investigation.

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CARNIVORE FECAL CHEMICALS SUPPRESS FEEDING BY ALPINE GOATS (*Capra hircus*)

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Abstract—The efficacy of carnivore and ungulate fecal chemicals in suppressing the feeding behavior of Alpine goats (*Capra hircus*) was examined. In the first four experiments, goats were offered food covered with paper strips treated with fecal extracts of the Bengal tiger, Siberian tiger, African lion, and brown bear, respectively; food covered with solvent-treated and untreated (plain) papers served as controls in each experiment. Goats made fewer head entries into, and ate less food from, buckets containing fecal extracts. In the fifth experiment, goats were offered food covered with paper strips treated with fecal extracts of the puma, Dorcas gazelle, white-bearded gnu, and conspecifics; food covered with solvent-treated and plain papers again served as controls. The amounts of food consumed from buckets containing puma, gazelle, gnu, and solvent treatments were statistically indistinguishable, but less food was consumed from them than from buckets containing the goat-scented or plain papers. No significant differences among treatments were detected with respect to head entries. Field experiments are needed on the use of predator-derived chemicals to reduce damage by goats to vegetation.

Key Words—Alpine goats, *Capra hircus*, predator chemicals, repellents.

INTRODUCTION

Chemicals that deter browsing by ungulates have been sought to control damage to crops and forest trees. Campaigns to identify ungulate repellents have focused on unpalatable compounds from plants and aversive chemicals from predators (see Müller-Schwarze, 1990).

Experimental studies of the responses of ungulates to predator scents have dealt primarily with the reactions of the North American black-tailed deer (*Odo-*

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coileus hemionus) to carnivores (Müller-Schwarze, 1972, 1983; Melchiors and Leslie, 1985; Sullivan et al., 1985; Swihart et al., 1991). These investigations indicate that chemicals from the feces and urine of canids and felids suppress feeding by deer when applied to or placed near food. The European red deer (*Cervus elaphus*) avoided food treated with a fecal extract of the African lion and a mixture of (unspecified) compounds characterized from it (Abbott et al., 1990).

Domesticated ungulates also are repelled by carnivore chemicals. Sheep (*Ovis aries*) and cattle (*Bos taurus*) consumed less food when coyote, fox, or puma feces were placed near food bins (Pfister et al., 1990). Bear feces repelled cattle, but were less effective with sheep. Sheep avoided bedding down in areas contaminated with carnivore feces, but they readily habituated to these odors. Sheep refused food treated with dog feces, and showed no sign of habituating to these materials during a nine-day test period (Arnould and Signoret, 1993).

We report here on responses by Alpine goats (*Capra hircus*) to the fecal scents of carnivores and ungulates. Our study indicates that carnivore fecal scents suppress feeding in this economically important ungulate.

METHODS AND MATERIALS

In the first four experiments, goats were tested for responses to extracts of the feces of the Bengal tiger (*Panthera tigris tigris*), Siberian tiger (*P. tigris altaica*), African lion (*P. leo*), and brown bear (*Ursus arctos*). These and other stimulus animals used in our study were maintained at the Houston Zoological Gardens, Houston, Texas. The tigers and lion were fed Frozen Feline Diet (Animal Spectrum Inc., Lincoln, Nebraska). The bear was fed Spectrum Omnivore Diet (Animal Spectrum Inc.), mackerel, apples, oranges, and carrots.

Fecal samples were obtained within 12 hr of defecation, placed into glass jars, and stored at −20°C. Seventy-five grams of feces from each species were extracted in 500 ml of chloroform–methanol–water (1:2:0.8, v/v/v). The slurries were stirred and passed through filter papers. Filter papers (diameter = 15 cm) were placed on a sheet of aluminum foil, treated with 1.5 ml of either fecal extract or extraction solvent, and allowed to air-dry for 20 min; plain papers were untreated. All filter papers were shredded in a Boston Electric paper shredder (Hunt Mfg. Co., Statesville, North Carolina).

Ten 1-year-old female subjects were randomly selected from a mixed herd of 150 goats at the International Dairy Goat Research Center at Prairie View A&M University, Prairie View, Texas. They were maintained on a complete mixed ration of cottonseed hull, corn, and alfalfa. Food was withheld from subjects for 15 hr before testing. Subjects were confined individually to 122 × 50 × 88-cm holding pens for 4 hr before testing. They were provided with water ad libitum before and during tests.

Subjects were presented with 907 g of their regular feed in a 3.8-liter bucket (diameter = 25 cm) covered with shreddings from fecal-scented, solvent-treated, and plain filter papers. Each treatment was presented three times during a trial, with 3 min/presentation (total = 9 min). Subjects were exposed to all these conditions in a Latin-square design. The goats' behavior was recorded on a video recorder positioned 2.5 m behind the stall. The numbers of head entries made by goats into buckets were scored, and the buckets were weighed at the end of each session to determine the amount of food removed from them.

In the fifth experiment, goats were tested for responses to extracts of the feces of the puma (*Felis concolor*), Dorcas gazelle (*Gazella dorcas*), white-bearded gnu (*Connochaetes taurinus albojubatus*), and conspecifics. The gazelle and gnu were maintained on alfalfa and 16% Special Zoo Diet (Bluebonnet Milling Co., Ardmore, Oklahoma). The gnu also received coastal Bermuda hay and bread. Goat fecal donors were fed their regular feed.

Thirty-three grams of feces from each stimulus species were extracted with 250 ml of chloroform–methanol–water (1:2:0.8, v/v/v) and filtered. Extracts were applied to filter papers, and behavioral tests were conducted as described above.

Differences between treatment means for head entries and food consumption in all experiments were examined using a Kruskal-Wallis test with Newman-Keuls multiple comparisons. Probability values less than or equal to 0.05 indicated significant effects.

RESULTS AND DISCUSSION

In each of the first four experiments, goats made fewer head entries into, and consumed less food from, buckets containing fecal extracts than from those containing plain or solvent-treated papers (Table 1). Responses to plain and solvent-treated papers were statistically indistinguishable in each case.

Goats in the fifth experiment made numerically fewer head entries into puma-scented buckets, but no significant effects were detected among treatments with this measure (Table 2). The amounts of food removed from buckets containing puma, gazelle, gnu, and solvent treatments were statistically indistinguishable, but less food was consumed from each of these than from buckets containing goat-scented or plain papers.

Results similar to ours with goats have been obtained with cattle and sheep: less was consumed when food was treated with carnivore extracts or placed near carnivore feces (Pfister et al., 1990; Arnould and Signoret, 1993). In cattle, the fecal scents of conspecifics also have been shown to suppress feeding (Dohi et al., 1991), an effect we failed to observe in goats during presentations of fecal extracts.

TABLE 1. MEAN (±SE) HEAD ENTRIES INTO, AND FOOD CONSUMED (g) FROM, BUCKETS CONTAINING FECAL-SCENTED, SOLVENT-TREATED, AND PLAIN FILTER PAPERS

	Feces	Solvent	Plain
Bengal tiger			
Head entries	2.9 ± 0.7 a[a]	7.1 ± 0.5 b	6.9 ± 0.4 b
Food consumed	30.0 ± 13.6 a	231.1 ± 36.5 b	259.1 ± 37.7 b
Siberian tiger			
Head entries	0.4 ± 0.2 a	5.9 ± 0.9 b	7.2 ± 1.0 b
Food consumed	9.1 ± 4.5 a	194.5 ± 33.1 b	178.2 ± 30.0 b
African lion			
Head entries	0.9 ± 0.3 a	5.2 ± 0.6 b	4.6 ± 0.4 b
Food consumed	8.2 ± 5.4 a	293.2 ± 20.3 b	302.2 ± 23.7 b
Brown bear			
Head entries	1.8 ± 0.7 a	5.1 ± 0.5 b	4.8 ± 0.4 b
Food consumed	22.7 ± 10.1 a	274.5 ± 30.3 b	228.2 ± 22.1 b

[a]Means with different letters are significantly different ($P \leq 0.05$).

TABLE 2. MEAN (±SE) HEAD ENTRIES INTO, AND FOOD CONSUMED (g) FROM, BUCKETS CONTAINING FECAL-SCENTED, SOLVENT-TREATED, AND PLAIN FILTER PAPERS

	Puma	Gazelle	Gnu	Goat	Solvent	Plain
Head entries	4.3 ± 0.5 a[a]	5.3 ± 0.6 a	5.4 ± 0.6 a	5.4 ± 0.5 a	5.7 ± 0.7 a	5.3 ± 0.5 a
Food consumed	145.1 ± 27.2 a	204.1 ± 13.6 a	158.7 ± 27.2 a	267.0 ± 27.2 b	217.7 ± 31.7 a	263.1 ± 22.6 b

[a]Means with different letters are significantly different ($P \leq 0.05$).

The cattle and sheep in the study of Pfister et al. (1990) made numerically fewer head entries into food bins containing carnivore scents than into control bins, but no statistically significant effects were detected. Since the five subjects of each species used by Pfister et al. had been exposed to fecal scents in a previous experiment, they may have habituated to the carnivore scents. The subjects in our study were chosen at random and not identified individually, thus it is uncertain whether our failure to obtain significant results in our fifth experiment with respect to head entries is due to habituation.

Neither our subjects nor the cattle and sheep in the study of Pfister et al. (1990) had had previous experience with the carnivores used as stimulus animals. Experience with these predators, therefore, appears unnecessary for the

initial elicitation of an aversive response. Our experiments also indicate that feeding in goats is suppressed by the scents of both New and Old World carnivores. These findings accord with other studies demonstrating that defensive responses are elicited in some species by chemicals from allopatric predators (see Weldon, 1990).

Our experiments showing that carnivore fecal scents suppress feeding in goats suggest a potential source of chemicals to deter browsing by this economically important species. Tests with other predator-derived materials, e.g., urine and epidermal extracts, may indicate other sources of aversive chemicals. Whether predator-derived chemicals can be used to protect vegetation against browsing by goats needs to be tested in the field where habituation effects, the persistence of predator scents, and other parameters likely to effect feeding suppression are assessed.

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DIEL RHYTHMS OF CALLING BEHAVIOR AND PHEROMONE PRODUCTION OF ORIENTAL TOBACCO BUDWORM MOTH, *Helicoverpa assulta* (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Both calling behavior and titer of (Z)-9-hexadecenal (Z9-16:Al), the major sex pheromone component of *Helicoverpa assulta*, in pheromone glands showed distinct diel periodicity, and these two were synchronous. Calling was most actively performed and the pheromone titer reached a maximum from 2 to 6 h after lights-off. During photophase, no calling was shown and only a relatively small amount of Z9-16:Al was detected. However, there was a time lag of a few days between peak calling activity and maximum pheromone titer. The pheromone titer was maximal from age 1 day to age 5 days and thereafter decreased while calling was most actively performed after age 3 days. Titers of three minor components, hexadecenal, (Z)-11-hexadecenal, and (Z)-9-hexadecenyl acetate, showed similar daily fluctuation patterns to that of Z9-16:Al, but relative to the titer of Z9-16:Al, the titer of the two aldehyde components remained relatively constant whereas that of Z9-16:Ac increased in the late scotophase.

Key Words—*Helicoverpa assulta*, sex pheromone, calling, diel periodicity, Lepidoptera, Noctuidae, (Z)-9-hexadecenal, hexadecanal, (Z)-11-hexadecenal, (Z)-9-hexadecenyl acetate.

INTRODUCTION

The production and the release behavior (calling behavior) of sex pheromone in female moths generally shows diel periodicity, which is modulated by several

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factors, such as age, photoperiod, and temperature (Fatzinger, 1973; Nordlund and Brady, 1974; Webster and Cardé, 1982; Delisle and McNeil, 1987). In many moths maximal pheromone titers in the pheromone gland coincide with peak calling activities (Fatzinger, 1973; Pope et al., 1982; Raina et al., 1986) or precede calling peaks (Webster and Cardé, 1982; Konno, 1986; Ono et al., 1990), but some moths show no distinct peak of pheromone titer in the glands (Coffelt et al., 1978; Schal et al., 1987).

Most moths use mixtures of several compounds as the sex pheromone, and their proportion is critical for attraction of males. The temporal fluctuation patterns of multiple components in gland extracts or emitted blends were reported for several species (Coffelt et al., 1978; Pope et al., 1982; Schal et al., 1987; Ono et al., 1990; Hunt and Haynes, 1990; Heath et al., 1991). These results show that in many cases blend ratios remain approximately constant but that the proportions among components that have different functional groups or different chain lengths may change.

The sex pheromone of *Helicoverpa assulta* was reported to consist of four components: hexadecanal (16:Al), (Z)-9-hexadecenal (Z9-16:Al), (Z)-11-hexadecenal (Z11-16:Al) and (Z)-9-hexadecenyl acetate (Z9-16:Ac) (Sugie et al., 1991; Cork et al., 1992). We report here hourly and daily changes of both calling activity and titers of the four sex pheromone components in the pheromone glands.

METHODS AND MATERIALS

Insects. Moths used in this study originated from a wild population collected in green pepper houses in Hasaki, Ibaraki Prefecture, Japan, and were maintained for successive generations in the laboratory at 23 ± 0.5°C under a reversed 15:9 hr light–dark photoperiod. Larvae were reared on an artificial diet for silkworm (Japan Chlorella, Tokyo: artificial diet for young larvae, wet type). Pupae were segregated by sex and adults were collected daily and maintained in separate cages according to emergence date and sex. Moths were supplied with 5% sugar solution. The age of moths was designated as age 0 on the day of emergence and age 1, age 2, etc., on subsequent days. All experiments were conducted under the same conditions as above.

Observation of Calling. Age 0 to age 7 virgin females were placed either in mesh cages (30 × 30 × 22 cm) in groups of marked moths (7–10 individuals of the same age in each cage) or held individually in clear plastic cups (200 ml). In both cases, moths were supplied with sugar solution in cottons. Since preliminary observations indicated that no moths called during the photophase, we observed moths only during the scotophase at 30-min intervals. For age 0

females, moths that emerged within the first 2 hr of the scotophase were observed from 2 hr after lights-off to lights-on. A female was scored as calling if the ovipositor was protruded from the abdomen, but females that showed obvious oviposition behavior were excluded. At least 20 moths were observed for each age.

More detailed observations were conducted using age 3 virgin females. Twenty-seven moths were placed in clear plastic cups individually with cotton containing sugar solution and observed during scotophase. The moths were observed at least once in every 5 min. Calling behavior of moths was classified into two categories: stationary calling and calling with wing-fanning, walking, or flight.

Pheromone Titer. Females were anesthetized with carbon dioxide, and the ovipositors were extruded by finger pressure and excised with a pair of fine scissors. A single ovipositor was extracted for an hour in 100 μl hexane containing 100 ng tridecyl acetate (13 : Ac) as internal standard and the solution was transferred into a hand-made glass conical vial by a Pasteur pipet. The ovipositor was rinsed twice with 50 μl hexane, and the rinses were combined. The gland extract was concentrated to ca. 1 μl under reduced pressure and injected onto a gas chromatograph (Shimazu GC-9A) with a FID and a splitless injector system. The column used was DB-23 (chemically bonded fused silica capillary column, 30 m $\times$ 0.25 mm ID, J&W Scientific). The column temperature was initially at 80°C for 2 min and then programmed to 200°C at 10°C/min. Nitrogen was used as a carrier gas at inlet pressure of 0.85 kg/cm^2. Each pheromone component in the extracts was identified from the retention time relative to that of 13 : Ac, previously measured with authentic compound, and quantified by comparing the peak area with that of 13 : Ac.

First, the hourly fluctuation pattern of pheromone titer in the pheromone gland was investigated. Individual ovipositors of age 3 to age 5 females were extracted at hourly intervals during scotophase and every other hour during photophase. More than 15 individuals were used for each hour. Quantification was conducted for the major component, Z9–16 : Al as well as the three minor components, 16 : Al, Z11–16 : Al, and Z9–16 : Ac. Quantification of minor components was conducted from lights-off to 2 hr after lights-on as these compounds were mostly below the detection limit for the rest of the time. Proportions of these compounds relative to that of Z9–16 : Al were also calculated.

Secondly, to investigate the daily changes in pheromone titer, individual ovipositors of age 0 to age 9 females were extracted between 3 and 5 hr after lights-off. More than 10 individuals were used for each age. The amount of pheromone present in gland of age 0 females was determined from extracts obtained 4 hr after lights-off. Quantification was conducted only for Z9–16 : Al.

RESULTS

Calling. Age 0 females rarely called and only after the next day were callings actively performed (Figure 1). Calling patterns of age 1 to age 7 females were similar to one another. In each case calling began within first 3 hr of scotophase and the percentage of females calling was maximal from 2 hr to 6 hr after lights-off and then decreased. A few moths performed calling near the end of scotophase but stopped calling as soon as the lights went on.

However, there were some differences in the calling behavior among females of age 1 to age 7. Firstly, age 5 females called longer than age 1 and age 2 females. We defined "calling count" as the total numbers of observations a day where the insect was calling (number of callings/day/individual). The average of calling counts of age 1 (5.8 $\pm$ 4.2) was not significantly different from that of age 2 (5.8 $\pm$ 4.2), and these were significantly lower than that of age 5 (8.1 $\pm$ 4.2). The averages of calling counts of age 3 (7.2 $\pm$ 3.8), age 4 (6.7 $\pm$ 3.5), age 6 (7.2 $\pm$ 3.7), and age 7 (7.1 $\pm$ 4.2) were neither significantly different

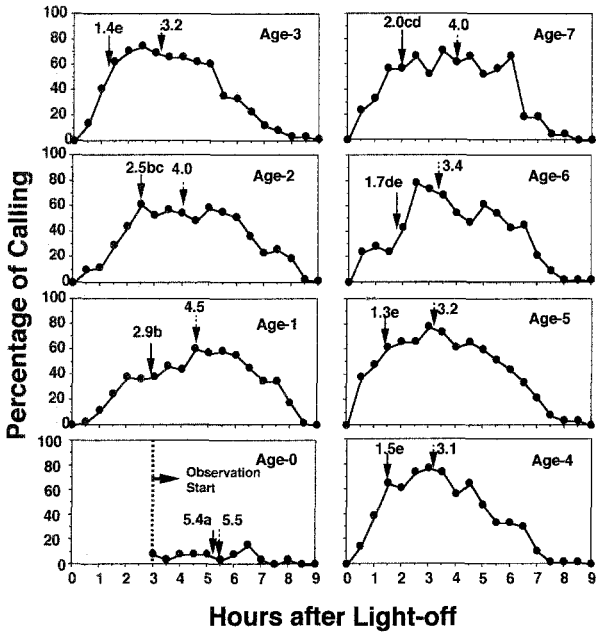

FIG. 1. Calling times of age 0 to age 7 *H. assulta* females.⟶ and the numbers above the arrows indicate the mean onset times of callings. Numbers followed by the same letters do not differ significantly (Duncan's new multiple range test, $P < 0.05$).⇢ and the numbers above the arrows indicate the mean calling times.

among themselves or the averages of groups of age 1, age 2 and age 5 (Duncan's new multiple rate test, $P < 0.05$).

Secondly, temporal calling patterns shifted with age. From age 1 to age 3, the onset calling time and the mean calling time advanced, and from age 5 to age 7 those two were delayed (Figure 1). The onset calling times were significantly different both between age 1, age 2, and age 3 and between age 5, age 6, and age 7 (Duncan's new multiple range test, $P < 0.05$).

This observation also showed that within a group of the same age there was a large variation in calling characteristics among individuals. To determine if this variation was inherent, the calling counts were recorded for successive days per individual. When moths of age 2 or age 3 were divided into two groups, one with calling counts higher than the average of their age (active groups), and the other with calling counts lower than the average (inactive groups) (9–19 individuals/group), the females in the active calling groups tended to continue active calling and those of the inactive groups demonstrated low calling counts for successive days (Figure 2). This result indicates that each moth had an inherent tendency toward the length of calling.

We conducted more detailed observations with age 3 females to investigate the temporal calling patterns of individuals. Considerable variation in the duration and spacing of calling bouts were observed. The total duration of calling (mean 243 min) varied greatly among individuals from 85.0 min to 472.5 min

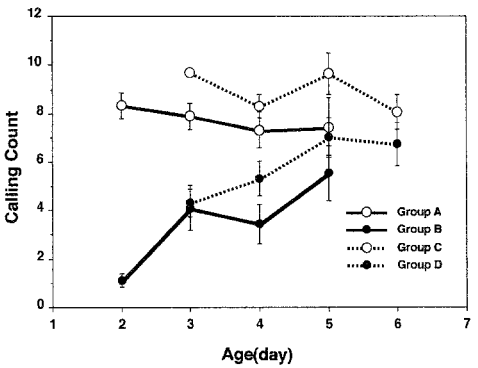

FIG. 2. Comparison of calling activity between active (A, C) and inactive (B, D) groups of *H. assulta* virgin females. Calling count represents the mean total number of callings counted in 19 observations in each day per individual. Group A: moths of which calling counts were more than the mean (5.8) at age 2 ($N = 9$). Group B: moths of which calling counts were less than the mean at age 2 ($N = 9$). Group C: moths of which calling counts were more than the mean (6.1) at age 3 ($N = 14$). Group D: moths of which calling counts were less than the mean at age 3 ($N = 19$). Calling counts of each group were recorded for successive four days. Vertical bars represent SE.

(moths that showed no calling behavior were excluded). The duration of each calling bout also varied greatly, ranging from shorter than 2.5 min to 400 min Calling bouts shorter than 5 min were most frequent and seen throughout the scotophase. Calling bouts longer than 60 min occupied more than half of the total time spent calling, although their frequency was small. These long calling bouts were seen mainly during late scotophase.

In the two calling categories (stationary calling and calling with wing-fanning, walking, or flight), stationary calling was most frequent, occupying 97.5% of total calling durations. In these cases, moths raised their wings at 45° angles to the body in a V shape. This may be a typical calling posture of this species. Callings with other movements were shorter than 5 min and those occupied only 2.5% of total calling duration.

Pheromone Titer. Extraction of pheromone glands at hourly intervals showed diel periodicity in the production of Z9-16:Al (Figure 3). Relatively small amounts of Z9-16:Al (19.8 ng/female, $N = 202$) were detected during photophase. The pheromone titer increased dramatically after lights-off and reached a maximum 2 hr later. The titer remained at this level for 4 hr and then decreased. The variation of quantities among individuals during the peak time was very large (Figure 4). The mean value was 135.7 ng ($N = 192$) and the titers ranged from 3.1 to 504.0 ng/female.

Quantification of the three minor components, 16:Al, Z11-16:Al and Z9-16:Ac, showed similar temporal patterns to that of Z9-16:Al (Figure 5A). Maximal titer was detected from 2 to 6 h after lights-off. However, the relative proportions of the minor components to Z9-16:Al were nearly constant for 16:Al (0.20) and Z11-16:Al (0.10). On the other hand, the proportion of Z9-16:Ac to Z9-16:Al increased from 5 hr after lights-off (0.05) to 2 hr after lights-on (0.36).

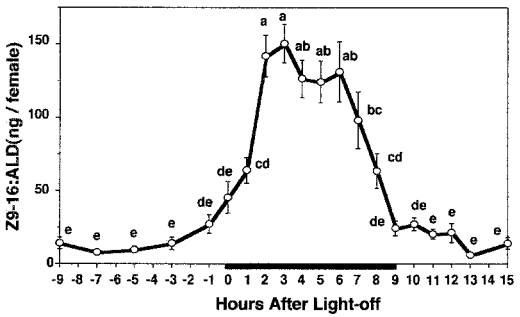

FIG. 3. Hourly change in titer of the major sex pheromone component, Z9-16:Al, of *H. assulta*, age 3 to age 5 females. Values followed by the same letters do not differ significantly (Duncan's new multiple range test, $P < 0.05$). Vertical bars represent SE. Solid bar under the abscissa indicates scotophase.

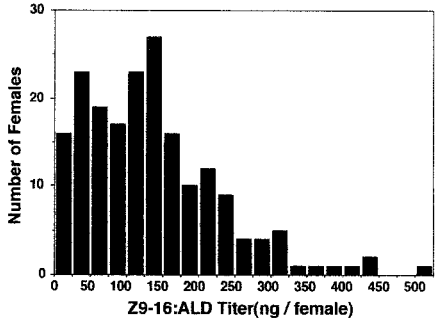

FIG. 4. Frequency distribution of Z9–16:Al titers in pheromone glands of *H. assulta*. Z9–16:Al titers of age 3 to age 5 virgin females were measured within 2–6 hr after lights-off, the peak time of pheromone titer. Titers of 192 females were measured.

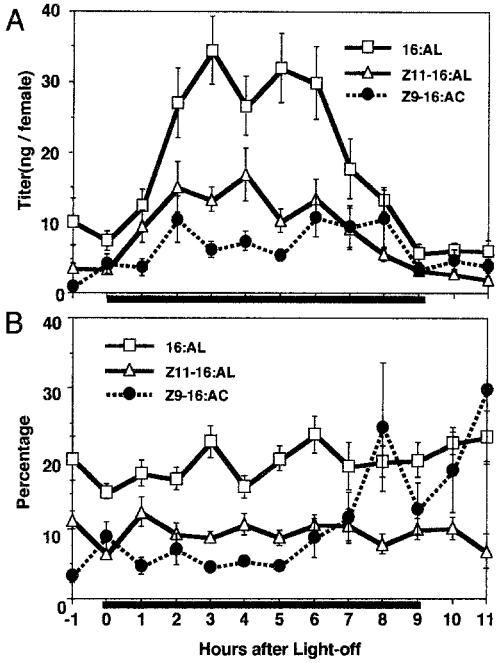

FIG. 5. Hourly changes in titers of the minor sex pheromone components of *H. assulta*. (B) Hourly changes in relative proportions of the titers of the minor components to the major component, Z9–16:Al. Vertical bars represent SE. Solid bars under the abscissa indicate scotophase.

The titer of Z9–16:Al significantly increased from age 0 to age 1, but there was no significant difference among the titers of age 1 to age 5 (Figure 6). After that, the titer decreased (Duncan's new multiple range test, $P < 0.05$).

DISCUSSION

Both calling activity and Z9–16:Al titer in pheromone glands showed distinct diel periodicity, and these two were synchronous (Figures 1 and 3). This is the case for many moth species (Fatzinger, 1973; Coffelt et al., 1978; Pope et al., 1982; Raina et al., 1986; Delisle and McNeil, 1987; Ramaswamy et al., 1988; Heath et al., 1991). Under a 15:9 hr light–dark photoperiod condition at 23 ± 0.5°C, *H. assulta* females actively called and the maximal pheromone titer was detected from 2 to 6 hr after lights-off. No calling was shown and only a relatively small amount of Z9–16:Al was present in the pheromone gland during photophase. This species seems to produce sex pheromone only during scotophase and releases it soon after production.

Nevertheless, there was a time lag of a few days between maxima of calling activity and pheromone titer. The pheromone titer was maximal from age 1 to age 5 and thereafter decreased (Figure 6), whereas calling was most actively performed after age 3 (Figure 1). Interestingly, age 0 females hardly performed calling and age 6 and age 7 females actively called, although they had approximately the same amount of Z9–16:Al. This time lag may be due to the difference of controlling systems of these two events (Hollander and Yin, 1982; Ohguchi et al., 1985).

The onset time of calling and the mean calling time advanced from age 0 to age 3 and were delayed from age 5 to age 7 (Figure 1). The advance of calling was commonly seen in moth species such as *Agrotis ipsilon* (Swier et

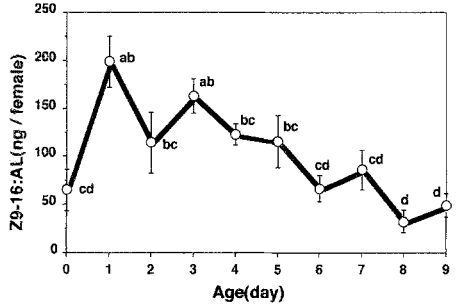

FIG. 6. Daily changes in titer of the major sex pheromone components, Z9–16:Al, of *H. assulta*. Values followed by the same letters do not differ significantly (Duncan's new multiple range test, $P < 0.05$). Vertical bars represent SE.

al., 1977), *Chilo suppressalis* (Kanno, 1979), *Pseudaletia unipuncta* (Turgeon and McNeil, 1982), and *Helicoverpa armigera* (Kou and Chow, 1987). This time shift is explained as the adaptation of older females to have more chances of mating than younger ones (Swier et al., 1977). This seems to be the case in *H. assulta*. We cannot explain why calling was delayed from age 5 to age 7 females. The old females may have some abnormality in calling behavior because *H. assulta* females usually mate within the first several days after emergence under laboratory conditions (unpublished data). The result that age 6 and 7 females had only small amounts of Z9–16:Al seems to support this assumption.

Detailed observations showed that stationary calling with the wings raised at a 45° angle to the body in a V shape was the typical calling posture of *H. assulta*. This is similar to that of *Helicoverpa zea* except for wing-fanning (Agee, 1969) and that of *Heliothis virescens* (Teal et al., 1981), but the scent-marking behavior that separates the first phase and the second phase of calling in *H. virescens* (Teal et al., 1981) was not seen in this species.

H. assulta utilizes four compounds, 16:Al, Z9–16:Al, Z11–16:Al, and Z9–16:Ac as the sex pheromone blend. Sugie et al. (1991) showed that the pheromone gland of *H. assulta* contained this blend in a ratio of 6:100:10:15 during mid-scotophase and this blend was effective to attract males. Daily fluctuation patterns of the minor components were similar to those of Z9–16:Al (Figure 5A). Maximal titers were detected during mid-scotophase. Nevertheless, the ratio of the minor components to Z9–16:Al was relatively constant for the aldehydes and increased in late scotophase for the acetate (Figure 5B). Cork et al. (1992) also reported a great increase of the Z9–16:Ac/Z9–16:Al ratio between mid-scotophase and late scotophase in the Korean strain of this species. In *Heliothis subflexa*, the alcohol–aldehyde ratio and acetate–aldehyde ratio in (Z)–11 isomers change temporally (Heath et al., 1991). In that study the changes were explained by the differences of activity of oxidase and esterase.The differences of activities of enzymes may also explain the temporal changes of the Z9–16:Ac/Z9–16:Al ratio and the lack of changes of 16:Al/Z9–16:Al and Z11–16:Al/Z9–16:Al ratios in *H. assulta*.

Both calling activity and pheromone titer showed very large variations among individuals. Similar large variations in calling behavior were also reported for *Pseudaletia unipuncta* (Turgeon and McNeil, 1982) and *Helicoverpa armigera* (Kou and Chow, 1987). In these two moths the first day of calling ranged widely, so durations of calling of individuals varied greatly with respect to chronological age. However, this assumption does not explain the variations of *H. assulta* because more than 85% of age 1 females performed calling at least once (data were not shown). On the other hand, when calling counts (see Results) were recorded individually for successive days, we could show that the females that performed calling for longer times at early ages continued to do so and vice versa (Figure 2). These results suggest that in this species the large variation of

calling time was not due to the variations of the first day of calling as seen in *P. unipuncta* and *H. armigera* or to the variation seen within one day but rather to inherent tendencies to perform calling for long or short durations.

It was found that both the calling activity and pheromone titer of *H. assulta* show distinct diel periodicity and these two are synchronous. Further experiments are in progress to investigate the controlling system of these two physiological events.

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REPELLENCY TO TWO-SPOTTED SPIDER MITE, *Tetranychus urticae* KOCH, AS RELATED TO LEAF SURFACE CHEMISTRY OF *Lycopersicon hirsutum* ACCESSIONS

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Abstract—Bioassays employing *Tetranychus urticae* Koch were used to examine repellency of leaves and trichome secretions of one cultivar of tomato, *Lycopersicon esculentum*, and 11 accessions of *L. hirsutum* consisting of seven accessions of *L. hirsutum* f. *typicum* and four accessions of *L. hirsutum* f. *glabratum*. Leaves of f. *typicum* were more repellent to mites than were those of f. *glabratum* or *L. esculentum*. Removal of trichomes and their secretions by wiping leaves of f. *typicum* accessions with 95% ethanol rendered them less repellent, indicating that trichomes or their secretions were potentially responsible for repellency. Gas and thin-layer chromatography of leaflet washes obtained by steeping leaflets in hexane indicated that the chemical composition of the washes differed among accessions. The main difference between botanical forms was the presence in leaflet washes from f. *typicum* accessions of compounds eluting late on the gas chromatograph and migrating further on reverse-phase thin-layer chromatography; compounds having these characteristics were absent in washes of f. *glabratum* and *L. esculentum*. The compositions of secretions taken directly from type I, type IV, and type VI trichomes were similar to that of the leaflet wash obtained from the same f. *typicum* plant, indicating that leaflet washes contained mainly compounds of trichomal origin. Leaflet washes from f. *typicum* plants were generally more repellent to *T. urticae* than were washes from f. *glabratum* when tested by choice and nonchoice bioassays. Taken together, the data indicate that tri-

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chome secretions of f. *typicum* are likely responsible for potent repellency to two-spotted spider mites observed in this taxon; the likely active compounds are sesquiterpene acids or other polar constituents of trichome secretions.

Key Words—Tomato, *Lycopersicon esculentum*, *L. hirsutum* f. *glabratum*, *L. hirsutum* f. *typicum*, deterrence, bioassay, sesquiterpene, 2-tridecanone, volatiles, host-resistance, trichomes, antixenosis, allomone.

INTRODUCTION

Lycopersicon hirsutum Humb. & Bonpl., a relative of the common tomato, *L. esculentum*, is a genetically diverse species (Miller and Tanksley, 1990) and is highly resistant to at least 19 species of arthropod pests of tomato (Farrar and Kennedy, 1992). In those cases where causes of resistance have been investigated in detail, trichomes and the chemical composition of trichome secretions have often been implicated (Farrar and Kennedy, 1992).

In the United States approximately 150 accessions of *L. hirsutum* are maintained for distribution to scientists. Of these accessions, approximately one third are maintained and distributed by the USDA Plant Introduction Station, formerly at Ames, Iowa (USDA) and now at Geneva, New York. The remainder are maintained and distributed by the Tomato Genetic Stock Center (TGSC) at Davis, California. Accessions distributed by the USDA carry a prefix of PI and those distributed by the TGSC carry a prefix of LA. Although resistance to many of the major insect and mite pests of tomato occurs in *L. hirsutum*, nearly 80% of the accessions available in the United States have never been systematically examined for insect resistance. In fact, a single accession of *L. hirsutum* f. *glabratum*, PI 134417, has received most of the attention related to pest resistance of *Lycopersicon* spp. (Farrar and Kennedy, 1992, and references therein). The presence of 2-tridecanone and 2-undecanone in type VI trichomes of PI 134417 has been associated with antibiosis to several major pests of tomato. Factors present in leaves have also been implicated in resistance.

Even though resistance of f. *glabratum* accession PI 134417 has been extensively researched, this accession does not represent the entire spectrum of resistance available in the species. Previously we reported that among 10 accessions of *L. hirsutum*, which exists as two botanical forms (f. *typicum* and f. *glabratum* Mull.), accessions of f. *glabratum* were less resistant to two-spotted spider mites (*Tetranychus urticae* Koch) than were accessions of f. *typicum* (Weston et al., 1989). Resistance was manifested by more movement of mites from an infested bean leaf onto leaves of f. *glabratum* accessions compared with movement onto leaves of the more resistant f. *typicum* accessions. Differences in resistance between botanic forms were not explained by differences in type IV trichome density, a primary determinant of mite resistance in f. *typicum* (Good and Snyder, 1988). Composition of trichome secretions, in terms of the

major components—2-undecanone and 2-tridecanone for f. *glabratum* accessions and the sesquiterpene hydrocarbons zingiberene and elemene for f. *typicum* accessions—differed between forms, but quantities of these major components of secretions were not highly correlated with resistance (Weston et al., 1989), indicating that these major components were probably not responsible for the greater resistance of these f. *typicum* accessions. Subsequently we demonstrated, by use of the thumbtack bioassay, that leaflets of one accession of f. *typicum*, PI 251303, were more repellent to spider mites than were leaflets of f. *glabratum* PI 134417 (Weston and Snyder, 1990).

Because of unanticipated similarity in composition of trichome secretions among accessions of a botanical form acquired from the USDA (Weston et al., 1989), we decided to investigate the potential for greater variation in the composition of trichome secretions among accessions available from the TGSC. An unpublished, limited survey of some of these accessions revealed that variation in composition of trichome secretions among these accessions was greater than that observed in accessions obtained from USDA. Greater variability among accessions acquired from the TGSC was not unexpected because these accessions were collected throughout the native range of *L. hirsutum* and have since been increased and maintained in a manner that ensures minimal loss of genetic variation. Similar assurances can not be provided for the accessions acquired from USDA.

Because we had identified heterogeneity in composition of trichome secretions among accessions acquired from the TGSC and had also demonstrated that leaves of one accession of f. *typicum*, PI 251303, were more repellent to *T. urticae* that were leaves from one accession of f. *glabratum*, PI 134417, we undertook this research to test the hypothesis that leaves of f. *typicum* are generally more repellent to spider mites than are those of f. *glabratum* and to determine whether or not trichome secretions were responsible for the differences in repellency between botanic forms of *L. hirsutum*.

METHODS AND MATERIALS

Plant Material. Seeds of seven f. *typicum* accessions (LA 1298, LA 1353, LA 1363, LA 1777, LA 1927, LA 2155, and LA 2329) and four f. *glabratum* accessions (LA 407, LA 1223, LA 1265, and LA 2144) were obtained from the TGSC and those of *L. esculentum* Ace 55 were obtained from W. Atlee Burpee Co. Seeds were sown in the greenhouse in April 1989 in styrofoam cells filled with Pro Mix BX (Premier Brands, New Rochelle, New York) and set in the field in May. In August, leaflet washes were obtained from these plants using procedures outlined below.

To obtain additional washes and to provide leaves for bioassay, the 11

accessions and Ace 55 were also grown in the greenhouse in fall 1990. Seeded on August 17, seedlings were set individually in 8-in pots filled with Pro Mix BX. Greenhouse temperature was set at 25/18°C (day/night). No artificial light was provided. Leaflet washes were prepared as outlined below. When leaflets from these plants were selected for bioassays, leaf development was noted by designating the youngest leaf with the smallest major leaflet 2 cm wide as position A, with each successively older leaf designated B, C, etc.

Mite Colony. Mites were lab-reared and handled using the methods of Weston and Snyder (1990). When needed, mites of uniform age (staged mites) were raised by transferring about 30 adult female mites to a mite-free bean seedling (*Phaseolus vulgaris* Dwarf Horticultural) having half-expanded unifoliate leaves. Transferred females were allowed to lay eggs for 5 hr and then were blown off with compressed air. Tanglefoot (The Tanglefoot Co., Grand Rapids, Michigan) applied to stems of these seedlings prevented uncontrolled mite infestation. Plants were examined once a day for three days after inoculation, and any adult mites discovered were removed. Mites hatched in about two days. Female mites 6 days old were used for the ring bioassay described later.

Thumbtack Bioassay. These bioassays followed procedures described by Weston and Snyder (1990). In August 1991, four leaves from position C were detached from plants of each of the 11 accessions and Ace 55 grown in the greenhouse. To maintain turgor during bioassay, leaf petioles were inserted into small vials filled with water. Leaves were placed abaxial surface down on styrofoam boards and were fastened to the board with a thumbtack in the middle area of each of four leaflets. Ten mites were transferred to each thumbtack. The number of mites remaining on the thumbtack at 30 min, 4 hr, and 8 hr was recorded. After bioassay, trichomes were counted on the adaxial surface of each assayed leaflet in four 1-mm^2 interveinal areas with the aid of microscope (100×). ANOVA was performed on transformed mite response data [$(X + 0.5)^{1/2}$)] and densities of type IV and VI trichomes. Least significant difference (LSD) was used to compare means among accessions.

Leaflet Washes. Fully expanded, healthy leaflets were collected from plants of each accession and steeped in hexane (3 ml/leaflet) for 60 min, with agitation every 15 min. After decantation, the leaflets were briefly steeped in hexane (1 ml/leaflet) twice. The washes were combined, filtered through Whatman No. 1 filter paper, and then dried with anhydrous Na_2SO_4. After evaporating solvent with a rotary evaporator, the final concentration was brought to 1 ml of hexane per leaflet. This concentration is referred to as 1×. Washes were stored in glass vials at −80°C.

Removal of Trichome Secretions from Leaflet Surfaces. Absorbent cotton balls were dipped in one of the following: 95% ethanol, 50% ethanol, pure methanol, 50% methanol, pure acetonitrile, 50% acetonitrile, pure acetone, or

50% acetone. Leaflet surfaces of LA 1363 were swabbed three times with each solution and then blotted with a Kimwipe (Kimberly-Clark Co.). Opposing leaflets of a pair, one wiped and the other intact, were then extracted as described above. Aliquots (2 μl) were analyzed by gas chromatography (detail follows) to determine whether or not hexane-soluble volatiles were removed from leaflets. Percentage removed by each solvent was calculated.

Repellency of Intact and Wiped Leaves. For this experiment, conducted during summer 1991, five f. *typicum* accessions were chosen because they had greatest resistance at the 4-hr sample in the previous thumbtack bioassay. Leaves from position C were removed from the plants, and the adaxial surface of one leaflet of a pair was wiped with cotton balls wetted with 95% ethanol. The wiped surface and the adaxial surface of the unwiped opposing leaflet were then bioassayed using the thumbtack bioassay. Ten pairs of leaflets from each accession served as replications. Numbers of mites on the thumbtacks at 10 min were recorded, and data were analyzed with the paired t test.

Spring-Board Bioassay. This bioassay, designed to assess repellency of leaflet washes, consisted of paper strips 5 mm wide and 5 cm long cut from Whatman No. 1 filter paper. In the middle area of the strips, two faint lines parallel to each other and 12 mm apart were drawn across both surfaces of the strip with a pencil. One end of each strip was clamped above a mirror, which allowed us to monitor movement of a mite on the lower surfaces of the strips. On two such strips, 2 μl of 1× wash was distributed with a micropipet along the pencil line nearer to the clamp on one strip, and another 2 μl was distributed on the line further away from the clamp on the other strip. Care was taken to ensure even distribution and minimal diffusion. Two microliters of hexane was distributed on the other line on each strip as control. After solvent evaporation, a mite was gently transferred with a brush to the area between the two lines on each strip. Movement of the mite was carefully observed, and the line over which it exited was recorded. The exiting mite was removed and another mite was immediately transferred to the area between the two lines. On each strip, 15–20 mites were assayed. Two such strips having the sample loaded on different positions (line nearer to or further away from the clamp) constituted an assay, so a total of 30–40 mites were used for assaying a sample. Exit ratios on the two strips were compared using χ^2. In all cases, performance was similar between the two strips, so the data were combined and the significance of the deviation from an expected 1 : 1 exit ratio was determined by χ^2.

Ring Bioassay. This procedure, also designed to assess the repellency of trichome secretions, consisted of paper rings (1.3 cm ID, 1.7 cm OD) cut from Whatman No. 1 filter paper. The area of such a ring is about 1 cm^2. Six microliters of 1× wash from field-grown plants was distributed evenly on each of three rings, and 6 μl of hexane was distributed on another ring as a control. After the hexane evaporated, the four rings were placed in the bottom of a glass

Petri dish lined with filter paper. One staged mite was transferred with a brush to the center of each ring. When a mite escaped over the ring or was inactive for more than 1 min, it was replaced by another. During the bioassay (minimum duration 30 min), mite activity was recorded on videotape. Afterwards the videotape was played and the retention time of each mite in each ring was determined. The data for the three rings loaded with leaflet wash were pooled, and a *t* test was used to determine if the average retention time of mites placed in rings loaded with 1× wash was statistically different from that of the hexane control.

GC Analyses. The volatile profiles of the 1× washes were determined by gas chromatography (GC). A Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector (FID) and a 30-m × 0.25-mm RTX-5 column (Restek, Bellefonte, Pennsylvania) was used. The carrier gas was He flowing at 10.0 ml/min and the head pressure was 90 kPa. The following temperatures were used: injector, 240°C; detector 300°C; oven program, 100°C for 4 min, then increasing at 10°C/min to 250°C. One-microliter samples were injected, and the split ratio was 1:8.

Leaflet wash from the f. *typicum* accession PI 251303 and purchased 2-methyl ketones (Alltech Associates, Deerfield, Illinois) served as standards. Three sesquiterpene hydrocarbons, zingiberene, curcumene, and γ-elemene (Weston et al., 1989), and two sesquiterpenoid acids have been identified in the leaflet washes of PI 251303 (unpublished data).

To obtain volatile profiles for individual types of trichomes, about 10 type I, 20 type IV, or 6 type VI trichome tips from 80-day-old plants grown in the greenhouse were collected with the aid of a 10-μl syringe and a stereomicroscope (100×). Trichome tips were picked up with the syringe needle, which was then rinsed in a 25-μl micropipet containing 3 μl of hexane. The volatile profile of the 3 μl was determined by GC-FID on a 1.2-μm, 10-m × 0.54-mm RSL-150 column (Alltech Associates). Helium was the carrier gas flowing at 3 ml/min. The following temperatures were used: injector, 240°C; detector, 300°C; oven, 100°C for 1 min, then increasing at 6°C/min to 170°C and then at 10°C/min to 250°C. In order to compare volatile profiles from different types of trichomes on the same accession, the chromatograms for the leaflet wash and trichome secretions from an accession were visually compared and the percentage abundances of the three most prevalent compounds detected in greatest abundance were calculated, based on integrator area units.

Thin-Layer Chromatography (TLC). Reverse-phase TLC was conducted on C_{18} plates (Whatman $KC_{18}F$). Samples were prepared by evaporating hexane from the 1× leaflet washes, weighing the residue, and then adding sufficient hexane to the residue to provide a concentration of 2 mg/ml. Forty micrograms of each sample was spotted on the TLC plate. After developing the plate with acetonitrile–water (80:20), visualization was accomplished by staining with anisaldehyde/H_2SO_4 (Krebs et al., 1963), which stains sesquiterpene hydrocar-

bons dark blue, 2-methyl ketones blue–green, and glucose and sucrose acyl esters and sesquiterpene acids brown. The R_f and color of each detected spot was recorded. Relative abundance of each detected spot was visually estimated using a three-point scale based on density of color of the spot and its area. Known sesquiterpene hydrocarbons and their acids, isolated from *L. hirsutum* f. *typicum* PI 251303, and purchased 2-undecanone and 2-tridecanone (Pfaltz & Bauer) served as standards. When compounds were present with R_f values and colors similar to those of known compounds, the R_f regions were scraped from replicate plates and then eluted with hexane. The composition of the R_f region was determined by coelution on the gas chromatograph with authentic compounds.

RESULTS

Thumbtack Bioassay. In general, mites remained on the thumbtacks longer on f. *typicum* accessions than on f. *glabratum* accessions or on the *L. esculentum* entry Ace 55 (Table 1). This indicated that leaflets of f. *typicum* were more

TABLE 1. DENSITIES OF TYPE IV AND VI TRICHOMES ON LEAFLETS AND REPELLENCY OF LEAFLETS FROM 11 *Lycopersicon hirsutum* ACCESSIONS AND ONE CULTIVAR OF *L. esculentum* AS MEASURED BY THUMBTACK BIOASSAY[a]

		Average number of mites remaining on thumbtacks at indicated time[c]			Trichome densities (N/mm^2)	
Accession	Taxon[b]	30 min	4 hr	8 hr	Type IV	Type VI
LA 1298	*typ*	9.5 A	8.5 A	8.5 AB	16 C	4 EF
LA 1353	*typ*	9.0 A	7.5 A	2.4 B	102 A	7 B
LA 1363	*typ*	8.5 A	6.0 B	5.5 B	6 DEF	6 C
LA 1777	*typ*	8.5 A	8.5 A	7.5 A	30 B	9 B
LA 1927	*typ*	8.0 A	3.5 C	3.0 C	10 CDE	10 B
LA 2155	*typ*	5.0 B	0.0 D	0.0 D	2 FG	5 D
LA 2329	*typ*	9.5 A	7.0 A	6.0 AB	8 DEF	8 B
LA 407	*gla*	4.0 B	0.0 D	0.0 D	11 CD	16 A
LA 1223	*gla*	0.5 C	0.0 D	0.0 D	1 FG	4 F
LA 1265	*gla*	1.0 C	0.0 D	0.0 D	4 EFG	2 F
LA 2144	*gla*	0.5 D	0.0 D	0.0 D	1 G	5 D
'Ace 55'	*esc*	0.0 D	0.0 D	0.0 D	0 G	4 E

[a] Ten mites were initially placed on each tack.
[b] *typ* = *L. hirsutum* f. *typicum*, *gla* = *L. hirsutum* f. *glabratum*, *esc* = *L. esculentum*.
[c] Means in a column sharing the same letter are not significantly different at 5%.

repellent than those of f. *glabratum* or Ace 55. Of all f. *typicum* accessions, leaflets of LA 2155 were least repellent and also had the lowest type IV trichome density. Type VI trichome densities ranged from 2 to 16/mm^2, and type IV trichome densities ranged from 0 to 102/mm^2.

Deterrence of Wiped and Intact Leaflets of f. typicum. Wiping leaflet surfaces of LA 1363 with cotton balls wetted with different solvents removed most of the hexane-soluble, volatile compounds. Acetonitrile, ethanol, methanol, and acetone, whether concentrated or diluted 50% with water, each removed more than 96% of leaflet volatiles (data not shown). Uniformly, mites left thumbtacks more quickly on leaflets that had been wiped with 95% ethanol than on intact leaflets (Table 2). This indicated that intact leaflets were more repellent than those having trichomes and secretions removed by wiping.

Chemical Profiles of Leaflet Washes and Trichomes. Chemical profiles, determined by GC and TLC, differed between forms and among accessions of a form. However, some components were similar among accessions. Washes from all f. *typicum* accessions except LA 1353 and from the f. *glabratum* accession LA 1223 possessed volatiles having retention times on GC-FID (data not shown) and R_f values from TLC identical to known sesquiterpene hydrocarbons (Table 3). Washes from f. *typicum* accession LA 1353 and from f. *glabratum* accessions LA 407 and LA 1223 contained compounds having retention times and R_f values identical to 2-undecanone and/or 2-tridecanone. Few compounds were detected in leaflet washes from f. *glabratum* accessions LA 2144 and 1265, or from Ace 55. In addition to the above differences, f. *typicum* accessions, with the exception of LA 1353, were distinguished from f. *glabratum* accessions by the presence of compounds in their leaflet washes having long retention times on the GC (data not shown) indicative of higher boiling

TABLE 2. AVERAGE NUMBER OF MITES REMAINING ON THUMBTACKS ON 10 PAIRS OF WIPED AND UNWIPED LEAFLETS FROM EACH OF 5 *Lycopersicon hirsutum* f. *typicum* ACCESSIONS[a]

Accession	Average number of mites on tack at 10 min		t value[b]
	Intact leaflet	Wiped leaflet	
LA 1298	6.3	0.4	2.8*
LA 1353	9.5	1.5	5.4**
LA 1363	5.3	1.3	3.5**
LA 1777	6.9	1.3	2.3*
LA 2329	5.1	0.8	2.2*

[a] Ten mites were initially placed on each tack.
[b] *, ** significant at 5% and 1%, respectively.

TABLE 3. R_f VALUES AND RELATIVE ABUNDANCE[a] OF COMPOUNDS PRESENT IN LEAFLET WASHES OF 11 *Lycopersion hirsutum* ACCESSIONS AND ONE CULTIVAR OF *L. esculentum* SEPARATED BY C_{18} REVERSE-PHASE THIN-LAYER CHROMATOGRAPHY

		Taxon[b]											
		typ							*gla*				*esc*
R_f	Tentative ID	LA 1298	LA 1353	LA 1363	LA 1777	LA 1927	LA 2155	LA 2329	LA 407	LA 2144	LA 1265	LA 1223	Ace 55
0.08	Sesquiterpene hydrocarbons[c]	+++	+		++	++	+	+				+	
0.13	2-tridecanone		+						++				
0.18													+
0.22		+		++	+	+	+		+	+	+	+	+
0.24	2-undecanone		+++						+++			+	
0.30			+				+		+	+	+	+	+
0.32	Sesquiterpene acid[d]				+++								
0.33				+++		++							
0.35	Sesquiterpene acid[d]	++			+++	+	++						
0.39								+					
0.45								++					
0.51								+					
0.63		+		+		+							

[a]The number of + symbols indicates relative abundance from low (+) to high (+++).
[b]*typ* = *L. hirsutum* f. *typicum*, *gla* = *L. hirsutum* f. *glabratum*, *esc* = *L. esculentum*.
[c]Determined by Weston et al. (1989).
[d]Determined by Coates et al. (1988).

points, compared to the sesquiterpene hydrocarbons or the methyl ketones, and larger R_f values on reverse-phase TLC, indicating their more polar character (Table 3).

The volatile profiles of type I, type IV, and type VI trichomes for an accession were similar to each other and to that of the leaflet wash from the same plant. Visual inspection of chromatograms indicated that chemical profiles of trichome tips closely matched those of the leaflet washes from the same plant (data not shown). Relative abundance of major components was similar among secretions obtained directly from the three types of trichomes and leaflet wash from the same accession (Table 4), suggesting that trichome secretions were similar among trichome types and supporting the finding of Weston et al. (1989) that volatiles present in leaflet washes contain compounds mainly associated with trichomes.

Spring-Board Bioassay. Leaflet washes from f. *typicum* accessions except LA 1353 were strongly repellent; all washes from f. *glabratum* accessions and that from Ace 55 were not repellent (Table 5). The amount of residue per leaflet differed among accessions, but no consistent trend between amount of residue per leaflet and repellency was detected.

TABLE 4. RETENTION TIME AS DETERMINED BY GAS CHROMATOGRAPHY AND RELATIVE ABUNDANCE OF THREE COMPOUNDS PRESENT IN GREATEST ABUNDANCE IN LEAFLET WASHES AND SECRETIONS TAKEN DIRECTLY FROM 3 DIFFERENT TYPES OF TRICHOMES ON 5 ACCESSIONS OF *L. hirsutum* f. *typicum*

		Origin of compound			
		Trichome source			
Accession	Retention time	I	IV	VI	Leaflet wash
		Percent abundance			
LA 1298	8.00	66	60	64	62
	9.03	13	9	9	11
	14.29	17	21	12	15
LA 1363	9.18	3	6	2	4
	12.51	89	65	73	75
	13.23	3	5	5	5
LA 1777	9.03	15	13	12	14
	13.38	30	33	33	32
	13.86	31	25	25	28
LA 1927	9.05	11	6	15	12
	12.49	81	65	66	78
	13.25	4	3	3	5

TABLE 5. REPELLENCY OF 1× LEAFLET WASHES FROM 11 *Lycopersicon hirsutum* ACCESSIONS AND ONE *L. esculentum* CULTIVAR AS MEASURED BY SPRING-BOARD BIOASSAY[a]

Accession	Taxon[b]	Residue per leaflet (mg)	Exit ratio	χ^2 value
LA 1298	*typ*	0.4	7:23	4.4**[c]
LA 1353	*typ*	0.3	11:19	1.1[ns]
LA 1363	*typ*	0.2	3:27	9.6**
LA 1777	*typ*	1.3	2:28	11.3**
LA 1927	*typ*	0.2	4:26	8.1**
LA 2155	*typ*	0.4	5:25	6.7**
LA 2329	*typ*	1.2	1:29	13.1**
LA 407	*gla*	0.2	11:19	1.1[ns]
LA 1223	*gla*	0.2	10:20	1.7[ns]
LA 1265	*gla*	0.2	12:18	<1[ns]
LA 2144	*gla*	0.3	14:16	<1[ns]
'Ace 55'	*esc*	0.2	14:16	<1[ns]

[a] The exit ratio is the ratio of the number of mites exiting over the leaflet wash and the number exiting over the hexane control. The expected exit ratio used for calculating χ^2 was 1:1.
[b] *typ* = *L. hirsutum* f. *typicum*, *gla* = *L. hirsutum* f. *glabratum*, *esc* = *L. esculentum*.
[c] ns, ** Not significant, and significant at 1%, respectively.

Ring Bioassay. Results from the ring bioassay (Table 6) generally coincided with those from the spring-board bioassay. Among the f. *typicum* accessions, the wash from the exceptional f. *typicum* accession LA 1353 was not repellent; washes from the other six f. *typicum* accessions were repellent. Washes from f. *glabratum* accessions except LA 2144 were not repellent, nor was that from Ace 55. The average time spent by mites inside rings treated with the wash from LA 2144 was very similar to those for other f. *glabratum* accessions and was much shorter than those for f. *typicum* accessions; the significant difference in time spent inside control and treated rings for this accession arose from the atypically short tenure time of mites within the control ring.

DISCUSSION

Results from the thumbtack bioassay indicated that, in general, leaflets of f. *typicum* accessions were more repellent to *T. urticae* than were leaflets of f. *glabratum* accessions or those of *L. esculentum*. LA 2155 was an exception to this generalization, having leaves that were less repellent than other f. *typicum* accessions and no more repellent than the most repellent f. *glabratum* accession, LA 407. These results confirm the observations of Weston et al. (1989) that f. *typicum* accessions are generally more repellent to *T. urticae* than are f. *gla-*

TABLE 6. REPELLENCY OF 1× LEAFLET WASHES OF 11 *Lycopersicon* ACCESSIONS AND ONE *L. esculentum* CULTIVAR AS MEASURED BY RING BIOASSAY[a]

		Leaflet wash		Control (hexane)		
Accession	Taxon[b]	*N*	Mean retention time ± SE (sec)	*N*	Mean retention time ± SE (sec)	*t* value
LA 1298	*typ*	9	257 ± 250	10	55 ± 39	2.4**[c]
LA 1353	*typ*	30	92 ± 78	11	73 ± 79	<1[ns]
LA 1363	*typ*	21	124 ± 120	26	32 ± 41	3.4**
LA 1777	*typ*	8	664 ± 585	19	53 ± 38	3.0**
LA 1927	*typ*	16	257 ± 242	10	28 ± 19	3.8**
LA 2155	*typ*	10	312 ± 218	14	29 ± 41	4.1**
LA 2329	*typ*	23	217 ± 189	10	28 ± 19	3.8**
LA 407	*gla*	22	100 ± 83	9	124 ± 143	<1[ns]
LA 1223	*gla*	28	110 ± 76	10	122 ± 105	<1[ns]
LA 1265	*gla*	32	61 ± 50	20	51 ± 33	<1[ns]
LA 2144	*gla*	26	84 ± 77	12	40 ± 34	2.5*
'Ace 55'	*esc*	15	38 ± 27	7	23 ± 12	1.6[ns]

[a] The number of mites assayed = *N*.
[b] *typ* = *L. hirsutum* f. *typicum*, *gla* = *L. hirsutum* f. *glabratum*, *esc* = *L. esculentum*.
[c] ns, ** Not significant, significant at 5% and 1%, respectively.

bratum accessions, extending the conclusion to a more diverse sample of germplasm.

A sequence of observations supports the conclusion that the quality and/or quantity of trichome secretions were at least partly responsible for the greater repellency of f. *typicum* leaflets. Wiped leaflets of f. *typicum* were less repellent than intact leaflets, implicating trichomes or trichome secretions as possible causes of repellency. The compositions of secretions collected directly from trichomes on an accession were similar to each other and similar to the composition of the leaflet wash from that accession. Leaflet washes from f. *typicum*, except that from LA 1353, were more repellent to *T. urticae* than were those from f. *glabratum* accessions or *L. esculentum*.

Because composition of leaflet washes differed among accessions and between botanical forms, it is likely that more than one compound was responsible for repellency. The main difference in the composition of washes between forms was the presence of compounds in washes from f. *typicum* accessions having higher boiling points, as indicated by their longer retention time on the GC column, and greater polarity, as indicated by their greater mobility on reverse-phase TLC. Compounds having these characteristics were largely absent,

as were compounds having characteristics of sesquiterpene hydrocarbons, in washes obtained from f. *glabratum* accessions. It seems likely that these more polar compounds account for the greater repellency of f. *typicum* accessions. Diversity in composition of washes from f. *typicum*, as judged by GC and TLC, was mainly associated with diversity among these more polar compounds. Diversity in composition of washes among f. *glabratum* accessions was mainly associated with presence or absence of 2-undecanone and 2-tridecanone. In fact, abundance of methyl ketones and volatiles in general was low in two f. *glabratum* accessions (LA 2144 and LA 1265).

The presence of compounds in washes from f. *typicum* accessions having greater polarity than the 2-methyl ketones was anticipated because carboxylic acids of sesquiterpene hydrocarbons are known to occur in trichome secretions of LA 1777 (Coates et al., 1988) and PI 251303 (unpublished data). The f. *typicum* accession LA 1353 was an exception to the generalization that leaflet washes from f. *typicum* leaves contained these more polar compounds. Washes from this accession contained 2-undecanone and probably a sesquiterpene hydrocarbon, indicating that the accession may have characteristics of both botanic forms. In fact, the flower structure on this accession is intermediate, lending credence to this hypothesis. Furthermore, like washes from all f. *glabratum* accessions tested, washes from this accession were not repellent, supporting the earlier suggestion that the presence of the polar compounds may be a cause of repellency.

Taken together, the data support the hypothesis that f. *typicum* accessions are more repellent to *T. urticae* by virtue of the quality of their trichome secretions, especially in regard to the presence of polar constituents in the secretions. The lack of a clear trend between quantity of secretions and repellency suggests that quantitative differences in trichome secretions among the accessions tested play little role in the degree of repellency. The relationships between quantity, composition, and repellency are under investigation and necessitate the development of a quantitative bioassay for repellency.

Repellency is probably not determined solely by the composition or amount of trichome secretion. There is likely an interaction between trichome density and repellency, especially if trichome secretions are mainly responsible for chemical repellency. For example, leaflets of LA 2155 were not highly repellent, but its secretions were. This accession had very few type IV trichomes, implying that some threshold of type IV density may exist for leaflet repellency. Furthermore, leaflets of LA 1353 had very high type IV trichome densities and were highly repellent, but leaflet washes of this accession were not repellent, suggesting that very high densities of type IV trichomes, even in the absence of repellent trichome secretions, may repel or deter mites.

A threshold response of mites to type IV trichome density has been reported previously (Carter and Snyder, 1985, 1986), and Good and Snyder (1988) dem-

onstrated that type IV density is a primary determinant of the interaction of *L. hirsutum* leaves and mites placed directly on the leaves. Furthermore, it is not surprising that there is an interaction between density and repellency if trichomes are the primary repository of chemical repellents.

The diversity of trichome secretions present in this small number of accessions of *L. hirsutum* is much greater than that reported by Weston et al. (1989) for a similar number of accessions. The reasons for the greater diversity remain unexplained but may relate to site of original collection, to how the accessions have been maintained since collection, or simply to which accessions were chosen for examination. These results confirm that composition of trichome secretions varies among accessions, a feature that needs additional investigation especially as it relates to modification of plant–insect interactions.

In summary, our data indicate that repellency is a mechanism of resistance in this species, which has been reported as immune to insect attack (Rick, 1982). The evidence also suggests that repellency is at least partially associated with trichomes and their secretions and may ultimately be related to the quality of trichome secretions as well as trichome density. Our data indicate that causes of repellency may not be similar among accessions. We must point out that repellency is likely one layer of resistance in this highly defended species, but may be a very useful mechanism for introgression into crops such as tomato, in which small amounts of insect damage can lead to great economic loss.

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2,3-DIHYDROFARNESOIC ACID, A UNIQUE TERPENE FROM TRICHOMES OF *Lycopersicon hirsutum*, REPELS SPIDER MITES

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Abstract—*Lycopersicon hirsutum*, a wild relative of the tomato, is highly resistant to arthropod herbivores. Both botanic forms of *L. hirsutum*, *L. hirsutum* f. *glabratum* (C.H. Mull.) and *L. hirsutum* f. *typicum* (Humb. & Bonpl.), are resistant to two-spotted spider mites, *Tetranychus urticae* Koch. However, leaves and trichome secretions from f. *typicum* repel mites more so than those from f. *glabratum*. We have previously demonstrated that trichome secretions from LA 1363 and LA 1927, accessions of f. *typicum*, repelled mites. In this paper we report the identification of the primary component of trichome secretions responsible for repellency. Leaflet washes having compositions similar to trichome secretions were collected and separated into neutral and acid fractions; repellency was mainly associated with the acid fraction, which, when applied to nonrepellent leaflets of f. *glabratum*, rendered them repellent. Separation of leaflet washes by HPLC allowed purification and subsequent identification by gas chromatography–mass spectrometry and nuclear magnetic resonance of 2,3-dihydrofarnesoic acid (3,7,11-trimethyl-6,10-dodecadienoic acid) as the primary chemical component responsible for repellency. Application of this acid to leaflets of *L. esculentum* rendered them repellent. Other volatile compounds present in minor amounts in the acid fractions were farnesoic acid and 16:0, 16:3, 18:0, 18:2, and 18:3 fatty acids. This is the first report of the natural occurrence of 2,3-dihydrofarnesoic acid.

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Key Words—Tomato, *Lycopersicon esculentum*, host resistance, *Tetranychus urticae*, Acari, antixenosis, sesquiterpene, trichomes, allomone, 3,7,11-trimethyl-6,10-dodecadienoic acid, farnesoic acid, 2,3-dihydrofarnesoic acid.

INTRODUCTION

Tomatoes (*Lycopersicon esculentum* Mill.), fill aesthetic and nutritive needs in the human diet worldwide. Insects and mites attack tomato plants and fruit and may cause complete crop loss (Berlinger, 1986). *L. hirsutum* Humb. & Bonpl., a wild, green-fruited relative of the tomato, is genetically diverse and nearly immune to infestation by arthropods (Rick, 1982). Even though the species *L. hirsutum* possesses high levels of resistance to insects and mites, this valuable genetic resource has not been used successfully to improve resistance of tomato (Tigchelaar and Foley, 1991). Understanding the intricacies of arthropod herbivore resistance in *L. hirsutum* may lead to genetic improvement of resistance in tomato.

Plant resistance to arthropods often results from the complex interplay of several plant-based characters (Ortman and Peters, 1980). Resistance of *L. hirsutum* to arthropods has been associated with long-chain methyl ketones, sesquiterpenoids, alkaloids, phenols, and unidentified chemical factors, many trichome borne (Farrar and Kennedy, 1992). Trichome density per se can also alter the interaction between plant and arthropod (Good and Snyder, 1988).

Reports of immunity of *L. hirsutum* to insects suggests that antixenosis underlies its resistance to arthropods. Neither of the two botanical forms of *L. hirsutum*, f. *typicum* or f. *glabratum*, supports high levels of mite infestation. Accessions of f. *typicum* have sesquiterpenes in their trichome secretions and are generally more resistant, or are resistant in a different manner, to two-spotted spider mites, *Tetranychus urticae* Koch, than are accessions of f. *glabratum*, which have long-chain methyl ketones in their secretions (Weston et al., 1989). In terms of mite behavior, this difference between botanic forms is manifested by greater movement of mites onto leaflets of f. *glabratum* compared with movement onto leaves of f. *typicum* (Weston et al., 1989). Trichome secretions from f. *typicum* are more repellent to mites than are those from f. *glabratum* (Guo et al., 1993). However, the compositions of trichome secretions among accessions of f. *typicum* are chemically diverse, implying that more than one factor or chemical is responsible for repellency.

Previously we identified two accessions of f. *typicum*, LA 1363 and LA 1927, having repellent trichome secretions with similar, simple composition (Guo, 1992; Guo et al., 1993). Consequently, the objectives of this research were to: (1) isolate the compounds present in trichome secretions from leaves of LA 1363 and LA 1927 that are repellent to spider mites; (2) identify the

major compound(s) responsible for repellency; and (3) demonstrate that isolated chemicals repel two-spotted spider mites ex planta and in planta.

METHODS AND MATERIALS

Plant Material and Leaflet Hexane Washes. Seed of *L. hirsutum* accessions were kindly provided by the Tomato Genetic Stocks Center at the University of California Davis, Davis, California. Seed of *L. esculentum* Mountain Delight were provided by Dr. R. Gardner, North Carolina State University. Plants were raised in the greenhouse. Leaflet washes were prepared by steeping fully expanded, healthy leaflets from plants of *L. hirsutum* f. *typicum* accessions LA 1927 and LA 1363 in hexane (1 ml/leaflet) for 60 min, with agitation every 15 min. After decantation, the leaflets were briefly steeped in hexane twice (0.3 ml hexane/leaflet). The combined washes were filtered through Whatman No. 1 filter paper and dried with anhydrous Na_2SO_4. After evaporating the solvent, the concentration was brought to 1 ml of hexane per leaflet. This concentration is referred to as 1×. Washes were stored in glass vials at −80°C. Compositions of these washes are very similar to those taken directly from trichomes (Guo et al., 1993).

Gas Chromatography–Mass Spectrometry (GC-MS). Most of the GC-MS analyses were performed with a Finnigan INCOS 50 instrument equipped with a DB-5ms column (15 m × 0.25 mm, 0.1 μm stationary phase). Electron impact mass spectra were acquired at 20 eV in the range of 40–440 amu for samples in the form of either methyl or trimethylsilyl esters. These esters, in turn, were prepared, respectively, by the treatment of the acid fraction with diazomethane–ethyl ether solution or bistrimethylsilyltrifluoroacetamide (BSFTA)–pyridine (1:1). GC oven temperature was programmed from 100°C for 1 min and then to 280°C at 10°C/min. Additional GC-MS analyses and high-resolution MS measurements were performed using a Kratos Concept IH two-sector instrument.

Nuclear Magnetic Resonance (NMR). All ^1H and ^{13}C NMR spectra were obtained with a Varian 300 MHz VXR instrument using $CDCl_3$ as solvent.

Partitioning Leaflet Washes. Washes were extracted three times with an equal volume of 0.05 N NaOH. The upper (neutral) phase was collected; the lower phases were pooled, acidified with 1 N HCl to pH 5 and then partitioned three times against a 1/3 volume of hexane. Combined hexane layers were reduced to the original volume of the leaflet wash. The leaflet wash and the acid and neutral fractions were analyzed by GC-FID (GC–flame ionization detection) and GC-MS and those from LA 1363 were assayed for repellent activity to mites using the quantitative choice bioassay (see below).

Gas Chromatography. Fractionation of leaflet washes and purification were monitored by GC-FID on a Hewlett Packard 5890A gas chromatograph equipped

with a flame ionization detector and a 10-m × 0.54-mm RSL-150 column (Alltech Associates). Helium was the carrier gas (3 ml/min). Injector temperature was 240°C and detector temperature was 300°C. The oven temperature was held at 100°C for 1 min and then programmed at 6°C/min to 170°C and 10°C/min to 250°C.

Mite Colony. Mites (*Tetranychus urticae* Koch) were maintained and handled as described previously (Weston et al., 1989). Female adult mites from bean seedlings moderately damaged by mites were used for bioassays.

Spring-Board Bioassay. This bioassay, a choice bioassay, was conducted according to the methods reported by Guo et al. (1993).

Quantitative Choice Bioassay. The concentration of repellents on filter paper in the spring-board bioassay could not be calculated precisely because the area wetted by the sample solution could only be roughly estimated. The quantitative choice bioassay was developed to overcome this lack of precision. The bioassay consisted of two filter paper rectangles (1.5 × 0.5 cm) held parallel to each other and 1.5 cm apart in a clamp (Guo, 1992). Ten microliters of test solution was loaded on one rectangle and, usually, 10 μl of solvent only was loaded on the other as a control. Ten microliters of solvent just wet the 0.75 cm^2 of filter paper. After the solvent evaporated, a small strip of filter paper (1.5 mm × 2.5 cm) was used to bridge the rectangles. A mite was then transferred to the middle of the bridge and its movement was observed. The rectangle over which the mite exited was recorded. A mite was replaced by another immediately after it left the bridge or if it was inactive for more than 2 min. Generally 15–20 adult female mites were assayed on a bridge, and two such bridges with the test sample loaded on opposing rectangles (left and right) constituted an assay. Thus, a total of 30–40 mites were usually assayed for a sample. Data were submitted to probit analysis.

Relative Repellency of Leaflet Washes and Acid and Neutral Fractions from LA 1363 and LA 1927. Leaflet washes and fractions were prepared as outlined above from 25 leaflets of each accession. Leaflet area was determined after extraction. Solvent was removed from each fraction, residues were weighed, and the amount of each fraction per square centimeter of leaflet surface and recoveries were calculated. Residues were redissolved in hexane (15 mg/ml), and serial dilutions (1 : 1) were tested for repellency with the quantitative choice bioassay. Data were submitted to probit analysis, and the EC_{50} (effective concentration) was determined for each fraction. Because data were obtained from a choice bioassay having an expected choice ratio of 1 : 1 in the presence of equivalent stimuli, calculation of an EC_{50} from raw data predicts the threshold concentration, the concentration at which mites begin to perceive the stimulus. In order to predict an EC_{50} representing a response by 50% of the mites tested, a response index (RI) for the data was first calculated (RI = [(No. of mites exiting over the control − No. of mites exiting over the suspected repellent) ÷

No. of mites tested]. The data submitted for probit analysis were concentration of the test solution, total number of mites tested ÷ 2, and RI × total number of mites tested ÷ 2.

Repellency of Acid Fraction from LA 1363 Applied to Leaflets of Nonrepellent L. hirsutum *f.* glabratum *accession, LA 2144.* The acid fraction from LA 1363 was dissolved in 50% ethanol and two-, four-, and eight-fold dilutions of this solution in 50% ethanol allowed application at varying rates to leaflets of LA 2144, a f. *glabratum* accession having low concentrations of nonrepellent, hexane-soluble volatiles on its leaflet surfaces (Guo et al., 1993). Application was accomplished by dipping cotton balls in a solution and then dabbing them on the adaxial surface of the leaflet, distributing the solution as evenly as possible. Adaxial surfaces of leaflets so treated were assayed for 2 hr with the thumbtack bioassay (Weston and Snyder, 1990). Numbers of mites on the thumbtack were counted and recorded at 30-min intervals. After bioassay, each leaflet was extracted with hexane as outlined previously and leaflet area was determined with a planimeter. The amount of 2,3-dihydrofarnesoic acid (see below) applied to the leaflet was quantified by GC-FID and expressed as micrograms per square centimeter of leaflet area. The number of mites remaining on the thumbtack at each observation time and the dosage on the leaflet were submitted to probit analysis to estimate an EC_{50}.

Isolation of Repellent Activity. In March 1991 7.6 mg of oleoresin was obtained from a leaflet wash prepared from 75 cm^2 of leaflets from LA 1927 plants grown in the greenhouse. Aliquots (1 mg) were separated by HPLC. Separation was accomplished with a 10-μm, C_{18} reverse-phase column (Waters) and a step gradient using acetonitrile–H_2O–phosphoric acid (60:40:1, vvv) at 1 ml/min for the first 15 min and 1% phosphoric acid in acetonitrile (vv) at 1 ml/min for the remaining time of each run. The separation was monitored by UV absorbance (218 nm). Detected peaks were collected from each run and pooled across runs. Pooled fractions were reduced to 2/3 of their original volume on a rotary evaporator and then extracted with 10 ml of hexane three times. The three hexane phases were combined and solvent was evaporated. The residue was weighed and resuspended in 0.5 ml hexane. Composition of each fraction was determined by GC-FID, and each fraction was then bioassayed using the quantitative choice bioassay at dosages equal to the quantity of material in that fraction recovered from 2 cm^2 leaflet area applied to 1 cm^2 of filter paper, assuming 100% recovery (dosage 2×). When a fraction was not repellent at this dose, it was retested at a dosage twice as high (dosage 4×) to further ensure that repellent compounds were not overlooked because of low concentration after separation by HPLC. Leaflet wash from LA 1363 was also separated by HPLC, but not bioassayed.

Purification of 2,3-Dihydrofarnesoic Acid. 2,3-Dihydrofarnesoic acid, the repellent component in greatest abundance as judged by GC-FID, was purified

by HPLC. Hexane was evaporated from the acid fraction of LA 1927 with an N_2 stream. Five milligrams of residue was dissolved in 1 ml of the carrier solvent (acetonitrile–H_2O–phosphoric acid 60:40:1, vvv). Aliquots of 100 μl were separated on a 10-μm, C_{18} reverse-phase HPLC column (Waters). The isocratic separation was monitored at 218 nm and took place for 40 min at a flow rate of 1.0 ml of sample solvent per minute. Collected fractions were reduced to 2/3 of their volume by evaporation on a rotary evaporator and then extracted with hexane. Purity, as judged by GC, exceeded 97%. This sample was used for bioassay, to construct a standard curve for quantitation by GC-FID, and for identification by GC-MS and ^1H and ^{13}C NMR.

Repellency of 2,3-Dihydrofarnesoic Acid Applied to Leaflets of L. esculentum *Mountain Delight.* Pure 2,3-dihydrofarnesoic acid (98.2%), isolated as outlined above, was diluted in acetone to provide concentrations of 0, 0.2, 0.5, 1.0, 1.5, and 2.0 mg/ml. Leaves of Mountain Delight were removed from 70-day-old plants raised in the greenhouse in summer 1991. To maintain turgor, petioles were inserted into glass vials filled with water. A square (1.4 × 1.4 cm) was marked on the adaxial surface of a leaflet by punching a pin hole at each corner. One-tenth milliliter of solution containing 2,3-dihydrofarnesoic acid was spread evenly over the square with a pipet. Rates of application were 0, 10, 25, 50, 75, and 100 $\mu g/cm^2$. For each rate, four squares on four leaflets provided replications. Thumbtack bioassays were conducted on the treated squares. Number of mites detained on each tack at 1, 2, 4, and 5 hr was recorded. Data were submitted to analysis of variance and probit analysis.

RESULTS

Identification of Components Present in Leaflet Washes. The leaflet washes from LA 1363 and LA 1927 and the corresponding neutral and acid fractions were analyzed by GC and, after treatment with diazomethane (to produce methyl esters of carboxylic acids) and BSTFA and pyridine (to produce trimethylsilyl esters), by GC-MS. A typical GC-MS analysis of methylated samples, illustrated by Figure 1, demonstrated the presence of nine distinct components in washes of LA 1363 and 11 components in those of LA 1927. The two early-eluting, neutral components (A and B), sesquiterpenoid hydrocarbons with the same molecular weight of 204, were abundant only in LA 1927 washes. Based on characteristic mass spectra, trace components C and H were phthalate impurities and component C was identified as diethylphthalate (the major plasticizer of Scotch Tape). Each of the other seven components, D–G and I–K, were common to both accessions, having identical R_ts and mass spectra. These compounds were methyl esters of sesquiterpenoid and aliphatic fatty acids. Specifically,

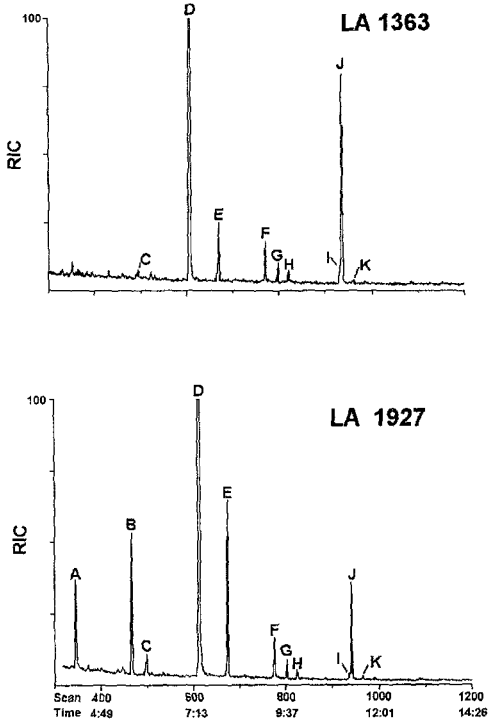
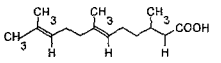
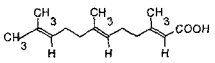


FIG. 1. GC-MS comparison of the methylated leaflet washes from two accessions of *L. hirsutum* f. *typicum*, LA 1927 and LA 1363. The vertical scale (RIC: reconstructed ion current) was multiplied by 10. Compounds identified are as follows: A,B—sesquiterpenoid hydrocarbons; C,H—phthalate impurities; D—methyl 2,3-dihydrofarnesoic acid; E—methyl farnesoic acid; F, G, I, J, and K—16:3, 16:0, 18:2, 18:3, and 18:0 fatty acids, respectively.



2,3-Dihydrofarnesoic Acid



Farnesoic Acid

FIG. 2. Sesquiterpene acids identified in leaflet washes from two accessions of *L. hirsutum* f. *typicum*, LA 1927 and LA 1363.

esters of sesquiterpenoid acids included the major component D, identified as methyl 2,3-dihydrofarnesoic acid, and E, identified as methyl farnesoic acid (Figure 2). The other five methyl esters (components F, G, I, J, and K) were identified as 16:3, 16:0, 18:2, 18:3, and 18:0 fatty acids, respectively, with 18:3 predominating. This identification was supported by the direct R_t and MS comparisons with the authentic standards of methyl esters of 16:0, 18:3, and 18:0 fatty acids.

2,3-Dihydrofarnesoic acid (3,7,11-trimethyl-6,10-dodecadienoic acid) was identified based on the characteristic electron impact mass spectra of the free acid [70 eV spectrum not shown: *m/z* (relative intensity) at 238 (M^+, 0.7), 223 (M-Me, 1.2), 195 (M-C_3H_7, 17), 123 (16), 109 (35), 95 (8), 81 (9), 69 (100)] and methyl ester and trimethylsilyl ester (Figure 3A and B). Molecular ions (M^+ at *m/z* 238, 252, and 310, respectively) were weak but clearly detected, and abundant pseudomolecular ions (MH^+) were present in the isobutane chemical ionization spectra of the free acid and trimethylsilyl esters at *m/z* 239 and 311, respectively. Moreover, the precise measurement performed for the molecular ion of the methyl ester was consistent with the expected elemental composition (measured 252.2088, expected for $C_{15}H_{26}O_2$ 252.2089). This acid was isolated by HPLC in an amount enabling its definitive ^1H and ^{13}C NMR characterization. Table 1 presents full spectral assignments obtained for the free acid and its methyl ester. The infrared spectrum of the methyl ester showed a major band at 1745 cm^{-1}, a saturated ester.

Farnesoic acid represented the minor component E (<5%). Although only mass spectral data were available (Figure 3C), this identification was fully confirmed by oxidation of farnesol with Jones' reagent (chromic acid) followed by methylation with diazomethane. Apart from the unsaturated aldehyde, the major product of this reaction sequence, methyl farnesoic acid with the same R_t and mass spectrum as the compound identified in washes from both accessions, was present as the minor oxidation product.

Repellency of Fractions from LA 1363. The leaflet wash and neutral and acid fractions from LA 1363 were repellent when assayed against hexane with the spring-board bioassay (Table 2). When tested against each other, the acid fraction was almost as repellent as the leaflet wash, and it took ca. 80 min to assay the 22 mites, an indication of strong repellency. The neutral fraction was less repellent than the acid and leaflet wash.

Relative Repellency of Leaflet Wash and Acid and Neutral Fractions. For each accession, the acid fraction comprised about 50–60% of weight of the leaflet wash (Table 3). The main differences between accessions were the weight and composition of the neutral fractions. Repellency of leaflet washes and acid fractions were similar regardless of source (Table 4). The neutral fractions were less repellent. Positive synergism for repellency between neutral and acid frac-

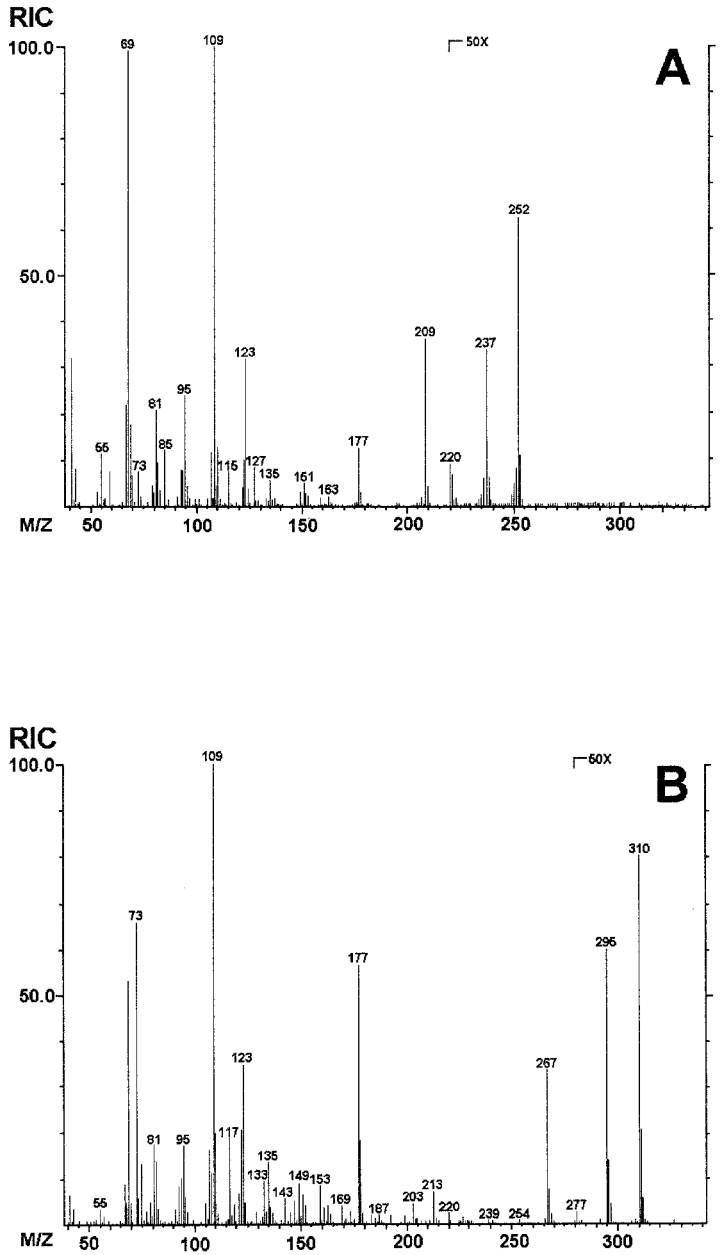


FIG. 3. Electron impact mass spectra (20 eV) of methyl 2,3-dihydrofarnesoic acid (A), trimethylsilyl 2,3-dihydrofarnesoic acid (B), and methyl farnesoic acid (C) identified in the accession LA 1927. Identical spectra were obtained for the accession LA 1363.

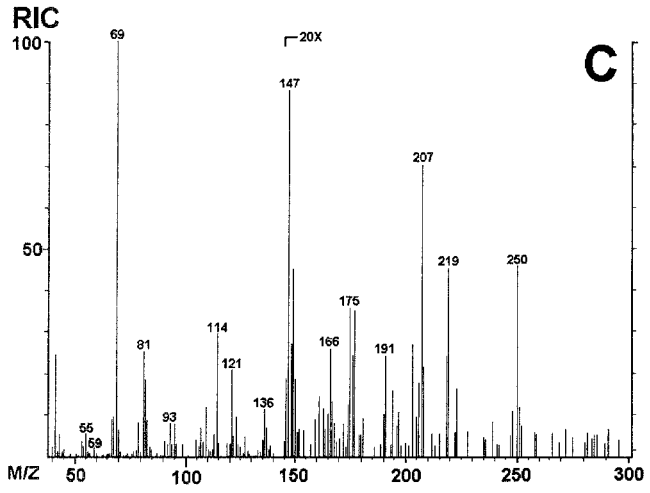


FIG. 3. Continued

tions did not seem likely because the EC_{50} values for acid fractions were less than those for the respective leaflet washes.

Repellency of Acid Fraction Applied to Leaflets of L. hirsutum *f.* glabratum *LA 2144.* EC_{50} values predicted from thumbtack bioassays of LA 2144 leaflets treated with the acid fraction from LA 1363 containing 2,3-dihydrofarnesoic acid and farnesoic acid were between 1 and 6 $\mu g/cm^2$, depending on the time after initiation of the bioassay (Table 5). These values were comparable to the EC_{50} obtained in the quantitative choice bioassay of this fraction, 10 $\mu g/cm^2$ (Table 4).

Repellency of HPLC Fractions and Pure 2,3-Dihydrofarnesoic Acid. HPLC provided adequate separation of compounds present in leaflet washes (Figure 4). Farnesoic acid eluted at $\approx$20 min and 2,3-dihydrofarnsoic acid, at $\approx$21 min. The compositions of leaflet washes of LA 1363 and LA 1927 were very similar except that LA 1927 contained more late-eluting hydrocarbons than did LA 1363 (Figure 4). Fraction 5 was highly repellent (Table 6) and was more than 90% 2,3-dihydrofarnesoic acid, as judged by GC-FID. The 3.2 mg recovered from this fraction (Table 6) was equivalent to 42.7 μg from 1 cm^2 leaflet area of LA 1927. The two other repellent fractions, fractions 4 and 6, also contained mainly 2,3-dihydrofarnesoic acid. Fraction 4 contained a significant amount of farnesoic acid as well, but the repellency of farnesoic acid could not be determined because no fraction contained just this compound. The abundances of the early eluting components were low. Their early elution from HPLC and nondetectability on GC-FID indicated that these components were less lipophilic and less volatile than farnesoic acid. The small amounts of these com-

TABLE 1. ^{13}C AND ^{1}H NMR DATA OF 2,3-DIHYDROFARNESOIC ACID (in $CDCl_3$) MEASURED AT 75.43 AND 300 MHz, RESPECTIVELY; FOR FREE ACID MULTIPLICITY ASSIGNED BY APT; AND Me ESTER ASSIGNMENT BY APT, COSI, AND HETCOR

	δ ^{13}C			^{1}H	
	Free acid	Me ester	$\Delta\delta^a$	Free acid	Me ester
C-1	178.33	173.83	−4.50		—
C-2	41.34	41.69	+0.35	2.370 dd[c]	2.31 dd[d]
				2.150 dd[e]	2.12 dd[f]
C-3	29.91	30.13	+0.22		(1.86 m)
C-3′	19.68	19.73	+0.05	0.981 d[g]	0.943 d[g]
C-4	36.74	36.82	+0.08		(1.2, 1.4 m)
C-5	25.37[b]	25.40[#]	+0.03		(1.90 m)
C-6	124.09*	124.20*	+0.11	5.085 tm	5.095 tm
C-7	135.35	135.23	−0.12		
C-7′	16.06	16.05	−0.01	1.598 bq	1.597 bq
C-8	39.80	39.80	0		(1.86 m)
C-9	26.75[#]	26.76[#]	+0.01		(1.97 m)
C-10	124.37*	124.38*	+0.01	5.100 tm	5.095 tm
C-11	131.45	131.42	−0.03		
C-11′	17.78	17.79	+0.01	1.598 bq	1.597 bq
C-12	25.79	25.80	+0.01	1.676 bq[h]	1.677 bq[h]
Me		51.47			3.667 s

[a] ^{13}C Chemical shift difference induced by methylation.
[b–#*] denotes uncertain, interchangeable assignments.
[c] J_{AB} = 15.0 Hz, J_{AX} = 6.1 Hz.
[d] J_{AB} = 14.6 Hz, J_{AX} = 5.9 Hz.
[e] J_{AB} = 15.0 Hz, J_{BX} = 8.2 Hz.
[f] J_{AB} = 14.6 Hz; J_{BX} = 8.2 Hz.
[g] J = 6.6 Hz.
[h] J = 1.2 Hz.

ponents in the leaflet wash may be related to low abundance or limited solubility in hexane; their abundance in or on the leaves may be high. Bioassays of these fractions at the 4× dose indicated no repellency. Fraction 8 contained sesquiterpene hydrocarbons that were not well separated, and this fraction was not repellent. No evidence was obtained indicating that compounds other than 2,3-dihydrofarnesoic acid or farnesoic acid were highly repellent to mites. The EC_{50} for pure 2,3-dihydrofarnesoic acid in the quantitative choice bioassay was 9 $\mu g/cm^2$ with 95% fiducial limits of 6 and 12 $\mu g/cm^2$. This value corresponded with the EC_{50} for the acid fraction tested similarly, 10 $\mu g/cm^2$ (Table 3), and the EC_{50} values for the acid fraction applied to leaflets of LA 2144, 1–6 $\mu g/cm^2$ (Table 5).

TABLE 2. REPELLENCY TO MITES OF LEAFLET WASHES FROM LA 1363 AND FRACTIONS DERIVED FROM THEM MEASURED BY SPRING-BOARD BIOASSAY

Test fractions[a]		Exit ratio[b]	
A	B	A:B	χ^2
Wash	Hexane	0:30	15.0**[c]
Acid	Hexane	5:25	6.7**
Neutral	Hexane	10:30	5.0*
Wash	Acid	6:16	2.3[ns]
Wash	Neutral	9:31	6.0*
Acid	Neutral	6:34	9.8**

[a]Test solution was loaded on one postion (A) of the spring-board and a control or another test solution was loaded at the other position (B) on the same spring-board.
[b]The exit ratio is the ratio of the number of mites exiting over position A and position B.
[c]*,** significant at 5% and 1%, respectively; [ns] not significant.

TABLE 3. QUANTITY OF RESIDUES IN LEAFLET WASHES AND NEUTRAL AND ACID FRACTIONS AND RECOVERY OF TRICHOME SECRETIONS FROM TWO ACCESSIONS OF *L. hirsutum* f. *typicum*

Accession	Leaflet wash ($\mu g/cm^2$ of leaflet surface)	Neutral fraction ($\mu g/cm^2$ of leaflet surface)	Acid fraction ($\mu g/cm^2$ of leaflet surface)	Recovery (%)
LA 1363	81	32	50	101
LA 1927	116	48	57	91

TABLE 4. REPELLENCY TO MITES AS INDICATED BY EC_{50}[a] OF LEAFLET WASH AND NEUTRAL AND ACID FRACTIONS FROM TWO ACCESSIONS OF *L. hirsutum* f. *typicum*

	Leaflet wash		Neutral fraction		Acid fraction	
Accession	EC_{50} ($\mu g/cm^2$)	95% fiducial limits	EC_{50} ($\mu g/cm^2$)	95% fiducial limits	EC_{50} ($\mu g/cm^2$)	95% fiducial limits
LA 1363	30	24–38	145	91–372	10	7–15
LA 1927	22	14–33	69	46–117	10	8–15

[a]Predicted concentration at which 75% of the spider mites respond to the stimulus.

TABLE 5. REPELLENCY TO MITES AS INDICATED BY EC_{50}[a] IN THUMBTACK BIOASSAY OF LEAFLETS FROM NONREPELLENT ACCESSION OF *L. hirsutum* f. *glabratum* (LA 2144) TREATED WITH ACID FRACTION OBTAINED FROM LEAFLETS OF *L. hirsutum* f. *typicum* ACCESSION LA 1363

Time after initiation of bioassay (min)	EC_{50} ($\mu g/cm^2$)	95% fiducial limits
30	0.7	0.1–1.8
60	2.9	1.4–4.8
90	4.5	1.2–7.9
120	5.7	3.5–8.8

[a]Predicted concentration at which 50% of the spider mites respond to the stimulus.

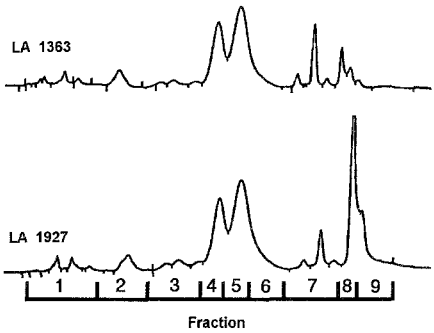


FIG. 4. HPLC fractionation of leaflet washes from two accessions of *L. hirsutum* f. *typicum*, LA 1363 (upper) and LA 1927 (lower).

Repellency of 2,3-Dihydrofarnesoic Acid Applied to Leaflet Surfaces of Tomato. Application of pure 2,3-dihydrofarnesoic acid on abaxial leaflet surfaces of Mt. Delight rendered them repellent (Figure 5). Repellency was related to dose, and the EC_{50} at 1 hr, 1.5 $\mu g/cm^2$, was similar to that obtained at 1 hr for the acid fraction applied to LA 2144 (2.9 $\mu g/cm^2$, Table 5). However, at 2 hr the predicted EC_{50} (33 $\mu g/cm^2$) was about fivefold greater than that obtained at 2 hr for the acid fraction applied to leaflets of LA 2144 (5.7 $\mu g/cm^2$, Table 5). The differences in the EC_{50} values at the 2-hr sampling periods in the bioassays may relate to the different plant material used for the two bioassays. LA 2144 has a trichome vesture distinct from that of Mt. Delight, which probably contributed to the greater durability of repellency on LA 2144. Nevertheless, both bioassays demonstrated that response to applied chemical repellents varied with dose.

TABLE 6. QUANTITY OF RESIDUE RECOVERED FROM AND REPELLENT ACTIVITY TO MITES ASSOCIATED WITH HPLC FRACTIONS OF LEAFLET WASHES FROM LA 1927[a]

Fraction	Weight of residue in fraction (mg)	Weight of residue ($\mu g/cm^2$ leaflet area)	Exit ratio (sample : control)	
			2× dose	4× dose
1	0.1	1	16:14	18:12
2	0.2	2	20:10	18:12
3	0.2	2	12:18	12:18
4	0.9	7	5:25[b]	NT[c]
5	3.2	43	0:30[b]	NT
6	0.9	7	5:25[b]	NT
7	0.4	3	15:15	14:16
8	1.1	8	19:11	8:22
9	1.4	10	17:13	13:17

[a] See Figure 4. The 2× and 4× dose indicate the concentration of the residue tested with the quantitative choice bioassay, assuming 100% recovery. Calculated recovery was 110%.
[b] Ratios significantly deviate from 1:1 at $P \leq 1\%$ as determined by χ^2.
[c] NT: the 4× dose was not tested because the 2× dose was repellent.

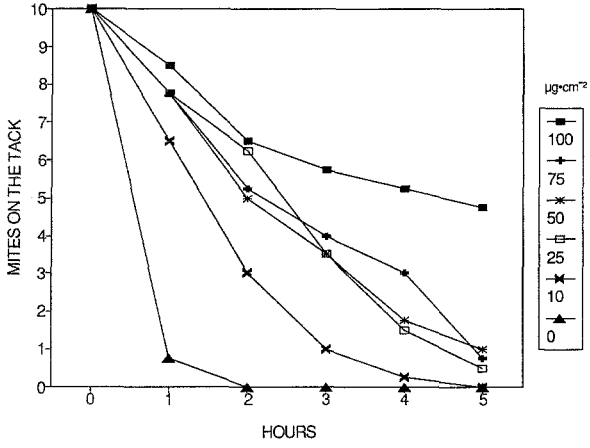


FIG. 5. Repellency to mites of 2,3-dihydrofarnesoic acid applied to leaves of *L. esculentum* Mt. Delight as measured by the thumbtack bioassay. Ten mites were initially placed on each tack.

DISCUSSION

Previously we reported that leaves and trichome secretions of LA 1927 and LA 1363 repelled mites and that removal of trichomes and secretions from leaflets by wiping reduced their repellency (Guo et al., 1993). Evidence presented herein substantiates that 2,3-dihydrofarnesoic acid is present in hexane washes of leaflets and is repellent to spider mites when encountered on filter paper or on leaflets treated with the acid. Because most, if not all, of the 2,3-dihydrofarnesoic acid is associated with trichomes (Guo, 1992) and because the concentration of the acid on the leaflet is well above its EC_{50} for repellency, it is likely that the presence of this acid in trichomes is responsible for repellency to mites of these accessions of *L. hirsutum* f. *typicum*.

The presence of 2,3-dihydrofarnesoic acid may confer resistance to other arthropods. Fatty acids, especially C_8–C_{12}, are toxic to arthropods (Siegler and Popenoe, 1925), and dodecanoic acid and (*E*)-β-farnesene are aphid alarm pheromones, which act by deterring settling of aphids (Bowers et al., 1972; Greenway et al., 1978). 2,3-Dihydrofarnesoic acid is structurally similar to (*E*)-β-farnesene and has physical and chemical properties similar to C_{12} fatty acids. Thus, it is reasonable to anticipate that the presence of 2,3-dihydrofarnesoic acid on plants may confer resistance to arthropods other than mites via toxicity and action similar to an alarm pheromone. Furthermore, farnesol serves as a precursor to juvenile hormone III, methyl farnesoic acid, a hormone regulating larval development of some insect species (Judy et al., 1973). It is possible that farnesoic acid or 2,3-dihydrofarnesoic acid may also modify plant–arthropod interactions by altering larval development.

Terpenes are known to affect mite behavior. Monoterpenes have been reported as attractive to predatory mites (Takabayashi et al., 1991) and to act as sex attractants for male two-spotted mites (Regev and Cone, 1980). Farnesol has been reported as a male sex attractant for two-spotted spider mites (Regev and Cone, 1975). Patterson et al. (1975) reported the presence of an unidentified sesquiterpene alcohol in plants of f. *hirsutum* PI 251303 that repelled female two-spotted spider mites. Our documentation that 2,3-dihydrofarnesoic acid repels mites adds to the list of terpenes reported to modify mite behavior. The relationships among chemical structure, response, mite species and sex, however, need additional research for greater delineation.

The presence of sesquiterpene acids has been reported in other accessions of *L. hirsutum* f. *typicum*. (+)-(*E*)-α-Santanlen-12-oic acid and (+)-(*E*)-endo-β-bergamoten-12-oic acid, present in trichomes, are ovipositional attractants for *Heliothis zea* in LA 1777 (Coates et al., 1988). These acids are also repellent to mites as are acids of zingiberene and curcumene in trichome secretions of f. *typicum* PI 251303 (unpublished). Thus, the presence of sesquiterpene acids in trichome secretions of *L. hirsutum* f. *typicum* appears to be relatively common,

but structures may be diverse. To the best of our knowledge this is the first demonstration of the natural occurrence of 2,3-dihydrofarnesoic acid, the compound known as a synthetic intermediate (Ahlquist and Stallberg-Stenhagen, 1971). Interestingly, the corresponding alcohol, terrestrol, has been found to occur in the bumble bee, *Bombus terrestris* (Stallberg-Stenhagen, 1970).

The evidence presented here strongly supports the idea that the presence of 2,3-dihydrofarnesoic acid on leaves of plants of LA 1927 and LA 1363 causes mites to avoid them. It is also reasonable to expect that the presence of 2,3-dihydrofarnesoic acid may alter the interaction of these accessions with other arthropods. We must emphasize, though, that 2,3-dihydrofarnesoic acid can not explain the repellency of all accessions of f. *typicum*, because this acid does not occur or occurs at very low levels in some highly repellent f. *typicum* accessions. Furthermore, the presence of 2,3-dihydrofarnesoic acid on leaves of LA 1363 and LA 1927 is likely just a single facet of a resistance complex that operates in these accessions, rendering them immune to mites.

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ATTRACTION OF BOTH SEXES OF MEXICAN FRUIT FLY, *Anastrepha ludens*, TO A MIXTURE OF AMMONIA, METHYLAMINE, AND PUTRESCINE

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Abstract—Eight chemicals were evaluated in laboratory experiments as attractants for sugar-fed adult Mexican fruit flies. Ammonium bicarbonate, methylamine HCl, ethanolamine, pyrrolidine, putrescine, and monomethyl succinate were slightly attractive when tested singly. A mixture containing all eight chemicals was much more attractive than any of the individual chemicals. Through a series of experiments, a mixture of three of the chemicals was found that was at least as attractive as the original eight-component mixture. The final mixture consisted of ammonium bicarbonate, methylamine HCl, and putrescine in a 10:10:1 ratio. Ratios were less important than actual concentrations of individual components over the range of component concentrations tested. The three-component mixture was equally attractive to male and female flies over at least a 1000-fold range of concentrations and was slightly more attractive than *Torula* yeast over the upper 10-fold range in competing McPhail traps in a greenhouse flight chamber.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, ammonia, methylamine, putrescine.

INTRODUCTION

Wakabayashi and Cunningham (1991) described a blend of chemicals that was attractive to both sexes of the melon fly, *Dacus cucurbitae* Coquillett. Their method was a novel approach to the problem of tephritid attractants. They

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hypothesized that fruit flies should be attracted to mixtures of volatile chemicals arising from biological degradation of amino acids and fats that are nutritionally important to the flies. They began with a mixture of nine such degradation products that proved highly attractive to adult melon flies. Through a series of experiments, they determined that only four of the compounds were necessary to retain the attractiveness of the original mixture. The final mixture consisted of ammonia, α-linolenic acid, putrescine, and pyrrolidine.

Robacker et al. (1993) provided evidence that the Mexican fruit fly, *Anastrepha ludens* Loew, is attracted to volatile metabolites produced by a bacterium isolated from the fly. Chemical characterization indicated that the most important of these chemicals contained ionizable nitrogen such as that found in amines. Thus, candidate attractant chemicals produced by bacteria may include some of the same amino acid degradation products studied by Wakabayashi and Cunningham (1991). As part of a continuing effort to develop new attractants for *Anastrepha*, we investigated the attractiveness to *A. ludens* of eight of the nine compounds tested by Wakabayashi and Cunningham (1991). This paper describes a mixture of three of those chemicals that is highly attractive to sugar-fed, adult *A. ludens*.

METHODS AND MATERIALS

Insects and Laboratory Test Conditions. Flies were from a culture that had been maintained on laboratory diet for about 90–100 generations with no wild-fly introductions. Mixed-sex groups of 180–200 flies were kept in 473 ml cardboard cartons with screen tops until used in tests. Flies were tested when 7–17 days old. Flies were fed sucrose and water up until the time of testing but were deprived of protein and other nutrients as adults. All tests were conducted between 0830 and 1530 hr. Laboratory tests were conducted under a combination of fluorescent and natural light. Laboratory conditions were 22 $\pm$ 2°C, 50 $\pm$ 20% relative humidity, and photophase from 0630 to 1930 hr.

Chemicals Evaluated in Bioassays. Eight of the nine chemicals evaluated by Wakabayashi and Cunningham (1991) as attractants for *D. cucurbitae* were obtained from commercial sources. Names, sources, and purities of the chemicals are listed in Table 1. Chemicals were evaluated without purification. One chemical tested by Wakabayashi and Cunningham (1991), 5-hydroxy-2-pyrrolidinone, was not tested because it was not commercially available. This chemical was not attractive to *D. cucurbitae* (Wakabayashi and Cunningham, 1991).

Cage-Top Bioassay Procedure. Cage-top bioassays were used to test attractiveness of chemicals and mixtures of chemicals in all experiments except the greenhouse evaluation of the final mixture. Cage-top bioassays were conducted by placing four filter paper triangles (3 cm/side) containing test chemicals or

TABLE 1. CHEMICAL NAMES, SOURCES, AND PURITIES OF EIGHT CHEMICALS EVALUATED FOR ATTRACTIVENESS TO *A. ludens* IN VARIOUS EXPERIMENTS

Chemical	Source[a]	Purity (%)[b]
Ammonium bicarbonate	Sigma	99
Methylamine HCl	Sigma	99
Ethanolamine	Aldrich	>99
Pyrrolidine	Sigma	99
Putrescine	Sigma	98
Phenethylamine	Aldrich	>99
Monomethyl succinate	Aldrich	95
α-linolenic acid	Sigma	98

[a] Sigma Chemical Company, St. Louis, Missouri; Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.
[b] From label information.

water (control) near the corners on the top of an insect cage (30 cm/side, aluminum-screened) and counting the numbers of flies beneath each filter paper once each minute for 10 min. The two papers containing test chemicals were positioned diagonally from each other on the cage top, as were the two papers containing water. The filter papers were raised 5 mm above the cage top using plastic rings as in Robacker et al. (1991) to ensure that olfaction and not contact chemoreception was solely responsible for the response of the flies. Two cartons containing 180–200 flies each were used in each bioassay cage in experiments 1–8, and one carton was used in each cage in experiments 9–17.

Experiments 1–8: Tests of Individual Chemicals. The purpose of this experiment was to determine the attractiveness of each of the eight chemicals over a 1000-fold range of odorant concentrations. Solutions of each of the eight chemicals were prepared at concentrations of 1, 10, 100, and 1000 ng/μl. Ammonium bicarbonate and methylamine HCl were prepared in water. The other chemicals were prepared in methanol. One drop of saturated aqueous sodium hydroxide was added to each solution of ammonium bicarbonate and methylamine HCl to produce free ammonia and methylamine. Quantities tested of each of the eight chemicals were 10 ng, 100 ng, 1 μg, and 10 μg, each in 10 μl of solvent.

The eight chemicals were tested in eight separate experiments. Each experiment was a randomized complete block design with blocks containing the four chemical quantities. Ten replications were conducted for all chemicals except methylamine HCl and pyrrolidine, for which nine and seven were conducted, respectively.

General Procedures for Mixture Experiments. Mixtures containing two to eight of the chemicals were tested for attractiveness to evaluate the importance

of each chemical in various combinations with the others. We began with the complete mixture and sequentially deleted those chemicals that contributed little attractiveness to determine the simplest combination that retained the attractiveness of the original mixture.

Unless stated differently under descriptions of specific experiments, the following procedures apply to all mixture experiments. Chemicals were incorporated into mixtures at the same concentrations at which they were most attractive in experiments 1–8. Mixtures were prepared in various proportions of methanol and water as needed for dissolution of the different components. The pH of solutions was adjusted to 8.8 with aqueous sodium hydroxide or phosphoric acid. Experiments were conducted as randomized complete blocks with each block containing all of the mixture treatments. Ten replications of most experiments were conducted.

Experiment 9: Tests of Eight- and Seven-Component Mixtures. The purposes of this experiment were: (1) to determine the attractiveness of the mixture of all eight chemicals, and (2) to determine the effect on attractiveness of deleting each of the chemicals from the eight-component mixture. α-Linolenic acid was incorporated at 100 ng/μl to approximate its concentration of 188 ng/μl used by Wakabayashi and Cunningham (1991), since it was not attractive at any concentration when tested singly in experiment 8. Initial bioassays showed that all of the mixtures containing putrescine at 1000 ng/μl, the concentration that was most attractive when it was tested singly, were much less attractive than the seven-component mixture that did not contain putrescine. Therefore, the concentration of putrescine in the mixtures was lowered to 100 ng/μl and the experiment was conducted again. The experiment was conducted without adjusting the pH of the solutions, which ranged from 8.3 to 9.3. Table 2 shows the quantities of the chemicals bioassayed in the 10-μl test volumes of the mixtures.

Experiment 10: Effect of pH on Attractiveness of Seven-Component Mixtures. The purpose of this experiment was to determine whether differences in attractiveness among several seven-component mixtures were due to pH differences or to deletion of components. Mixes 7A, 7F, and 7G were prepared as before and divided into two parts. The pH of one part of each mixture was adjusted to 8.8 with phosphoric acid or sodium hydroxide to equal the pH of Mix 7D, the most attractive mixture from experiment 9. Table 3 shows the quantities of the chemicals bioassayed in the 10 μl test volumes of the mixtures (suffix 'adj' indicates pH adjusted to 8.8.).

Experiment 11: Tests of Six-Component Mixtures. Mix 7D was chosen as the new "complete" mixture, since it was more attractive than mix 8 in experiment 9. The purposes of experiment 11 were: (1) to verify the greater attractiveness of mix 7D relative to mix 8, and (2) to determine the effect on attractiveness of deleting each of the chemicals from mix 7D, except ammonium

TABLE 2. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS (Experiment 9)

	Mix								
	8	7A	7B	7C	7D	7E	7F	7G	7H
Chemical (μg)									
Ammonium bicarbonate	10		10	10	10	10	10	10	10
Methylamine HCl	10	10		10	10	10	10	10	10
Ethanolamine	1	1	1		1	1	1	1	1
Pyrrolidine	1	1	1	1		1	1	1	1
Putrescine	1	1	1	1	1		1	1	1
Phenethylamine	10	10	10	10	10	10		10	10
Monomethyl succinate	10	10	10	10	10	10	10		10
α-Linolenic acid	1	1	1	1	1	1	1	1	
Mean count at mix	167.4	75.0	184.2	161.0	198.7	159.4	157.2	124.4	166.3
Mean count at water	16.4	21.0	16.1	12.8	18.5	14.6	16.5	15.0	17.8
Mix—water[a]	151.0 bc	54.0 e	168.1 ab	148.2 c	180.2 a	144.8 c	140.7 c	109.4 d	148.5 c

[a]Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

TABLE 3. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS (Experiment 10)

	Mix						
	8	7A	7A adj	7F	7F adj	7G	7G adj
Chemical (μg)							
Ammonium bicarbonate	10			10	10	10	10
Methylamine HCl	10	10	10	10	10	10	10
Ethanolamine	1	1	1	1	1	1	1
Pyrrolidine	1	1	1	1	1	1	1
Putrescine	1	1	1	1	1	1	1
Phenethylamine	10	10	10			10	10
Monomethyl succinate	10	10	10	10	10		
α-Linolenic acid	1	1	1	1	1	1	1
Mean count at mix	188.0	96.1	89.2	162.9	162.6	149.1	188.9
Mean count at water	22.3	24.7	25.0	21.8	21.0	24.6	20.7
Mix–water[a]	165.7 a	71.4 c	64.2 c	141.1 b	141.6 b	124.5 b	168.2 a

[a]Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

bicarbonate, which proved essential in experiment 9. Table 4 shows the quantities of the chemicals bioassayed in the 10-μl test volumes of the mixtures.

Experiment 12: Tests of Five-Component Mixtures. Mix 6B was chosen as the new "complete" mixture based on attractiveness in experiment 11 that was statistically equivalent to that of mix 7D. The purposes of experiment 12 were: (1) to verify the attractiveness of mix 6B relative to mix 7D, and (2) to determine the effect on attractiveness of deleting each of the chemicals from mix 6B, except ammonium bicarbonate and methylamine HCl, which proved important in previous experiments. Table 5 shows the quantities of the chemicals bioassayed in the 10-μl test volumes of the mixtures.

Experiment 13: Tests of Four- and Three-Component Mixtures. Mix 5B was chosen as the new "complete" mixture based on attractiveness in experiment 12 that was statistically equivalent to that of mix 6B. The purpose of experiment 13 was to test the effect on attractiveness of deleting phenethylamine, α-linolenic acid, or both from mix 5B. Table 6 shows the quantities of the

TABLE 4. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS (Experiment 11)

	Mix							
	8	7D	6A	6B	6C	6D	6E	6F
Chemical (μg)								
Ammonium bicarbonate	10	10	10	10	10	10	10	10
Methylamine HCl	10	10		10	10	10	10	10
Ethanolamine	1	1	1		1	1	1	1
Pyrrolidine	1							
Putrescine	1	1	1	1		1	1	1
Phenethylamine	10	10	10	10	10		10	10
Monomethyl succinate	10	10	10	10	10	10		10
α-Linolenic acid	1	1	1	1	1	1	1	
Mean count at mix	196.0	238.6	136.9	231.7	200.0	218.6	222.1	225.6
Mean count at water	21.8	20.8	25.4	23.6	26.7	21.5	20.4	22.7
Mix—water[a]	174.2 c	217.8 a	111.5 d	208.1 ab	173.3 c	197.1 b	201.7 ab	202.9 ab

[a]Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

TABLE 5. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS (Experiment 12)

	Mix				
	7D	6B	5A	5B	5C
Chemical (μg)					
Ammonium bicarbonate	10	10	10	10	10
Methylamine HCl	10	10	10	10	10
Ethanolamine	1				
Putrescine	1	1	1	1	1
Phenethylamine	10	10		10	10
Monomethyl succinate	10	10	10		10
α-Linolenic acid	1	1	1	1	
Mean count at mix	203.5	201.3	190.7	208.4	195.7
Mean count at water	20.3	19.5	23.4	24.1	22.0
Mix—water[a]	183.2 a	181.8 a	167.3 a	184.3 a	173.7 a

[a]Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

TABLE 6. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS (Experiment 13)

	Mix			
	5B	4A	4B	3A
Chemical (μg)				
Ammonium bicarbonate	10	10	10	10
Methylamine HCl	10	10	10	10
Putrescine	1	1	1	1
Phenethylamine	10		10	
α-Linolenic acid	1	1		
Mean count at mix	187.4	168.9	179.0	186.1
Mean count at water	21.2	21.8	20.4	22.5
Mix–water[a]	166.2 a	147.1 b	158.6 ab	163.6 a

[a]Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

chemicals bioassayed in the 10-μl test volumes of the mixtures. Nineteen replications of the experiment were conducted.

Experiment 14: Tests of Two-Component Mixtures and Increased Linolenic Acid. Mix 3A was chosen as the new "complete" mixture based on attractiveness in experiment 13 that was statistically equivalent to that of mix 5B. The purposes of experiment 14 were: (1) to verify the attractiveness of mix 3A relative to mix 5B; (2) to test the effect on attractiveness of deleting ammonium bicarbonate, methylamine HCl, or putrescine from mix 3A; and (3) to reevaluate α-linolenic acid as a fourth component, but with the concentration of α-linolenic acid 10-fold higher than was used in earlier experiments. This last objective was carried out because Wakabayashi and Cunningham (1991) found α-linolenic acid to be such an important component in their attractant for *D. cucurbitae*. Table 7 shows the quantities of the chemicals bioassayed in the 10-μl test volumes of the mixtures.

Experiment 15: Substitution of Ammonia for Methylamine. The purpose of this experiment was to determine if ammonium bicarbonate could substitute for methylamine HCl in mix 3A. Two new mixtures were prepared in which additional ammonium bicarbonate was substituted for methylamine HCl. A third new mixture was prepared containing the usual concentration of methylamine HCl but with an increased concentration of ammonium bicarbonate. Table 8 shows the quantities of the chemicals bioassayed in the 10-μl test volumes of the mixtures. Twelve replications of the experiment were conducted.

TABLE 7. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS
(Experiment 14)

	Mix					
	5B	4C	3A	2A	2B	2C
Chemical (μg)						
Ammonium bicarbonate	10	10	10		10	10
Methylamine HCl	10	10	10	10		10
Putrescine	1	1	1	1	1	
Phenethylamine	10					
α-Linolenic acid	1	10				
Mean count at mix	137.7	131.6	127.8	29.1	90.3	114.5
Mean count at water	37.7	12.9	12.2	13.5	17.8	15.5
Mix—water[a]	124.0 a	118.7 a	115.6 a	15.6 d	72.5 c	99.0 b

[a] Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

TABLE 8. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS (Experiment 15)

	Mix			
	3A	3B	2D	2E
Chemical (μg)				
Ammonium bicarbonate	10	20	15	20
Methylamine HCl	10	10		
Putrescine	1	1	1	1
Mean count at mix	140.9	161.4	105.2	106.1
Mean count at water	15.8	19.8	15.8	18.1
Mix—water[a]	125.1 a	141.6 a	89.4 b	88.0 b

[a]Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

Experiment 16: Effect of pH on Attractiveness of Mix 3A. Experiment 10 demonstrated that pH of the mixtures affected their attractiveness. The purpose of experiment 16 was to test the effect of pH on attractiveness of mix 3A from pH 7.6 to 9.4 in increments of 0.2 pH units. Mix 3A was prepared as before and its pH adjusted with phosphoric acid or sodium hydroxide to the various treatment levels. Sixteen replications of the experiment were conducted.

Experiment 17: Tests of Ratios of Mix 3A Components. The purpose of this experiment was to determine the most attractive ratios of the three components of mix 3A. Eight mixtures were prepared in which the concentration of each component was either 100 or 1000 ng/μl. Table 9 shows the quantities of the chemicals bioassayed in the 10-μl test volumes of the mixtures, and the test ratios of the components to each other can be ascertained readily from this table. Eleven replications of the experiment were conducted.

Experiment 18: Comparison of Torula *Yeast and AMPu in McPhail Traps.* The components of mix 3A were ammonium bicarbonate, methylamine HCl, and putrescine. The purpose of experiment 18 was to compare the attractiveness of various concentrations of mixtures of these three components (AMPu) to the attractiveness of an aqueous suspension of *Torula* dried yeast and borax (TY). TY is a common bait used for detection of *A. ludens* and other *Anastrepha*. TY traps were prepared using three *Torula* yeast/borax "bait pellets" (Sit-Khem Corp., Michigan City, Indiana) in 200 ml of water per McPhail trap (Baker et al., 1944). TY traps were used in tests on the day they were prepared.

AMPu was prepared in 200 ml of water at various concentrations but with constant ratios of the three components at 10:10:1, respectively, for ammonium

TABLE 9. CHEMICAL QUANTITIES TESTED IN MIXTURES AND MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING VARIOUS MIXTURES OF CHEMICALS OR WATER IN CAGE-TOP BIOASSAYS (Experiment 17)

	Mix							
	R1	R2	R3	R4	R5	R6	R7[a]	R8
Chemical (μg)	1	1	1	1	10	10	10	10
Ammonium bicarbonate								
Methylamine HCl	1	1	10	10	1	1	10	10
Putrescine	1	10	1	10	1	10	1	10
Mean count at mix	28.7	33.1	43.7	50.5	91.4	90.6	109.0	105.2
Mean count at water	11.9	12.7	14.3	13.7	14.2	15.2	12.6	9.7
Mix–water[b]	16.8 d	20.4 d	29.4 cd	36.8 c	77.2 b	75.4 b	96.4 a	95.5 a

[a] Mix R7 is identical to mix 3A used in previous experiments.
[b] Mean differences followed by the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$).

bicarbonate, methylamine HCl, and putrescine. This ratio proved most attractive to flies in previous experiments. Concentrations of the components ranged from 2, 2, 0.2 ng/μl to 2, 2, 0.2 μg/μl in steps that approximately doubled each previous concentration. The 200 ml of each AMPu concentration was colored amber using a combination of red, yellow, and green McCormick food colors (McCormick & Co., Inc., Baltimore, Maryland) to mimic the color of TY. AMPu traps also contained 0.01% Triton X-100 (Rohm and Haas Co., Philadelphia, Pennsylvania) as a wetting agent. The pH of each AMPu solution was adjusted to 8.3–8.8.

For each test, a McPhail trap containing TY was suspended side by side (30 cm apart) with a trap containing one concentration of AMPu in the upwind end of an aluminum-screened flight chamber (2.0 m long × 0.7 m wide × 1.3 m high) with an airflow of 0.1–1 m/sec in a greenhouse. The two traps were alternated between left and right sides of the chamber every 15 min. Test duration was 1 hr. Approximately 200 flies were released into the downwind end of the cage at the beginning of each test. Flies used in this experiment were irradiated with 7000–9200 rads (cobalt-60 source) one to three days before adult eclosion because *A. ludens* is a quarantined insect in Texas and the greenhouse was not secure. Six to eight replications of each AMPu concentration were tested in a completely random design over a period of six weeks. Interspersed with tests of the AMPu treatments were seven replications testing a TY trap vs. a trap containing only amber-colored water and 0.01% Triton X-100.

Statistical Analyses. Experiments 1–8 were analyzed by paired *t* tests to compare total counts beneath the treatment papers in each bioassay to total

counts beneath the control papers. Experiments 9–17 were analyzed by analyses of variance (ANOVA) to compare treatments with each other. This was done by subtracting total counts at control papers from total counts at treatment papers for each bioassay to obtain differences that were then subjected to analyses of variance. Means were compared by Fisher's protected least significant difference (LSD) method. The treatment effect in experiment 17 was partitioned using a 2^3 factorial analysis of the eight ratio treatments. Experiment 18 counts of males and females captured in AMPu traps were converted into proportions of total males and females captured in both traps (AMPu + TY), transformed by arcsin of the square root, and subjected to separate ANOVAs for data sets with or without the seven replications of the water traps. The treatment effect from the ANOVA without the water-trap data was partitioned into linear regression and deviations from linear regression. For experiment 18, paired *t* tests were used to compare the numbers of flies captured in AMPu traps to those captured in TY traps for various AMPu concentrations.

RESULTS AND DISCUSSION

Experiments 1–8. Attractiveness of Individual Chemicals. Six of the eight chemicals were more attractive than water controls (Table 10). At the 10-μg test quantity, ammonium bicarbonate, methylamine HCl, ethanolamine, pyrrol-

TABLE 10. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING 10 μl OF WATER OR VARIOUS QUANTITIES OF EIGHT CHEMICALS IN CAGE-TOP BIOASSAYS (Experiment 1–8)

	10 ng		100 ng		1 μg		10 μg	
Chemical	Chemical	Water	Chemical	Water	Chemical	Water	Chemical	Water
Ammonium bicarbonate	24.7	32.7	29.3	31.3	43.8[a]	33.9	83.5[b]	33.0
Methylamine HCl	42.6	44.0	39.1	41.0	62.5[b]	40.7	105.2[b]	42.0
Ethanolamine	44.0	42.5	40.8	37.6	63.2[b]	37.9	70.0[b]	45.5
Pyrrolidine	35.8	35.4	45.7	41.0	72.1[b]	41.1	51.6[a]	38.0
Putrescine	57.6	50.1	61.9[b]	50.5	61.7[a]	48.9	80.6[b]	60.3
Phenethylamine	43.1	44.6	36.8	44.2	46.5	48.1	49.0	43.8
Monomethyl succinate	44.3	39.1	42.2	37.6	39.8	39.3	59.8[b]	43.5
α-linolenic acid	36.5	33.7	34.1	36.1	29.0	32.2	33.0	36.9

[a] The mean response to a chemical is significantly greater than the response to water by paired *t* tests ($P < 0.05$).

[b] The mean response to a chemical is significantly greater than the response to water by paired *t* tests ($P < 0.01$).

idine, putrescine, and monmethyl succinate were significantly attractive at least at the 5% level by paired t tests (smallest $t = 2.6$, $df = 6$). At the 1-μg test quantity, ammonium bicarbonate, methylamine HCl, ethanolamine, pyrrolidine, and putrescine were significantly attractive at least at the 5% level (smallest $t = 2.5$, $df = 9$). At the 100-ng test quantity, only putrescine was significantly attractive ($P < 0.01$, $t = 3.8$, $df = 9$) and no chemicals were attractive at the 10-ng quantity. Phenethylamine and α-linolenic acid were not attractive at any test quantity. None of the chemicals was more than 2.5-fold more attractive than water.

Experiment 9: Attractiveness of Eight- and Seven-Component Mixtures. The eight-component mixture (mix 8) was 10-fold more attractive than water (Table 2). Removing ammonium bicarbonate or monomethyl succinate from mix 8 significantly reduced attractiveness of the resulting mixes 7A and 7G relative to mix 8. Removing pyrrolidine significantly increased attractiveness of the resulting mix 7D.

Results suggested that ammonium bicarbonate was critical to the attractiveness of mix 8 and that monomethyl succinate was also important. However, a possible confounding factor was the pH of the mixtures. Most mixtures had pH near 8.8. Exceptions were mix 7A with pH 9.0, mix 7G with pH 9.3, and mix 7H with pH 8.3. Therefore, no conclusions regarding ammonium bicarbonate or monomethyl succinate were possible until the effect of pH was investigated in experiment 10.

Although pyrrolidine was slightly repellent in mixtures used in experiment 9, we concluded that it should be further evaluated in experiment 11, owing to its great attractiveness when tested by itself at the same concentration (Table 10).

Experiment 10: Effect of pH on Attractiveness of Seven-Component Mixtures. Adjusting the pH of mix 7A to 8.8 did not restore attractiveness of the resulting mix 7Aadj to the level of mix 8 (Table 3). This indicates that ammonium bicarbonate contributes attractiveness to mix 8. Adjusting the pH of mix 7G to 8.8 did restore attractiveness of the resulting mix 7Gadj to the level of mix 8. Thus, the loss of attractiveness that occurred when monomethyl succinate was removed from mix 8 apparently was due only to the accompanying increase in pH. Finally, mix 7F, which did not contain phenethylamine, was significantly less attractive than mix 8 in this experiment regardless of its pH. This result differed slightly from that of experiment 9 in which mix 7F was not significantly less attractive than mix 8 (Table 2).

Based on results of experiments 9 and 10, we concluded that ammonium bicarbonate was essential to attractiveness, and the decision was made that it would be retained in all mixtures; also, the conservative decision was made to retain phenethylamine and monomethyl succinate for further testing in experiment 11.

Experiment 11: Attractiveness of Six-Component Mixtures. Mix 7D again was more attractive than mix 8, indicating that pyrrolidine was repellent in mix 8 (Table 4). We concluded that pyrrolidine should be eliminated from further consideration in mixtures.

Mixes 6A, 6C, and 6D all were significantly less attractive than mix 7D (Table 4). These results indicated that methylamine HCl, putrescine, and phenethylamine contributed to the attractiveness of mix 7D. The decision was made to retain both methylamine HCl and putrescine in all mixtures.

Mix 6B was 95% as attractive as mix 7D, indicating that ethanolamine was contributing little to the attractiveness of mix 7D. The conservative decision was made to retain ethanolamine for a retest of mix 6B against 7D. Mixes 6E and 6F were not significantly less attractive than mix 7D. Nevertheless, the conservative decision was made to retain monomethyl succinate and α-linolenic acid for further testing in five-component mixtures.

Experiment 12: Attractiveness of Five-Component Mixtures. Mix 6B again was statistically equivalent to mix 7D, verifying that ethanolamine was not contributing attractiveness to the mixtures (Table 5). Ethanolamine was eliminated from further consideration. Deletion of phenethylamine, monomethyl succinate, or α-linolenic acid had no significant effect on attractiveness of the resulting mixes 5A, 5B, and 5C. Mix 5B was chosen as the best mixture, and the decision was made to eliminate monomethyl succinate from further consideration. The conservative decision was made to continue evaluation of phenethylamine and α-linolenic acid.

Experiment 13: Attractiveness of Three- and Four-Component Mixtures. Mix 4A, which did not obtain phenethylamine, was less attractive than mix 5B (Table 6). However, mix 3A, which contained neither phenethylamine nor α-linolenic acid was statistically equivalent to mix 5B. The decision was made to eliminate phenethylamine from further consideration except to retest mix 3A vs mix 5B. We also decided to test α-linolenic acid at a higher concentration. The reason for this latter choice was that Wakabayashi and Cunningham (1991) demonstrated that α-linolenic acid was the most important component in their attractive mixtures.

Experiment 14: Attractiveness of Two-Component Mixtures and Increased α-Linolenic Acid. Mix 3A again was equally attractive as mix 5B (Table 7). Thus, phenethylamine was eliminated from further consideration. Mix 4C, which contained α-linolenic acid at high concentration, was not more attractive than mix 3A. α-Linolenic acid was eliminated from further consideration.

All three two-component mixtures were significantly less attractive than mix 3A (Table 7). Deletion of ammonium bicarbonate decreased attractiveness the most, followed by methylamine HCl and putresine. The decision was made to retain ammonium bicarbonate, methylamine HCl and putrescine for further testing.

Experiment 15: Substitution of Ammonia for Methylamine. Because ammonia and methylamine are both of very low molecular weight and obviously have many properties in common, we decided to test whether the increase in attractiveness contributed by methylamine was due to its similarity to ammonia or to properties unique to methylamine. Table 8 shows that mixes 2D and 2E, which contained elevated amounts of ammonium bicarbonate in place of methylamine HCl, were significantly less attractive than mix 3A, which contained both chemicals. Thus, attractiveness of methylamine does not come from its similarity to ammonia. These results suggest there may be separate receptor sites on the antennae for ammonia and methylamine. The decision was made to retain both ammonium bicarbonate and methylamine HCl as well as putrescine as components of the final mixture.

Experiment 16: Effect of pH on Attractiveness of Mix 3A. pH had little effect on attractiveness of mix 3A within the tested range of 7.6–9.4. Generally, the most attractive solutions had pH between 7.8 and 9.0. Only the solution with pH 9.4 was significantly less attractive than most of the other solutions by LSD at the 5% level. We concluded that pH between 7.6 and 9.2 had little effect on attractiveness of mix 3A, at least under our conditions.

Experiment 17: Effects on Attractiveness of Ratios of Ammonium Bicarbonate, Methylamine HCl, and Putrescine. No significant interactions of the three chemicals occurred over the two test quantities of each, by factorial analysis. This indicates that the effect of test quantity of each chemical was more or less independent of the test quantities of the other two. Thus, ratios were relatively unimportant.

Table 9 shows responses to the eight mixtures. All mixtures with the higher test quantity of ammonium bicarbonate were more attractive than those with the lower test quantity. All mixtures with the higher test quantity of methylamine HCl were more attractive than the corresponding mixtures with the lower test quantities, although the difference was not statistically significant in one case (R3 vs. R1). The test quantity of putrescine had little effect in this experiment.

Mix R7, which is the same as mix 3A, and mix R8 were the most attractive mixtures (Table 9). The combination (mix 3A or R7) containing ammonium bicarbonate, methylamine HCl, and putrescine in the ratio 10:10:1, respectively, was chosen as the best combination of chemicals out of the original eight test chemicals.

Impurities of Putrescine. The putrescine used in this work was at best 98% pure (Table 1). In addition to impurities that may have been present in the commercial preparation when it arrived at our lab, it is also possible that other impurities formed from the putrescine after we opened it. Amoore et al. (1975) presented convincing anecdotal evidence that small amounts of 3,4-dihydro-2H-pyrrole (1-pyrroline) and perhaps 4-aminobutanal form by spontaneous oxidation of putrescine exposed to air. Amoore et al. (1975) also reported that 1-pyrroline

has a strong "semenlike" odor while pure putrescine has only a very "feeble" odor. A semenlike odor was very evident in our putrescine preparations indicating that 1-pyrroline was present.

Assuming that 4-aminobutanal and 1-pyrroline were present in our preparations, it is possible that the attractive effect we have attributed to putrescine may in fact be due to 4-aminobutanal, 1-pyrroline, or both. However, 4-aminobutanal is probably a very transient species under our conditions and undoubtedly rapidly cyclizes to form 1-pyrroline (Amoore et al., 1975). Baker et al. (1992) showed that 1-pyrroline is also unstable due to oxidation when exposed to air, although it appears to be considerably more stable than 4-aminobutanal. Despite the instability of 1-pyrroline, it could nevertheless be responsible for the attractiveness of our putrescine solutions since more 1-pyrroline may simply form from excess putrescine to replace that lost by oxidation. 1-Pyrroline previously has been implicated as a pheromone produced by the male Mediterranean fruit fly (*Ceratitis capitata* Wiedemann) (Baker et al., 1985; Jang et al., 1989).

Because of the instability of 4-aminobutanal and 1-pyrroline, they are not commercially available. Synthesis of 1-pyrroline in trimeric form is relatively easy (Nomura et al., 1977), but this form also is not stable (Baker et al., 1992). Therefore, should 4-aminobutanal and/or 1-pyrroline eventually prove to be the active principles responsible for the attractiveness ascribed to putrescine in this work, the easiest way to formulate them may be to use putrescine, from which they can evolve at low rates for a long period of time.

Experiment 18: Comparison of Torula *Yeast and AMPu in McPhail Traps.* Figure 1 shows relative captures of males and females in traps containing either amber-colored water (0 ng/μl) or various concentrations of AMPu in a greenhouse flight chamber. Data are mean percentages of the sum of the flies captured in both traps used in each test (flies in AMPu or water trap divided by flies in TY trap + flies in AMPu or water trap). Thus, 50% indicates equal captures by AMPu and TY traps.

Traps baited with various concentrations of AMPu captured higher percentages of male and female flies than those containing only amber-colored water (Figure 1). The difference is significant by LSD ($P < 0.05$) in all treatments except 2 and 8 ng/μl for males ($F = 4.5$; $df = 11, 77$; $P < 0.01$), and in all treatments for females ($F = 6.8$; $df = 11, 77$; $P < 0.01$).

The percentages of males and females captured increased with concentration of the AMPu components (Figure 1). The effect was largely linear on the $\log_2$ scale from 2 to 2000 ng/μl of the two major components, ammonium bicarbonate and methylamine HCl (0.2–200 ng/μl of putrescine). Captures in AMPu traps relative to TY traps increased about 2.2% per $\log_2$ concentration step for males ($F = 19.4$; $df = 1, 60$; $P < 0.01$) and 2.0% per $\log_2$ step for females ($F = 20.5$; $df = 1, 60$; $P < 0.01$). Deviations from linear regression were not significant for either males or females ($F < 1$; $df = 9, 60$; $P > 0.5$).

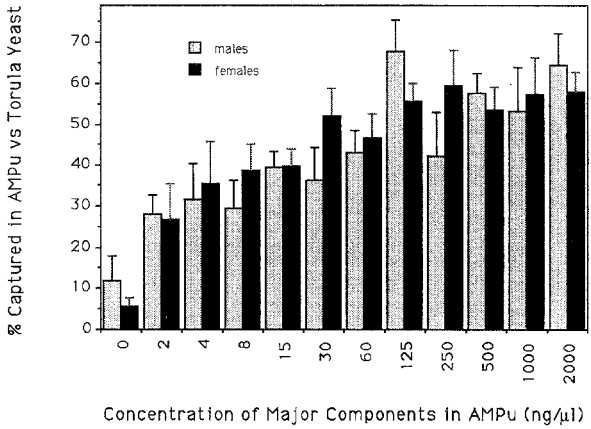

FIG. 1. Captures of adult *A. ludens* by McPhail traps baited with various concentrations of a mixture of ammonium bicarbonate, methylamine HCl, and putrescine (AMPu), expressed as mean percentages (±SE) of the sum of the flies captured by the AMPu (or colored water) trap and the *Torula* yeast trap. A value of 50% indicates equal captures by the AMPu and *Torula* yeast traps.

AMPu traps were not more attractive than TY traps for any AMPu concentration by paired t tests. However, for AMPu concentrations 125–2000 ng/µl taken as a group, AMPu traps captured significantly more males ($P < 0.01$, $t = 2.8$, $df = 34$) and females ($P < 0.05$, $t = 2.6$, $df = 34$) than TY traps.

Fifty-nine percent of flies captured in AMPu traps were females, and 58% of flies captured in TY traps were females. These percentages were not significantly different from the percentage of females actually released into the chamber (56%). Thus, both AMPu and TY were about equally attractive to males and females.

These data demonstrate that the three-component combination of ammonium bicarbonate, methylamine HCl, and putrescine at a ratio 10:10:1, respectively, has potential as an attractant for the Mexican fruit fly. Although the combination was not strikingly more attractive than *Torula* yeast in our tests, the fact that the attractant is chemically defined opens the door to formulating the chemicals into slow-release dispensers. Such dispensers could be used in common sticky traps or other newly designed traps other than the fragile, bulky McPhail traps commonly in use today with proteinaceous baits such as *Torula* yeast.

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NONFLORAL SOURCES OF CHEMICALS THAT ATTRACT MALE EUGLOSSINE BEES (APIDAE: EUGLOSSINI)

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Abstract—We present chemical analysis of four rotten or fungus-infected logs that attracted fragrance-collecting male euglossine bees. Eight of the 10 volatile compounds detected have never been found in the fragrances of orchids pollinated by male euglossine bees. Nonfloral sources of chemicals such as rotting wood may constitute an important fragrance resource for male bees. Since rotten logs produce large quantities of chemicals over long periods of time, such nonfloral sources might be more important than flowers as a source of certain fragrances for some euglossine bee species. Fragrance collecting in euglossine bees might have evolved originally in relation with rotting wood rather than flowers.

Key Words—Hymenoptera, Apidae, Euglossini, floral fragrance, fungi, skatole, chemical ecology, orchid, rotting wood.

INTRODUCTION

Euglossine bees are important pollinators of many plant species in neotropical forests (Janzen, 1971; Dressler, 1982; Williams, 1982; Ackerman, 1985; Roubik and Ackerman, 1987). Euglossines are best known for the unique fragrance-

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collecting behavior of the male bees (Kimsey, 1984) that are specialized pollinators of certain orchids, aroids, and other plants. Male euglossines use their modified front- and mid-tarsi and their abundant labial gland secretions to collect volatile chemicals and to transfer the chemicals to highly specialized hind tibial organs (Whitten et al., 1989). The fate of these stored fragrances and their role in euglossine biology are still unclear (Stern, 1991), although current hypotheses suggest that they play a role in chemically-mediated sexual selection (Kimsey, 1982; Whitten et al., 1989; Lunau, 1992). The identification of compounds present in orchid fragrances has yielded a set of common, easily-obtained chemicals that attract male euglossines in large numbers in simple field bioassays ("baiting"); such bioassays led to the discovery of many new species of euglossines and allow easy censusing of male euglossine populations (e.g., Ackerman, 1989; Janzen et al., 1981). Females remain difficult to census, and many euglossine species are known only from male specimens.

Flowers are not the only source of fragrance chemicals for male euglossines. Male euglossines collect fragrances from a variety of sources, including rotting logs, tree wounds, rotting fruits, and leaf litter (Dressler, 1967; Ackerman, 1983; Whitten et al., 19890. *Eufriesea purpurata* (Mocsáry) is attracted in large numbers to wooden surfaces sprayed with Aldrin (Dressler, 1967) and DDT (Roberts et al., 1982), although it is not clear whether bees were attracted to the pesticide, to minor constituents, or to breakdown products. Reports of such nonfloral euglossine attractants are uncommon, primarily because most studies of euglossines have been performed by orchid biologists. Bees visiting showy orchids attract more attention than bees brushing on rotten logs or bark, and hence such nonfloral sources are probably underreported by field biologists. Are orchids and other flowers the primary source of fragrances for euglossines, or do male bees obtain a large portion of their fragrances from other sources such as rotting wood, bark, and fruits? Do both floral and nonfloral attractants produce the same sets of chemicals? Several surveys have provided analyses of orchid fragrances that attract male euglossines (Williams and Whitten, 1983; Gerlach and Schill, 1991; Kaiser 1993). Unfortunately, few data exist on the chemical composition of the nonfloral sources that attract male bees.

In this paper, we present chemical analyses of four non-floral substrates that attract male euglossines, and we discuss the relative importance of floral vs. nonfloral sources of chemicals to euglossine chemical ecology. These four substrates were encountered by chance in different localities in Central America. These instances are described separately below, and then the results are discussed and compared.

METHODS AND MATERIALS

Samples were placed in either hexane or methylene chloride (HPLC grade) and shipped to Gainesville, Florida. Samples were stored at $-4°C$ until analyzed. The extracts were analyzed by gas chromatography-mass spectrometry

(GC-MS) using a Hewlett-Packard 5995 GC-MS equipped with a 30 m DB-5 column. Oven temperature was programmed from 25°C to 290°C at 3°C/min. Compounds were identified by comparison of mass spectra and retention times with synthetic standards. Spectra of unknowns were searched against the NIST/EPA/NIH mass spectral library (Ausloos et al., 1992).

Site 1. This study was conducted at a logging stockpile bordering the Sarapiqui River, near La Virgen de Socorro, in northeastern Costa Rica (Heredia Province). On February 27, 1990, one of us (A.M.Y.) discovered dozens of male *Euglossa purpurea* Friese collecting volatiles (brushing) along a darkly-stained crevice within the heartwood of the transversely cut ends of a large log. The stained heartwood where the bees were brushing emitted a strong fecal odor, similar to skatole (3-methyl indole). Local loggers called this tree "Quizarrá caca" (caca = feces), named after the characteristic odor of the wood. The tree was tentatively identified as *Ocotea leucoxylon* (Sw.) Laness. (Lauraceae). This determination is tentative because foliage was lacking; the tree might belong to the closely related genus *Nectandra*. As many as 50 bees gathered at each area of split, exposed heartwood (Figure 1), and a much lower number hovered above equally fragrant wood chips on the ground near the log. No bees were seen at other logs stacked nearby. Voucher specimens of bees were collected; only *E. purpurea* was present. Samples collected for chemical analysis were: (1) hind tibiae of 14 bees (pooled); (2) heads of same 14 bees (pooled); (3) wood shavings from surface of log where bees were brushing; (4) wood shavings from face of log where bees were not seen brushing; and (5) wood chips on ground that attracted bees. All samples were immediately placed in hexane or methylene chloride for later analysis. The site was revisited on March 5, when bees were still observed on the log. Two squares of filter paper impregnated with skatole were tacked to a nearby log; the skatole attracted dozens of *E. purpurea*.

Site 2. On February 29, 1988, one of us (D.L.S.) observed several male *Eulaema bombiformis* (Packard) brushing on the surface of a rotten log on Barro Colorado Island, Panama. Fragments of wood from the brushed surface were placed in vials of hexane.

Site 3. In April 1984, along the Río Iguanita, near Portobelo, Panama, we found a dead tree trunk that had recently been knocked down and broken open by the fall of an adjacent tree. The log was covered by 100–150 male *Euglossa* of several species that were brushing on the punky, decayed wood near the center. The wood had a faint aromatic odor. Of 21 bees captured, 18 were *E. variabilis* Friese and three were *E. despecta* Moure; at least two additional species were present but were not captured. Samples of wood from where bees were brushing were collected and extracted in hexane.

Site 4. In July 1986, we observed numerous *Eulaema cingulata* (Fabricius) brushing on fungus-infected machete wounds of the trunk of *Dalbergia cub-*

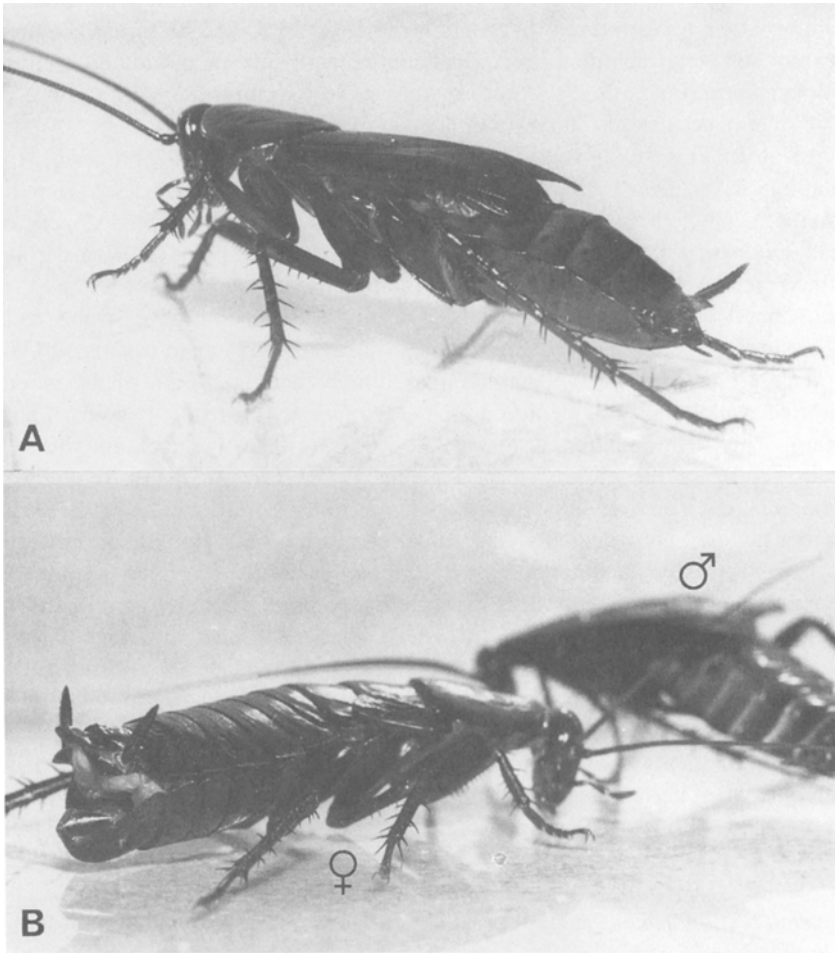

FIG. 1. Male *Euglossa purpurea* collecting volatiles from crack in sawn end of skatole-producing *Ocotea* log in Sarapiqui District, Costa Rica.

ilquitensis Pittier (Leguminosae) at Estación Experimental La Lola, near Siquirres, Costa Rica. During irregular visits to this site over a three-year period, we frequently observed bees brushing on the same tree wounds. This site was discussed by Whitten et al. 1989. Samples of tree wounds and undamaged bark and wood were collected and extracted in hexane.

RESULTS

Site 1. GC-MS analyses of the extracts of the samples revealed large quantities of skatole in the wood sampled where the bees were brushing (Figure 2A). These surface wood extracts (of discolored areas where bees were brushing) also contained large amounts of high molecular weight hydrocarbons and esters that occur in *E. purpurea* labial glands (Figure 2), the most characteristic being eicos-9-enyl-1,20-diacetate. The presence of the labial secretions on the surface of the wood suggests that the bees apply the labial secretions to the wood, using the secretion as a solvent to extract and collect volatiles from the wood (Whitten et al., 1989). Areas of the log that did not attract bees contained no skatole or other hexane-extractable compounds. The pooled hind tibial extracts (Figure 3) contained large amounts of skatole, as well as several other fragrance compounds including *p*-anis alcohol, *p*-anisaldehyde, anisyl acetate, *trans*-nerolidol, *p*-dimethoxybenzene, and several sesquiterpenes. We presume that the bees obtained these compounds from other sources, either flowers or nonfloral sources.

Skatole has long been known to be a strong attractant of certain species of

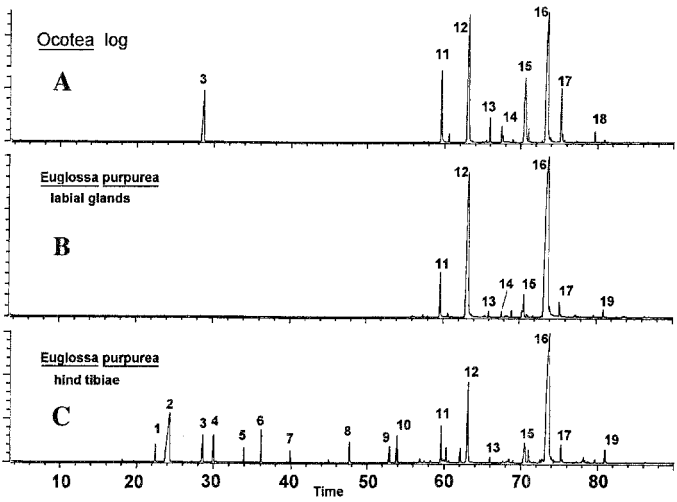

FIG. 2. Total ion chromatograms of methylene chloride extracts of: *Ocotea* log attracting male bees; pooled heads of 14 male *Euglossa purpurea*; and pooled hind tibiae of 14 male *Euglossa purpurea*. Numbered peak identifications are: **1**, *p*-anisaldehyde; **2**, *p*-anis alcohol; **3**, skatole; **4**, anisyl acetate; **5**, beta-bisabolene; **6**, *trans*-nerolidol; **7**, unidentified sesquiterpene; **8**, 16:1; **9**, unidentified; **10**, unidentified; **11**, 20:2; **12**, eicosenyl acetate; **13**, 15:0; **14**, eicosenol; **15**, 16:1 + coeluting acetate; **16**, eicos-9-enyl-1,20-diacetate; **17**, 29:1; **18**, 31:1; **19**, unidentified. Aliphatic hydrocarbons are identified as # of carbons:# of double bonds. Unlabeled peaks are unidentified.

TABLE 1. FRAGRANCE COMPOUNDS PRESENT IN EUGLOSSINE-ATTRACTING WOOD SAMPLES[a]

Source	Compound	Relative abundance	Present in orchid fragrances?	Attractive to male euglossines?
Ocotea	skatole (**3**)	+++	no	yes
unidentified wood, BCI	2,5-dimethyl-3-methoxy phenol (**20**)	+++	no	unknown
unidentified wood, Rio Iguanita	trans-methyl cinnamate (**34**)	+	yes	yes
	unknown **35**	+	no	unknown
	unknown **36**	+++	no	unknown
	unknown **37**	+	no	unknown
	unknown **39**	++	no	unknown
Dalbergia wood	unknown **50**	+	no	unknown
	unknown **51**	+++	no	unknown
	trans-nerolidol (**52**)	+++	yes	unknown (never tested)

[a]Mass spectra data of unknowns: **20**—152(M+, 100), 137(53), 121(42), 107(25), 91(30), 79(26), 77(27), 65(15), 53(21); **35**—178(M+, 13), 124(100), 123(23), 109(64), 95(14), 81(12), 55(32), 53(13), 41(15); **36**—176(M+, 20), 161(19), 145(6), 124(89), 123(24), 109(100), 95(22), 81(20), 53(50); **37**—spectrum weak; **39**—207(16), 204(35), 161(18), 135(34), 107(54), 93(51), 81(53), 67(57), 43(60), 41(100); **50**—156(M+, 2), 128(2), 114(3), 99(100), 87(15), 43(42), 42(42), 41(50); **51**—223(1), 155(6), 138(10), 127(7), 111(23), 109(88), 93(28), 69(77), 43(100), 41(70).

male euglossines. Its attractant properties were discovered by trial-and-error bioassays of fragrant compounds during the 1960s by C. Dodson, H. Hills, and Williams (N. Williams, personal communication). Chemical analyses of the hind tibial organs of various euglossine species have shown that certain species may contain large amounts of skatole (Whitten, unpublished observation). However, fragrance analyses of over 350 species of orchids have failed to reveal any orchid sources of skatole (Whitten, unpublished observation; Gerlach and Schill, 1991; Kaiser, 1993). The only known floral sources of skatole are *Arum* and *Hydrosme* (Araceae) (Smith and Meeuse, 1966). Two possible explanations exist: (1) euglossines obtain skatole from unknown floral sources, and/or (2) euglossines obtain skatole from other, nonfloral sources.

These data show that: (1) skatole is a naturally-occurring substance within the habitat of *Euglossa purpurea*; and (2) *E. purpurea* obtains skatole, probably in large amounts, from a non floral source. The role of fungi in the production of the skatole is not clear. The restriction of bee activity to the crack in the tree trunk suggests that healthy heartwood is not attractive and that skatole is pro-

duced by fungi and/or bacteria or by the wood in response to microbial infection. Dressler (1967) also reported *E. purpurea* and two other species brushing on a "large malodorous seeping area" on the trunk of a tree near Puerto Viejo, Costa Rica; the seep smelled strongly of skatole (R. Dressler, personal communication).

Site 2. Hexane extracts of the wood (Fig. 3) contained a single volatile, compound **20,** plus a variety of normal acetates, alcohols, alkenes, and alkanes. Compound **20** is tentatively identified as 2,5-dimethyl-3-methoxy phenol (R. Kaiser, personal communication). It is not known from orchid fragrances, but is known from oak wood and "oak moss" lichens [*Evernia prunastri* (L.) Ach.] (Tabacchi and Nicollier, 1979; R. Kaiser, personal communication). The lipid compounds (peaks between 36 and 66 minutes, Figure 2) are qualitatively and quantitatively similar to the labial secretions of *E. bombiformis* (Williams and Whitten, 1983). Although no samples of *E. bombiformis* were collected at this site, we did analyze tibial extracts of one male from near Portobelo, Panama, and one male from Ecuador. In both tibial extracts, compound **20** was present in trace quantities ($>1\%$ of integrated total ion chromatogram area).

Site 3. The wood extracts (Figure 4) contained five compounds, plus large quantities of lipids characteristic of *Euglossa* and *Eulaema* labial gland secretions. One of the five compounds is trans-methyl cinnamate, a common floral fragrance compound that attracts many euglossine species. Three of the five compounds are unidentified but appear structurally related **(35–37);** the mass spectra of all three contain prominent ions of *m/z* 109 and 124, suggestive of a methoxyphenyl fragment. Several known euglossine attractants possess this structure, including *p*-methoxyphenylethyl alcohol and methyl *p*-methoxycin-

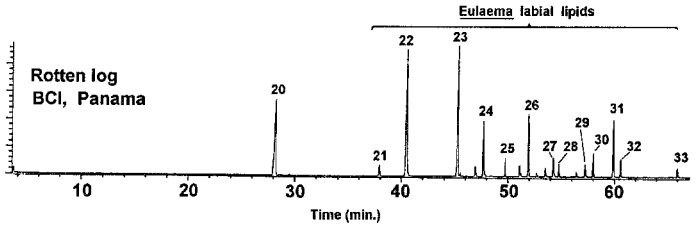

FIG. 3. Total ion chromatogram of hexane extract of the surface of an unidentified rotten log at Barro Colorado Island, Panama, that attracted *Eulaema bombiformis*. Compounds 21–33 are typical of labial gland secretions of *E. bombiformis* and were probably applied to the surface of the log by fragrance-foraging males. Numbered peak identifications are: **20**, 2,5-dimethyl-3-methoxyphenol; **21**, tetradecanal; **22**, tetradecanol; **23**, tetradecyl-1-acetate; **24**, 1-hexadecanol; **25**, 1-hexadecenyl acetate; **26**, hexadecyl acetate; **27**, octadecanol; **28**, 21:0; **29**, octadecenyl acetate; **30**, octadecyl acetate; **31**, 23:1; **32**, 23:0; **33**, 25:0.

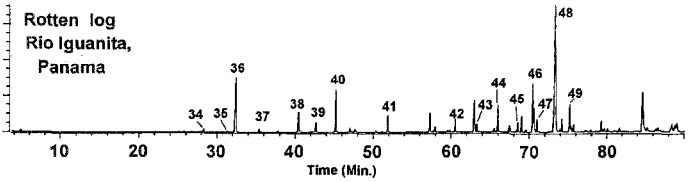

FIG. 4. Total ion chromatogram of hexane extract of the surface of an unidentified log from Rio Iguanita, Panama, that attracted a variety of euglossine species. Numbered peak identifications are: **34**, *trans*-methyl cinnamate; **35**, unidentified; **36**, unidentified; **37**, unidentified; **38**, 12:1; **39**, unidentified; **40**, tetradecyl acetate; **41**, hexadecyl acetate; **42**, 23:0; **43**, 24:0, **44**, 25:0; **45**, 26:0; **46**, heptacosanol; **47**, 27:0; **48**, eicos-9-enyl-1,20-diacetate; **49**, 29:1.

namate. However, none of the four unknowns is reported from any orchid fragrance. The lipid fraction of the extract (retention times from 40 to 90 min) contains a variety of normal alkanes, alkenes, acetates, and eicos-9-enyl-1,20-diacetate. The lipids presumedly were applied to the surface of the wood by fragrance-collecting male bees.

Site 4. In an earlier study of this site (Whitten et al., 1989), we were unable to identify the volatiles present in the bee-attracting wounds. Subsequent analyses revealed three volatile compounds in the extracts of bee-attracting wood. The most abundant is trans-nerolidol, a compound known from orchid fragrances. The remaining two unidentified compounds (**50** and **51**) have never been detected in any orchid fragrance. The attractiveness of these compounds is unknown; nerolidol has never been used in bioassays for euglossines.

DISCUSSION

The wood extracts contain two sets of compounds: (1) aliphatic alkanes, alkenes, alcohols, acetates, and diacetates that are typical of euglossine labial gland secretions; and (2) lower-molecular-weight terpenoids and aromatic compounds. The latter group probably is produced in the rotting wood, whereas the lipids are applied to the surface of the wood by fragrance-foraging male bees. Whitten et al. (1989) demonstrated that when euglossines are allowed to brush on the surface of filter paper impregnated with a chemical bait, the paper quickly becomes saturated with labial gland lipids applied by the bees. We hypothesized that the labial lipids serve as a nonpolar solvent that serves to increase fragrance collection efficiency

The fragrance-collecting behavior of male euglossine bees and their role as pollinators are well-documented, although how male euglossines utilize the collected fragrances is still unknown. Earlier hypotheses suggested that the fra-

grances served as precursors of sex pheromones or were used to produce leks (Dodson, 1975; Kimsey, 1980), but current hypotheses postulate that female bees choose mates based upon the amount or number of chemicals in each male's tibial organ (chemical-based sexual selection) (Kimsey, 1982; Kiester et al., 1984; Schemske and Lande, 1984; Stern, 1991; Lunau, 1992). Roubik (1989) noted that females of several species of *Eulaema* collect skatole-containing dung for use in nest construction; he speculated that males might collect skatole and then pass the chemical to the females during copulation, as a nuptial gift. However, there is no evidence that males can expel the chemicals in quantity or that the females can accept or store fragrances from males. Whatever the role of fragrance chemicals, male bees appear to spend a large amount of their time and energy seeking and collecting fragrances.

What is the relative importance of flowers vs. rotting wood as chemical sources for male euglossines? We suggest several plausible and possibly overlapping scenarios.

1. Male euglossines seek certain chemicals and obtain them from any available source, floral or nonfloral. Some compounds are obtained only from flowers, others only from wood (e.g., skatole), and some might be obtained from both flowers and wood (e.g. methyl cinnamate, nerolidol).

2. Flowers are the primary source of fragrance chemicals. Some rotting logs might produce chemical analogs that deceive male bees and trigger fragrance-collecting behavior, but the analogs play no role in the biology of the bees.

3. Rotting wood and other plant secretions are the primary source of fragrance chemicals. Certain orchids and other plants also produce the same chemicals and take advantage of the bees' behavior to utilize them as pollinators, but provide relatively minor amounts of chemicals for the bees.

Most workers have assumed that flowers are the principal source of chemicals collected by male bees (e.g., Williams, 1982). Chemical analyses of euglossine tibial organs cannot determine whether the chemicals present were obtained from flowers or from nonfloral sources. However, most orchids pollinated by male euglossines have low population densities and short-lived flowers and therefore might be unreliable sources of chemicals for long-lived euglossines. Ackerman (1983) found that one third of the euglossine species on Barro Colorado Island, Panama, have never been observed to visit or carry pollinaria of any orchid species on the island; these euglossines apparently obtain chemicals from sources other than orchids. Our observations suggest that rotting wood may provide a long-lasting and abundant source of at least some chemicals sought by some male euglossines. This is certainly the case for skatole-collecting *Euglossa purpurea* in Costa Rica. However, we do not know whether skatole is actually used by the bees in their courtship or whether it is an analog of indole (indole is fairly common in orchid fragrances).

Since the orchid–euglossine–wood interaction is complex, involving many species and many chemicals, we suggest that all three hypothesis may be correct to some degree, depending upon the particular bee and plant species involved. Our observations document that nonfloral sources may be important resources for at least some euglossine species.

The relative importance of nonfloral sources for male euglossines also bears upon the evolution of fragrance-collecting behavior and the extent to which euglossines and plants have coevolved. Some workers have assumed that orchids and other flowers are a major source of chemicals for the bees and that orchids and euglossines have coevolved (Kiester et al., 1984), albeit somewhat loosely. An alternative hypothesis (Ackerman, 1983) is that euglossine fragrance-collection evolved independent of the orchids and that orchids later adapted to use euglossines as pollinators, taking advantage of a preexisting behavior of male bees. In the latter hypothesis, the initial sources of fragrance chemicals for bees would have been nonfloral sources such as plant essential oils and resins and fungal secretions. Rotting logs and tree resins certainly predated the evolution of orchids, and the origin of euglossine fragrance collection might have involved rotting wood as a source of chemicals. Therefore, male euglossine-pollinated orchids might have evolved to take advantage of this pre-existing interaction involving the bees, rotting wood, and possibly fungi.

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DAMAGE-INDUCED ROOT NITROGEN METABOLISM IN *Nicotiana sylvestris*: TESTING C/N PREDICTIONS FOR ALKALOID PRODUCTION

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Abstract—"Nitrogen surplus" models for nicotine production induced by leaf damage predict that the observed increase in root nicotine synthesis after leaf damage results from "overflow" metabolism; reduced nitrogen existing in excess of growth requirements is shunted into nicotine biosynthesis. To test the nitrogen surplus model for induced nicotine production, we measured the concentrations of the major N-containing metabolites exported from the roots and the nitrate reductase activity (NRA) of roots and shoots in damaged and undamaged *Nicotiana sylvestris* plants. Leaf damage: (1) had no significant effect on root or shoot NRA, (2) increased nicotine export and decreased amino-acid and amide export from the roots of NO_3-fertilized plants, and (3) had no significant effect on the export of any N-containing metabolite from the roots of NH_4-fertilized plants. These results are not consistent with the nitrogen surplus model and indicate that leaf damage has a direct influence on root alkaloid metabolism.

Key Words—damage-induced responses, *Nicotiana sylvestris*, nicotine, nitrate, glutamine, xylem fluid, nitrate reductase.

INTRODUCTION

While roots are the principal site of nitrogen uptake, the amount of nitrogen that a plant reduces and assimilates in its roots varies greatly among species (Andrews, 1986). Since reduction and assimilation of nitrogen is thought to be performed

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more efficiently in the shoot than in the root (Oaks and Hirel, 1985), it seems paradoxical that some plants reduce nitrate in their roots and export nitrogen transport compounds to the shoot. Moreover, when root nitrogen metabolism is inhibited, plants may attain higher rates of growth (Oaks, 1992). Many explanations are possible for the maintenance of the apparently less efficient nitrogen metabolism in the roots (Oaks, 1992); however, for *Nicotiana* species that produce damage-inducible alkaloids in their roots (Baldwin and Ohnmeiss, 1993), the explanation includes supplying the nitrogen requirements of these defensive responses.

In *N. sylvestris* plants grown with nitrate as the principal nitrogen source, leaf damage increases the nicotine concentration of undamaged leaves fourfold; this increase is due to a large increase in the nicotine concentration of the xylem fluid exported from the roots (Baldwin, 1989). The biosynthesis of nicotine is well understood and is thought to be restricted to root tissues (Saunders and Bush, 1979). The xylem fluid exuding from decapitated *Nicotiana tabacum* plants is also known to contain nitrate (MacKown et al., 1990), amino acids and amides (Reuter, 1957), in addition to nicotine. The relative importance of these nitrogen transport compounds will be determined first by the amount and activity of root nitrogen reductase, which, in turn, will determine the ratio of reduced nitrogen (i.e., amino acids, amides and alkaloids) to nitrate transported in the xylem and, second, by the flux of reduced nitrogen into the nicotine biosynthetic pathway.

Understanding the effect of leaf damage on nitrate reductase activity (NRA) and the export of nicotine, amino acids and amides from the root in the xylem fluid can lead to important insights into the mechanism responsible for this damage-induced response. Five partially overlapping mechanistic models have been proposed to explain how secondary metabolites change after damage (reviewed in Baldwin, 1993). Three of these models argue that secondary metabolites accumulate in response to imbalances existing between growth-related processes and metabolite production, but these three hypotheses differ in how this imbalance is perceived; two additional models argue that damage results in signals that directly control secondary metabolism, but they differ in regard to the specificity of the signalling system. According to two of the "balance" theories, specifically, the carbon–nutrient (C/N) theory (Bryant et al., 1983) and the substrate–enzyme imbalance theory (Haslam, 1986; Waterman and Mole, 1989), increased nicotine synthesis in the roots after leaf damage might result from "overflow" metabolism; reduced nitrogen existing in excess of growth requirements will be shunted into nicotine biosynthesis. A surplus of reduced nitrogen could occur if leaf damage increased: (1) rates of nitrate uptake and root NRA was substrate limited; (2) rates of root nitrate reductase; and (3) shoot-to-root transport of reduced nitrogen. If these "nitrogen surplus" models of induced nicotine production are correct, leaf damage should increase the export

of nicotine from the roots with a concomitant increase in amino acids and amides exported from the roots regardless of how this "surplus" of reduced nitrogen was produced. Accordingly, the proportion of reduced nitrogen exported from roots in nicotine should remain unaltered by leaf damage.

To test this model, we examined the effect of leaf damage on root and shoot NRA and on the export of nitrate and reduced nitrogen compounds from the root as estimated by their respective concentrations in xylem fluid (Figure 1). We utilized two techniques to collect xylem fluid: expression of xylem fluid with a pressure bomb (Scholander et al., 1965) and by root pressure (Reuter, 1957). Both techniques have their drawbacks. While the latter technique may produce a pure xylem fluid sample, it necessitates the ultimate form of leaf damage, decapitation. Pressure bomb expression of xylem fluid allows for the facile comparison of damaged and undamaged plants, but is likely to produce xylem fluid contaminated by other apo- and symplastic constituents. We therefore use both techniques to estimate the effect of leaf damage on the export of nitrogenous compounds from the root.

METHODS AND MATERIALS

Plant Growth, Damage, and Sampling Schedules. Full-sib seeds from one *Nicotiana sylvestris* plant were germinated in Cornell mix A (Boodley and Sheldrake, 1977), grown for 17 days, and planted into either Jiffy-7 (Jiffy Products Ltd., Shippegan, Canada) peat plugs (experiments I and III) and subsequently planted into sand-containing pots, or into an aerated complete nutrient hydroponic solution (Koch et al., 1987) containing 1 mM NO_3 (experiment II). All sand-grown plants were placed in individual trays so that water-soluble nutrients would not be lost by leaching. Seeds were from the same source as those used in the experiments of Baldwin et al. (1990). All plants were grown in a greenhouse under supplemental lighting from 1000-W metal halide lamps for 13 hr/day. All plants were in the rosette stage of growth for the duration of all exper-

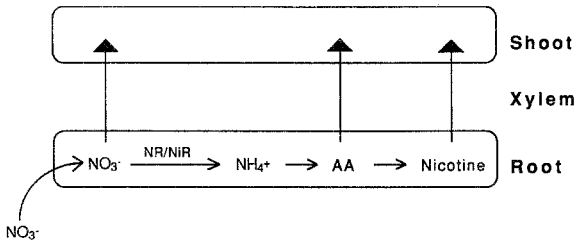

FIG. 1. Overview of nitrogen metabolism from nitrate to nicotine in *Nicotiana* roots. Abbreviations: NR/NiR, nitrate and nitrite reductase; AA, amino acids and amides.

iments. For each experiment, plants were assigned to treatment groups (damage, sampling day, and fertilization) and positions on the growing tables using a random numbers table.

The first two fully expanded leaves occupying positions 4 and 5 from the apical bud were designated as sample leaves on all plants. On day 0, herbivory was simulated with scissors on the plants in the damage treatments of each experiment. All fully expanded leaves excluding the two sample leaves were removed at the base of the petiole; no leaves were removed from any of the undamaged plants. Regrowth on damaged plants was not removed except in experiment III. Sample leaves were not damaged except for the removal of leaf disks for chemical and specific leaf mass measures. Leaf disks (0.32 cm^2, circular) were taken from the middle half of the leaf lamina; veins and margins were avoided.

Experiment I: Effect of Leaf Damage on NRA. Forty-eight 59-day-old plants growing in sand were assigned to one of the four following fertilization treatments: (1) no N, (2) NO_3, (3) NH_4, or (4) NO_3NH_4. For 15 days prior to leaf damage, all plants were watered with a half-strength hydroponic nutrient solution (Koch et al., 1987) containing all required plant nutrients except nitrogen. Plants in treatments 2 and 3 were watered daily with 50 ml of either a 7.16 mM KNO_3 or a 7.16 mM NH_3SO_4 solution; plants in treatment 4 were watered with 50 ml of a 3.58 mM NH_3NO_3 solution. Hence plants in treatments 2–4 received the same amount of nitrogen, while those in treatment 1 received no additional nitrogen in the 15 days prior to damage. Plants in the damage treatment from each of the fertilization treatments were damaged on day 0. All plants were analyzed for root and shoot nitrate reductase activity, specific leaf mass, and leaf nicotine contents on day 4.

Experiment II: Change in N Metabolites over Time in Xylem Fluid Produced by Root Pressure. Ten 47-day-old plants growing in a 40 liter hydroponic tank were decapitated with a razor and fitted with a glass collection tube for xylem fluid collection at time 0. After 4 hr, nine plants had produced sufficient xylem fluid sample for chemical analysis. A second sample was collected from these nine plants at 20 hr.

Experiment III: Change in N Metabolites after Damage in Xylem Fluid Expressed from Plants Growing under Varying N Nutrition. Two hundred ten 110-day-old plants growing in sand were transplanted from their 7.6-cm pots into 20.3-cm pots to avoid pot-binding inhibition of the alkaloidal response to damage (Baldwin, 1988) and assigned to one of the three following fertilization treatments: (1) low NO_3, (2) high NO_3, and (3) high NH_4. For 10 days prior to leaf damage and for the duration of the experiment, plants were watered daily with a 20% hydroponic nutrient solution (Koch et al., 1987) containing either 0.05 mM KNO_3, 3 mM KNO_3, or 3 mM NH_3SO_4 for plants in fertilization treatments 1, 2, and 3, respectively. Within each fertilization treatment five

replicate plants were randomly assigned to each of two damage and seven sampling-day treatments on day 0. Regrowth was removed from all plants in the damaged treatments on day 10. Plants were destructively harvested on the seven sampling days, the timing of which is designated in Figure 4 below. Two leaf disks were removed from each of the two sample leaves and immediately placed in the alkaloid extraction solution. Two additional leaf disks were removed, weighed (to 0.1 mg), dried for 24 hr at 65°C, and reweighed for estimates of leaf dry mass extracted. The shoot was removed, roots were irrigated in tap water, blotted dry, and placed in the pressure bomb for xylem fluid collection.

Chemical Analysis. Nicotine was separated and quantified in leaf and xylem extracts by high-pressure liquid chromatography (Baldwin, 1988). Leaf nicotine values were expressed as percentage leaf dry mass and xylem nicotine values were expressed as micrograms nitrogen in nicotine per 100 μl of xylem fluid.

Xylem nitrate measures were performed on 50 μl of freshly collected xylem fluid. Nitrate was quantified with a salicylic acid-based colorimetric technique (Cataldo et al., 1975). Salicylic acid reagents and NO_3 standards were prepared fresh daily. Values are expressed as micrograms nitrogen in NO_3/100 μl of xylem fluid.

Amino acids and amides in xylem fluid were measured by gas chromatography (0.531 mm, 15 m DB-1 bonded-phase capillary column, H_2 carrier gas, and flame ionization detection) after derivitization (as *N*-trimethylsilyl TMS amino acid esters) with an internal standard (phenanthrene) technique following the procedure of Gehrke et al. (1969). Xylem fluid (200–350 μl) was weighed (0.1 mg) and freeze-dried, and the dried residue was taken up in 200 μl of phenanthrene-containing (0.488 mM) acetonitrile and 200 μl of BSTFA with 1% TMCS (Sigma Chemical Co., St. Louis, Missouri) and heated at 90°C for 1 hr. The TMS derivatives of aspartic acid, asparagine, glutamic acid, glutamine, and 2-pyrrolidone 5-carboxylic acid were quantified for all samples; peak identity was confirmed with standard compounds and by GC-MS. Relative molar response factors for the two amino acids and amides were determined each day. Glutamine was consistently the dominant component, representing more than 60% of the nitrogen in all detected amino acids and amides, and it was frequently the only compound detected in addition to nitrate and nicotine. Therefore, to simplify the data analysis, we report glutamine and total amino acids and amides concentrations expressed as micrograms of nitrogen in glutamine (or amino acids and amides) per 100 μl of xylem fluid so that they can be directly compared with the nitrate and nicotine values.

Nitrate Reductase Assay. Weighed (to 0.1 mg) leaf disks (approx. 20 mg fresh mass) and root sections (approx. 1 g fresh mass) cut into 5-mm pieces were placed in 5 ml of an incubation medium described in Jaworski (1971) containing *n*-propanol at a concentration of 3% v/v. We chose this concentration of *n*-propanol after varying its concentration from 1 to 7% in 1% increments

and finding that a 3% level gave the highest NRA for both root and shoot samples. Because NRA is known to vary over leaf ontogeny in *N. tabacum* (Wakhloo and Staudt, 1988), we analyzed only leaf disks taken from the same-aged sample leaves from all plants. Whole leaf disks gave consistently higher NRA activity than leaf disks that had been cut into 4-mm strips, so in our assays we used whole disks. We examined the NRA activity in the root sections of differing size and found the highest activity in the thickest root portions near the stem. This portion of the root was used in all samples. Aryan and Wallace (1983) argue that NRA assay is reductant-limited and that true NRA cannot be measured in the absence of excess NADH. Because we found no consistent increase in measured NRA activity when the incubation medium was supplemented with 0.2 mM NADH, we did not add extra reductant to the incubation medium.

After placing the sample into the incubation medium, samples were kept in a darkroom at 25–27°C. After 20, 40, 60, and 80 min, 400-μl aliquots of incubation medium were analyzed for nitrite as described in Jaworski (1971). Concentrations of nitrite per gram fresh mass sample were calculated for each of the four samplings and regressed against sampling time. The slopes of this regression (micrograms of $N\text{-}NO_2$ per gram fresh mass per minute) were our estimates of NRA.

Xylem Fluid Collection. A minimum volume of 300 μl of xylem fluid was required to measure the concentrations of nicotine, nitrate, amino acids, and amides from one plant. In order to collect this volume, one of two techniques was employed: expression of xylem fluid with a pressure bomb (Scholander et al., 1965) and root pressure. While the latter technique has been used with soil-grown *N. tobaccum* plants (Reuter, 1957; MacKowan et al., 1990), we found in preliminary experiments that only one in 10 *N. sylvestris* plants grown in sand produced sufficient quantities of xylem fluid after decapitation. Hydroponically grown plants, on the other hand, once decapitated, would continue to produce copious amounts of xylem fluid for up to seven days after decapitation. Before collection of xylem fluid with either technique, the cut stem was rinsed with a 100 mM aqueous solution of $CaCl_2$ for approximately 1 min in order to minimize contamination by phloem exudate (Kallarackal et al., 1989). For xylem samples produced with root pressure, a 1-cm piece of surgical tubing that held a glass collection tube was fitted snugly to the cut stump. The collection tube was covered with a wax film to minimize evaporation of the xylem fluid between collections. For xylem samples produced with a pressure bomb, roots were placed in a PMS-650 pressure bomb (PMS Instrument Co., Corvallis, Oregon) so that the cut stem protruded from the bomb. The stem was recut with a razor and rinsed in $CaCl_2$. A pressure of 0.5 MPa was required to consistently produce a 400-μl xylem sample within 5 min. The first 100 μl was discarded to minimize symplastic contamination from the stem. We examined the effect of increasing

bomb pressure from 0.5 to 2.0 MPa on xylem fluid composition and found that both glutamine and nicotine concentrations decreased with increasing pressure. Hence, all samples were collected at 0.5 MPa of pressure. After collection, xylem fluid was weighed and immediately processed for nicotine, nitrate, and glutamine analysis.

Statistical Methods. Two-way ANOVAs with the damage and fertilization treatments as main effects were used to analyze measures of leaf nicotine, NRA, and the N-containing constituents of the xylem fluid. Paired *t* tests were used to compare the nicotine, nitrate, and amino acid composition of xylem fluid collected from the same plants in experiment II. Percentages were arcsine transformed. Analysis was performed with the MGLH ANOVA module from Systat Inc. (Evanston, Illinois).

RESULTS

Experiment I. Leaf damage significantly (Table 1; Figure 2) increased leaf nicotine concentrations in the three fertilization treatments that received nitrogen; nitrogen-deprived plants did not significantly increase their leaf nicotine pools in response to damage. While NH_4-fertilized plants attained the highest absolute leaf nicotine concentrations, these plants exhibited the smallest proportional increase above undamaged plants (2.6-fold increase) compared with the NO_3- (3.1-fold increase) and NO_3NH_4-fertilized (3.5-fold increase) plants.

TABLE 1. TWO-WAY ANOVAS FOR ROOT AND SHOOT NITRATE REDUCTASE ACTIVITY AND LEAF NICOTINE CONTENT DEPICTED IN FIGURE. 2

	df	*F*	*P*
Shoot nitrate reductase			
Fertilization (F)	3	6.399	0.001
Damage (D)	1	1.197	0.281
F * D	3	0.090	0.965
Error	39		
Root nitrate reductase			
Fertilization (F)	3	2.235	0.099
Damage (D)	1	2.275	0.139
F * D	3	1.263	0.300
Error	40		
Nicotine % leaf dry mass			
Fertilization (F)	3	31.722	0.000
Damage (D)	1	164.341	0.000
F * D	3	16.427	0.000
Error	40		

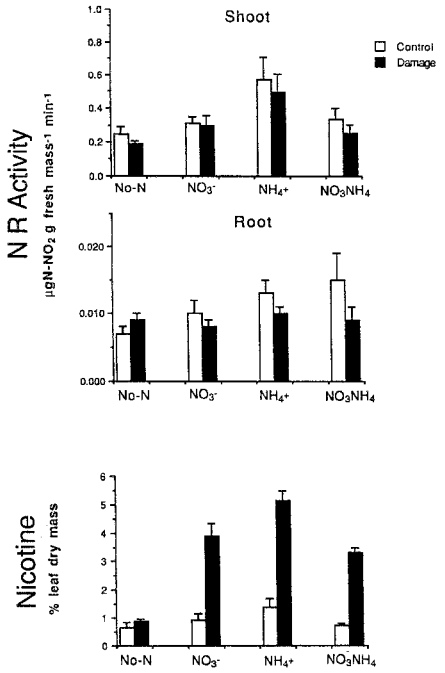

FIG. 2. Mean (±SEM) shoot and root nitrate reductase (NR) activity and leaf nicotine concentrations in six damaged and six undamaged *N. sylvestris* plants in each of four fertilization treatments four days after leaf damage. See text for descriptions of the fertilization treatments for experiment I and Table 1 for statistical analysis.

In contrast to its effect on leaf nicotine pools, leaf damage had no significant effect on either root or shoot NRA (Table 1; Figure 2). As has reported in *N. tabacum* (Wakhloo and Staudt, 1988), shoot NRA was substantially larger than root NRA, with the latter representing only 2.8–4.8% of the former.

Experiment II. The composition of all N-containing compounds in xylem fluid exuding from decapitated hydroponically grown plants changed significantly ($ts = 6.7$–17.3; all $Ps < 0.0001$; $df = 8$) from the first 400 µl of xylem fluid collected 4 hr after decapitation compared to a sample collected 20 hr after decapitation (Figure 3). Glutamine represented 95% or more of all the amino acids and amides found in the xylem fluid and therefore we report only glutamine values. Glutamine concentrations decreased by 96%, nicotine concentrations increased by 134%, and nitrate concentrations decreased by 50% from hour 4 to 20 of sampling.

Experiment III: Leaf damage on days 0 and 10 significantly (Table 2; Figure 4) increased leaf nicotine concentrations in all three fertilization treat-

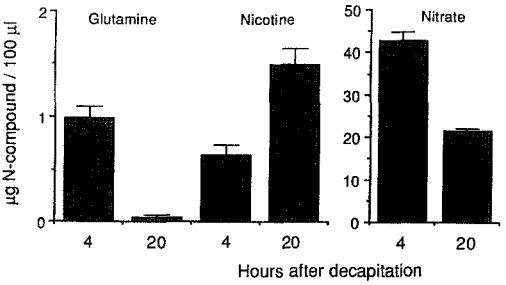

FIG. 3. Mean (±SEM) glutamine, nicotine, and nitrate concentrations of xylem fluid samples exuding from 10 hydroponically grown *N. sylvestris* plants that were decapitated at time 0. See text for details of experiment II.

ments. The concentrations of leaf nicotine attained in response to damage were largely similar among the three fertilization treatments. However, the response was greater in NH_4-fertilized plants after the second damage event, and the damage-induced nicotine concentrations returned to levels found in undamaged plants between the two damage events in the NH_4-fertilized plants, but not in the other two fertilization treatments.

Given that xylem fluid represents the transport pool shuttling nicotine between the site of synthesis (roots) and an important accumulation site (the shoot), it was not surprising to find that leaf damage resulted in significantly more complex patterns of responses in xylem fluid constituents (Figure 4 and 5). As expected, for xylem fluid constituents containing reduced nitrogen (nicotine, amino acids and amides), the concentrations were generally highest in NH_4-fertilized plants and decreased with nitrate supply rate. Similarly, xylem fluid nitrate concentrations were highest in plants in the high nitrate supply rate treatment (Figures 4 and 5).

The effect of leaf damage depended strongly on the fertilization treatment. Leaf damage did not significantly alter any of the measured xylem fluid constituents in NH_4-fertilized plants ($F_{1,58}$s = 0.2–1.6; all Ps > 0.21) when samples were pooled across all days (Figure 5). Nicotine concentrations on day 3 and glutamine concentrations on day 20 were significantly elevated in damaged plants (Figure 4, Table 2). The percentage of reduced nitrogen in xylem fluid that was in nicotine (17–20%) was not significantly influenced by leaf damage in NH_4-fertilized plants.

In contrast to these results, leaf damage resulted in significant changes in the composition of xylem fluid when plants were fertilized with 3 mM NO_3. Leaf damage had the following significant effects on xylem fluid composition: (1) increased nicotine concentrations ($F_{1,65} = 7.95$; $P = 0.006$; Figure 5) when pooled over all days and during all days analyzed individually except days 6

TABLE 2. *P* VALUES AND DF_{error} FROM TWO-WAY ANOVAS FOR LEAF AND XYLEM FLUID NICOTINE CONTENTS AND XYLEM FLUID GLUTAMINE AND NITRATE CONTENTS DEPICTED IN FIGURE 4[a]

	Leaf nicotine	Xylem		
		Nicotine	Glutamine	Nitrate
Day 1				
P Fertilization (F)	0.225	0.083	0.283	0.004
P Damage (D)	0.001	0.224	0.430	0.740
P F * D	0.133	0.966	0.283	0.896
DF_{error}	24	18	22	8
Day 3				
P Fertilization (F)	0.321	0.126	0.715	0.238
P Damage (D)	0.000	0.015	0.192	0.690
P F * D	0.793	0.806	0.958	0.710
DF_{error}	24	23	24	20
Day 6				
P Fertilization (F)	0.217	0.502	0.022	0.000
P Damage (D)	0.000	0.569	0.433	0.921
P F * D	0.469	0.394	0.310	0.254
DF_{error}	23	23	23	31
Day 8				
P Fertilization (F)	0.085	0.004	0.314	0.000
P Damage (D)	0.006	0.029	0.659	0.122
P F * D	0.051	0.061	0.429	0.003
DF_{error}	24	24	24	24
Day 13				
P Fertilization (F)	0.039	0.000	0.221	0.000
P Damage (D)	0.000	0.016	0.073	0.000
P F * D	0.160	0.643	0.377	0.000
DF_{error}	24	24	24	24
Day 16				
P Fertilization (F)	0.026	0.002	0.016	0.000
P Damage (D)	0.000	0.501	0.953	0.000
P F * D	0.094	0.457	0.616	0.000
DF_{error}	23	21	24	24
Day 20				
P Fertilization (F)	0.385		0.073	
P Damage (D)	0.003		0.078	
P F * D	0.596		0.038	
DF_{error}	19		17	

[a]DF for F, D and F * D treatments were 2,1,2 respectively for all days.

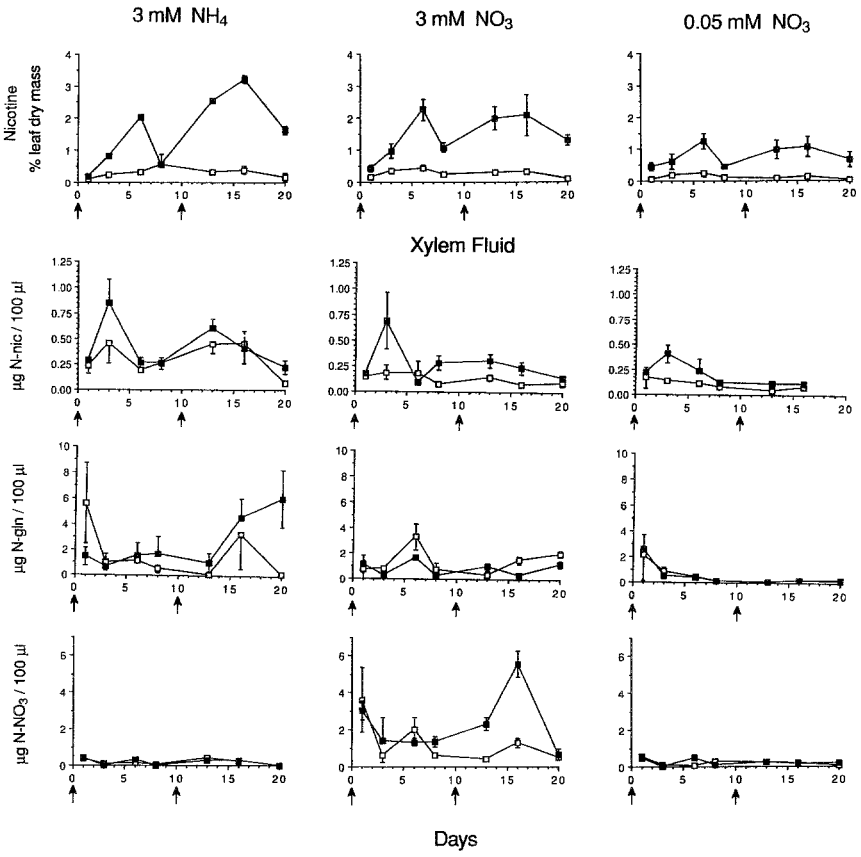

FIG. 4. Mean (±SEM) leaf and xylem nicotine and xylem glutamine and nitrate concentrations taken from five damaged (■) and five undamaged (□) *N. sylvestris* plants harvested during seven samplings over 20 days after an initial leaf damage event on day 0 and a subsequent removal of regrowth on day 10 (as indicated by arrows) in three different fertilization treatments. Experiment III therefore consisted of 5 replicates × 3 fertilization treatments × 2 damage treatments (damaged and undamaged) × 7 sampling days. See text for descriptions of the fertilization treatments for experiment III and Table 2 for statistical analysis.

and 30 (Figure 4; Table 2); (2) decreased total amino acid and amide composition ($F_{1,65} = 3.78$; $P = 0.056$; Figure 5), which was in large part due to decreases in glutamine concentrations ($F_{1,65} = 3.57$; $P = 0.063$; Figure 5); (3) increased xylem nitrate concentrations ($F_{1,60} = 6.18$; $P = 0.016$; Figure 5). The percentage of reduced nitrogen in xylem fluid that was in nicotine increased

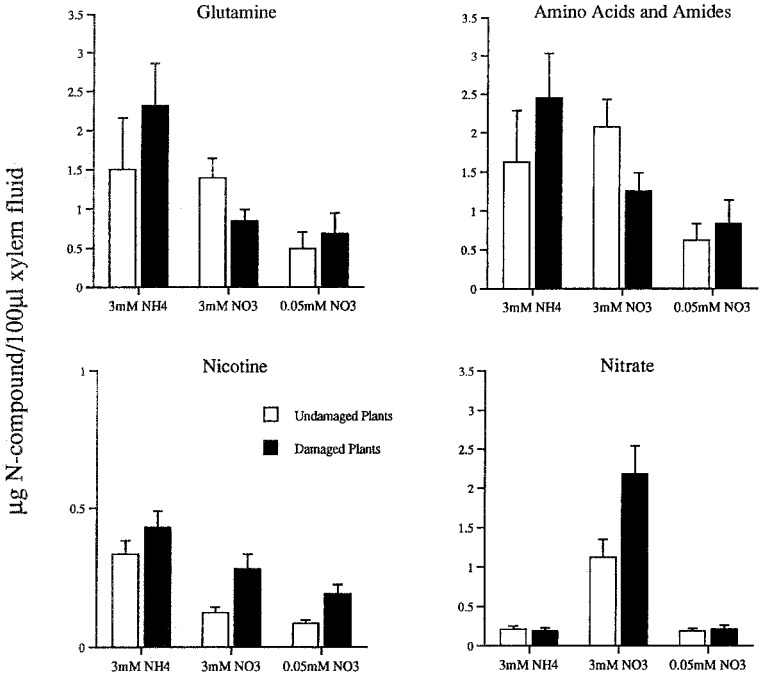

FIG. 5. Mean (±SEM) glutamine, total amino acids and amides, nicotine, and nitrate concentrations of xylem fluid samples depicted in Figure 4 pooled over all sampling dates by damage and fertilization treatment.

significantly from 6.1% in undamaged plants to 22.6% in response to leaf damage.

In plants fertilized with 0.05 mM NO_3, leaf damage significantly increased xylem fluid nicotine concentrations ($F_{1,57} = 10.901$; $P = 0.002$; Figure 5) but did not change any of the other constituents significantly (all $F_{1,57}$s = 0.25–0.35; Ps > 0.55; Figure 5). The percentage of reduced nitrogen in xylem fluid that was in nicotine increased from 14.1% in undamaged plants to 23.4% in damaged plants.

DISCUSSION

A number of authors have argued that nitrogen-containing metabolites, such as nicotine, accumulate in response to stress in order to detoxify excess free ammonia (Rabe, 1990, and references therein). Nowacki et al. (1976) proposed that enhanced alkaloid production under high nitrogen supply rates is a means

of avoiding amino acid toxicity. While the C/N theory was originally formulated to explain changes in carbon-containing metabolites, these arguments are consistent with the application of C/N theory to the production of nitrogen-containing metabolites. The patterns of constitutive (as opposed to induced) nitrogen-containing secondary metabolite production appear to be consistent with a "nitrogen surplus" model. High nitrogen supply rates increase the concentration of some (Gershenzon, 1984; Johnson et al., 1987; Baldwin, 1988) but not all (Nowacki et al., 1976) of these metabolites. Low nitrogen supply rates, by lowering both the substrate availability and the concentrations of alkaloid biosynthetic enzymes, can lower the constitutive concentrations (Waterman and Mole, 1989; Gershenzon, 1984). However, the results of this study are not consistent with the "nitrogen surplus" model's predictions for damage-induced root nicotine production.

Since leaf damage did not significantly increase the concentration of reduced nitrogen in the xylem fluid, our experiments provide little evidence for the existence of a nitrogen surplus resulting from the leaf damage. However, since we did not measure the amount of reduced nitrogen transported from root to shoot, this remains a weak test of the model. We examined one of the mechanisms by which a root nitrogen surplus may be produced by leaf damage, specifically, that leaf damage could increase root NRA, and found no evidence for such a mechanism.

We tested the "nitrogen surplus" model's prediction that leaf damage would increase the export of nicotine from roots with a concomitant increase in amino acids and amides exported, leaving the proportion of reduced nitrogen exported as nicotine unaltered by damage. The results of both experiments II and III with nitrate-fertilized plants were not consistent with this prediction. While the export of nicotine increased after leaf damage, the export of amino acids and amides decreased, resulting in a greater proportion of reduced nitrogen exported in nicotine from the roots of damaged plants. The nitrogen-containing constituents in the xylem fluid of plants fertilized with ammonium were not significantly altered by leaf damage since both control and damaged plants had xylem nicotine concentrations representing 17–20% of the total reduced nitrogen in xylem fluid, which approximates that found in damaged, nitrate-grown plants. An upper concentration limit may exist for the transport of reduced-nitrogen in the xylem fluid, and ammonium-grown plants may be functioning at these limits.

The concentration of nitrate in xylem fluid exuding from decapitated plants growing in a hydroponic solution containing nitrate at 1 mM was 20 times greater (Figure 3) than that expressed from damaged plants grown in sand and fertilized with a 3 mM nitrate solutions (Figures 4 and 5). Similarly high nitrate concentrations have been reported for the xylem fluid exuding from decapitated soybean plants, which similarly declined as the plant continued to produce xylem fluid (Rufty et al., 1982). These high concentrations of nitrate may be an osmotic

response to decapitation as the plant attempts to regain normal root pressure. Since the amount of reduced nitrogen exported from the roots remains relatively unchanged despite the large increase in nitrate uptake, it appears that root NRA is not substrate limited. Consequently, the increases in root-specific nitrate uptake rates that accompany folivory in some plants (Ruess, 1988) may not alter the pool size of reduced nitrogen in the roots.

While these results are not consistent with the predictions of a nitrogen surplus model, they are consistent with models proposing that damage results in signals that directly control secondary metabolism. In experiment II, the observed decrease in nitrogen exported from the root in the form of glutamine is sufficient to account for the increase in nitrogen exported in nicotine. This apparent trade-off between glutamine and nicotine export suggests that leaf damage increases the flux of nitrogen into root nicotine biosynthesis, and we have recently demonstrated that this is in fact the case (Baldwin et al., 1994).

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QUANTITATIVE ANALYSIS OF PHEROMONE PRODUCTION IN IRRADIATED CARIBBEAN FRUIT FLY MALES, *Anastrepha suspensa* (LOEW)[1]

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Abstract—Pheromonal volatiles emitted by irradiated and control 5- to 11-day-old Caribbean fruit flies, *Anastrepha suspensa* (Loew), were collected on Tenax adsorbent filters and quantified by gas chromatography (GC). The components released were identified by comparison of retention times on GC and by mass spectrometry (MS) with authentic synthetic standards. Pharate adults were irradiated with gamma rays from a ^{60}Co source at a dose rate of 10.3 Gray (Gy)/min. The total dosages given were 30, 50, 70, and 100 Gy. Pheromone volatiles were collected from adult males when they were between 5 and 11 days of age. The compounds quantified were (*Z*)-3-nonenol and (*Z*, *Z*)-3,6-nonadienol, which eluted from the GC column together and were quantified as one peak, β-bisabolene, suspensolide, anastrephin, and epianastrephin. Irradiation with 30 Gy did not significantly reduce any pheromonal components, nor did it change the pheromonal blend. In contrast, suspensolide and bisabolene were significantly reduced in flies irradiated with 50 Gy, while the nonenols and epianastrephin were reduced at the 70-Gy dose. Irradiation with the 100-Gy dose reduced all components with the exception of suspensolide.

Key Words—*Anastrepha suspensa*, fruit fly, Tephritidae, Diptera, phero-

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mone, irradiation, β-bisabolene, anastrephin, epianastrephin, suspensolide, (*Z*)-3-nonenol, (*Z*, *Z*)-3,6-nonadienol.

INTRODUCTION

The Caribbean fruit fly, *Anastrepha suspensa* (Loew), is indigenous to the Caribbean islands of the West Indies, and a population became permanently established in Florida following an apparently accidental introduction near the Miami International Airport in 1965 (Weems, 1965). Although they are not preferred hosts, citrus fruits are sometimes an oviposition resource for female flies, and the fly has a significant economic impact in Florida upon export of citrus.

The state of Florida is considering use of irradiation in a sterile insect technique (SIT) effort against the Caribbean fruit fly (von Windeguth et al., 1973; Brown and Holler, 1988; Minno, 1990). The SIT to maintain fly-free zones in Florida has high potential and is a relatively benign procedure for the environment. SIT programs have demonstrated their usefulness for fruit fly suppression or eradication in Japan and Okinawa (Gilmore, 1989; Iwahashi, 1977; Kawasaki, 1991; Nakamori, 1991) and in Hawaii (Vargas and Spencer, 1991). The efficacy of SIT in reducing the feral population is directly proportional to male competitiveness in attracting and mating with feral females. Males of the Caribbean fruit fly produce sex pheromone (Nation, 1972), and nine volatile components have been identified (Nation, 1983; Battiste et al., 1983; Chuman et al., 1988; Rocca et al., 1992). Behavioral roles in the attraction of females have been demonstrated for most of the compounds (Nation, 1975, 1991).

The objective of the research reported here was to evaluate what effect a series of irradiation doses had upon the quantity and blend of pheromonal volatiles released by mature male flies, in order to determine if an irradiation dosage could be identified that would sterilize both males and females while leaving pheromone production and release unaffected.

METHODS AND MATERIALS

Insects. The fruit flies, under laboratory colonization for about 10 years, were obtained from the State of Florida Sterile Fly Laboratory, Agriculture and Consumer Services Department, Division of Plant Industry, Gainesville, Florida. Flies were irradiated aerobically as pharate adults about 24–36 hr prior to their emergence from puparia. Time to emergence was judged from eye iridescence viewed after dissection of the puparium. In some cases flies were allowed to start to emerge and then the unemerged pharate adults were irradiated. Adults not emerging within 36 hr after irradiation were not collected for exper-

iments. Irradiation was performed in a cobalt-60 irradiator at the USDA/ARS Insects Affecting Man and Animals Laboratory in Gainesville, Florida. Pharate adults were irradiated by placing approximately 1000 pupae in a circular plastic dish (360 ml capacity, 11 cm diameter × 4 cm high). Radiation doses of 30, 50, 70, and 100 Gy (1 Gy = 100 rads) were given at a dose rate of 10.3 Gy/min (Hall, 1984). Control pupae were treated exactly as experimental ones, except that no radiation was given.

One day after adults had emerged, males were collected by a pump-driven aspirator and placed into cloth-covered, aluminum frame cages (25 × 15 × 24 cm) with about 500 males per cage. The males were then kept in environmental chambers with a light–dark (LD) cycle of 14:10 hr, 26 ± 1°C, and allowed to feed ad libitum on dry yeast hydrolysate, dry sucrose, and water.

Collection of Volatiles. The volatiles were collected by published procedures (Nation, 1989, 1990). Cages of male flies were removed from the incubators about 15–20 min prior to collection to allow flies to adjust to room conditions. Volatiles were collected from males 5–9 days old for 3 hr between the 9th and 12th hours of the photophase, when pheromone release peaks (Nation, 1990). At the end of a daily collection, the males were removed from the collection devices and put back into the environmental chamber to be used again on subsequent days. Usually collections were made with 10 males in each collection apparatus, and simultaneous collections were made from four control and four experimental groups. In the 100-Gy treatment, pheromone was collected from 20 flies, with appropriate controls, for 1 hr when flies were 9–11 days old. During all collections of volatiles, overhead lighting was provided by a combination of fluorescent and halogen bulbs, with room temperature at 25 ± 1°C and 75–80% relative humidity.

Analysis and Quantitation of Volatile Components. A published procedure (Nation, 1990) for the analysis of the volatiles from Caribbean fruit flies was followed. Trapped volatiles were eluted from Tenax traps with pentane (final eluted volume = 10 ml) and a measured volume of an internal standard solution in pentane was added before any further manipulation of samples. The internal standard contained decanol to estimate the nonenols, tetradecane to estimate bisabolene, and methyl tridecanoate to estimate the three lactones. The suitability of these particular internal standards for quantitation has been established (Nation, 1990). (*Z*)-β-Ocimine, α-bergamotene, and (*E*, *E*)-α-farnesene are minor components with no known behavioral role in the volatiles of the males (Rocca et al., 1992), and they were not quantified. Immediately prior to chromatography, samples were concentrated to about 0.5 ml with a gentle jet of nitrogen gas. Quantitative data from peak areas were taken from chromatography of samples on a fused silica 25 m × 0.25 mm capillary column containing bonded RSL 150 (Alltech, Deerfield, Illinois) in a Shimadzu (Kyoto, Japan) GC-14A chromatograph with FID. Chromatographic data were recorded with a

Spectra Physics (Santa Clara, California) SP 4400 integrator and analyzed with Spectra Physics Chrom-Jet software. The detector response was 1:1 between the pheromonal component and its designated standard. Confirmation of identity and mass spectral data were obtained after chromatography on a Perkin Elmer (Norwalk, Connecticut) GC interfaced directly with a (Finnigan, San Jose, California) ion trap detector system (ITDS). The column was a 30-m × 0.25-mm fused silica capillary containing bonded DB-1 (J&W, Folsom, California) phase. Mass spectral data were recorded with the ITDS data software package. Mass spectra could be compared with about 40,000 spectra in the National Bureau of Standards mass spectral library and our own library of pheromonal synthetic standards.

The same chromatographic conditions were used in each instrument, as follows. Samples were injected splitless into a capillary injector port at 180°C, followed after 30 sec by a split-purge flow of helium. The initial column temperature was 60°C, with a programmed rise in temperature of 10°C/min beginning immediately after injection. When the temperature reached 120°C, the programmed rise was changed to 6°C/min until a final temperature of 150°C was reached. The linear flow of helium through each capillary column was adjusted to 25 cm/sec.

Data Analysis. For analysis, the quantity of volatiles released by control flies versus experimental flies in simultaneous collections from flies of a given age were subjected to Student's paired t test. Collections were paired in this way because it has been demonstrated that the release of pheromone varies daily with age and with different cohorts of flies (Nation, 1990). The 0.05 probability level was chosen in all tests to indicate significance. Linear regression was used to test for the relationship between components of the pheromonal blend. All statistical analyses were done with JMP-IN software (SAS Institute, Inc., Cary, North Carolina) on a Macintosh PC.

RESULTS

Males irradiated with 30 Gy had no statistically significant reduction in the volatile components when compared with unirradiated controls (Figure 1). The combined, mean quantity of all pheromone components collected from irradiated flies during five days was 1135 ng/male/hr, whereas control flies produced 1244 ng/male/hr. The proportions of the volatiles in control and experimental flies were almost identical.

Flies irradiated with a dose of 50 Gy showed some reduction in all components when compared with untreated flies (Figure 2), but only the reductions in suspensolide ($t = 3.34$, df 19, $P = 0.02$) and bisabolene ($t = 2.53$, df 19, $P = 0.02$) were statistically significant. Irradiation of males with 70-Gy doses

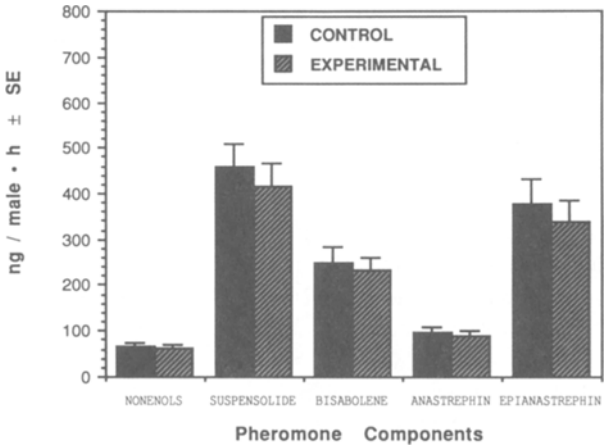

FIG. 1. Pheromone collected (mean ±SE) from control Caribbean fruit flies and those irradiated with 30 Gy as pharate adults 24–36 hr prior to emergence. Means for irradiated flies were not significantly different from those of control flies at the 0.05 significance level. Each bar represents 20 replicate collections made when flies were 5–9 days old.

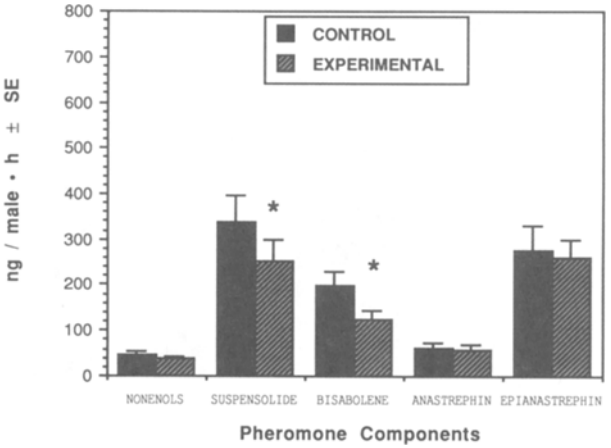

FIG. 2. Pheromone collected (mean ±SE) from control Caribbean fruit flies and those irradiated with 50 Gy as pharate adults 24–36 hr prior to emergence. An asterisk above an experimental bar indicates that the mean was significantly different (0.05 level) from the control mean for that component of the pheromone. Each bar represents 20 replicate collections from flies that were 5–9 days old.

(Figure 3) caused significant reduction in nonenols (t = 4.81, df 19, P < 0.0001) and epianastrephin (t = 2.27, df 19, P = 0.03). Experimental flies irradiated with 70 Gy produced 1519.8 ng/male/hr, whereas control flies produced 1771 ng/male/hr. Thus, the 70-Gy dosage caused 14.2% reduction in volatiles released by the experimental flies.

Males irradiated with 100-Gy doses showed the greatest indication of damage to their pheromone-producing ability (Figure 4). The totals of all quantified volatiles from control and experimental flies after three days of collection were 2044 ng/male/hr and 1536 ng/male/hr, respectively. The total overall reduction of volatiles in the irradiated flies was 24.9%, with reduction of constituents in irradiated insects as follows: nonenols 31.6% (t = 6.69, df 11, P < 0.0001), bisabolene 15.9% (t = 2.61, df 11, P = 0.02), anastrephin 31.4% (t = 3.14, df 11, P = 0.009), and epianastrephin 28.3% (t = 3.03, df 11, P = 0.01). Although there was about an 18% reduction in suspensolide, it was not significant at the 0.05 significance level, possibly because of high variance from collection to collection. High variance in all components has been shown previously (Nation, 1990).

Regression analyses of data from irradiated flies that had reduced pheromone release (i.e., those males irradiated with 50 or more Gy) (Figure 5A) and their controls (figure 5B) showed a linear relationship between amounts of anastrephin and epianastrephin released, but there was greater spread of the data in

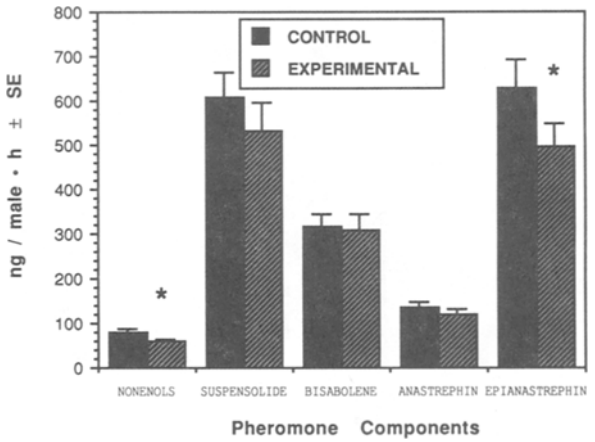

FIG. 3. Pheromone collected (mean ±SE) from control Caribbean fruit flies and those irradiated with 70 Gy as pharate adults 24–36 hr prior to emergence. An asterisk above an experimental bar indicates that the mean was significantly different (0.05 level) from the control mean for that component of the pheromone. Each bar represents 20 replicate collections made when flies were 5–9 days old.

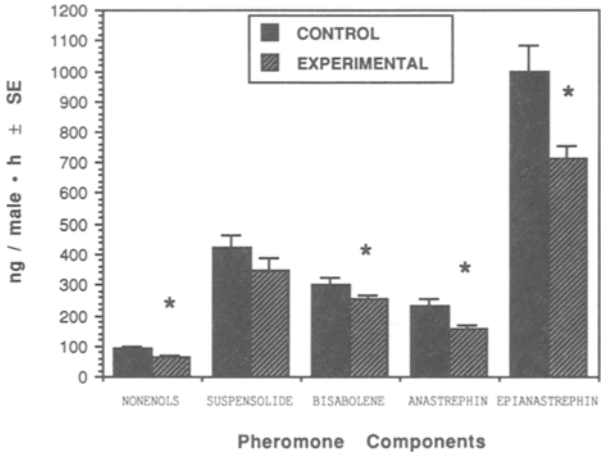

FIG. 4. Pheromone collected (mean ±SE) from control Caribbean fruit flies and those irradiated with 100 Gy as pharate adults 24–36 hr prior to emergence. An asterisk above an experimental bar indicates that the mean was significantly different (0.05 level) from the control mean for that component of the pheromone. Each bar represents 12 replicate collections made when flies were 9–11 days old.

the case of the irradiated flies. No relationships between other components were discovered.

DISCUSSION

Effect of Irradiation on Pheromone Production There was no statistically significant reduction in the quantity or blend of pheromonal components released by males irradiated with 30 Gy. Previous work has shown that doses of 30 Gy or greater will sterilize both sexes of the Caribbean fruit fly (Burditt et al., 1975). Calkins et al. (1988) reported that females were completely sterilized even at 10 Gy and that males were 95% sterile at 13.14 Gy and 99% sterile at 16.22 Gy. Another recent report showed that 25 Gy caused complete atrophy of the ovarian tissues, with no evidence of regeneration after 30 days (Walder and Calkins, 1992). A method to sex Caribbean fruit fly pupae is not available, and sterile females may have to be released along with sterile males.

A number of workers have found that overall reduction in mating competitiveness in fruit flies is correlated with radiation dose (Hooper and Katiyar, 1971; Hooper, 1972, 1975; Anwar et al., 1975; Teruya et al., 1975; Teruya and Zukeyama, 1979; Velasco and Enkerlin, 1982; Morena et al., 1991), although none of those studies actually measured pheromone released by the

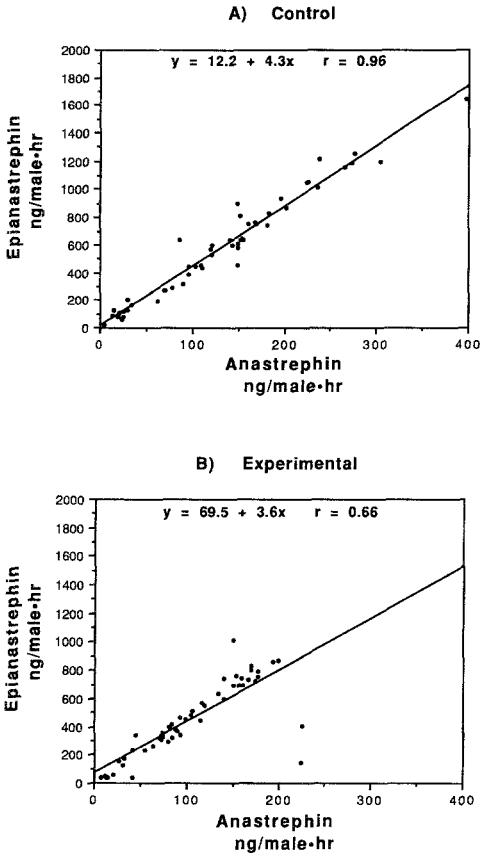

FIG. 5. The relationship between the two pheromone components, anastrephin and epianastrephin. Data from all controls or all experimentally irradiated males were lumped, and each figure represents a total of 72 collections. (A) control data; (B) experimental data.

flies. Moreno et al. (1991) found that whole body extracts contained about 35% less pheromonal compounds than extracts of control males in the case of Mexican fruit flies irradiated with 70–116 Gy. Haynes et al. (1977) measured the pheromone that irradiated boll weevils, *Anthonomus grandis* Boh., released in their frass and found that fractionated doses totaling 62.5 Gy reduced pheromone production by two thirds for the first five days of adult life. Recovery apparently occurred, however, so that between days 5–11 posttreatment the treated weevils were equal to the controls.

Knipling (1955) stressed that competitiveness of sterile insects is perhaps the most important factor for the success of a SIT program. Calkins et al. (1988)

reported some reduction in mating competitiveness of sterilized *A. suspensa* males. Irradiation may damage any of several sensory modes that insects rely upon for successful mating. Tephritid fruit flies utilize pheromonal, visual, and auditory cues in courtship and mating (Fletcher, 1968; Haniotakis, 1974; Enkerlin et al., 1975; Landolt et al., 1992; Robacker and Hart, 1985; Prokopy, 1975; Prokopy and Hendrichs, 1979; Nation, 1972; Webb et al., 1976).

Fletcher and Gianakakis (1973), however, found no differences in responses of *Bactrocera tryoni* (Froggatt) females to extracts from pheromone glands of males irradiated as pharate adults with 80 and 200 Gy. Irradiation of flies in an anaerobic atmosphere increased competitiveness in sterile males, even though higher radiation doses were required to achieve sterilization (Zumreoglu et al., 1979; Ohinata et al., 1971; Hooper, 1971). Mating competitiveness of irradiated Mediterranean fruit flies, *Ceratitis capitata* Wiedemann, improved when young, emerged adults were irradiated instead of pupae (Chambers et al., 1970; Hooper, 1971; Ohinata et al., 1971). A large program for SIT, however, is likely to require irradiation of pharate adults within the pupal cases.

Irradiation altered slightly the relationship of anastrephin and epianastrephin, as evidenced by the greater variability in the data of Figure 5 for irradiated flies. Chuman et al. (1988), Mori and Nakazono (1988), and Rocca et al. (1992) suggested that the diastereomers anastrephin and epianastrephin are biosynthesized via a common pathway until the last few steps. Irradiation may have influenced one or more of the last steps in the synthesis.

Our data show that an irradiation dose of 30 Gy can be delivered to pharate adults just prior to their emergence without significantly reducing male ability to produce pheromone. The mating behavior of the Caribbean fruit fly is complex, however, involving the formation of leks (Burk, 1983), and additional evaluation of irradiated males in competition tests with feral males is needed.

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Announcement

ELEVENTH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY

The International Society of Chemical Ecology will hold its 11th Annual Meeting at the College of Environmental Science and Forestry, Syracuse, New York, June 4 through noon of June 8, 1994. Registration will start in the afternoon of June 4, and a barbecue will be held the same evening.

The plenary lecturers are May Berenbaum (Univ. of Illinois) and Rodrigo Gamez (Inst. National de Bioversidad, Costa Rica).

Symposia topics, organizers, and speakers are as follows:

Plant-Herbivore Interactions (D. Müller-Schwarze, J. Schultz)
Jack Schultz — Ian Baldwin
John Bryant — Frederick Provenza

Chemical Prospecting (T. Eisner, J. Meinwald)
Paul Anderson — Thomas Eisner
Prabhavathi Fernandes — Francisco Grifo
Janice Thompson

Chemical Ecology of Forest Insects (S. Teale, D. Wood)
Steve Teale — Steve Seybold
Desiree Vanderwel — Wittko Francke

Chemical Ecology and Chemical Analysis: Advances in Detection and Identification (W. Roelofs, F. Webster)
Ring Cardé — Richard Durst — Susan Pachapski
Terry Acree — Scott Smedley

Interspecific Chemical Recognition (J.A. Renwick)
Olen Yoder — Erich Staedler
Joe Lewis — Jelle Atema

Contributed papers (12 minutes) and poster presentations throughout the range of chemical ecology are invited. Deadline for receipt, May 1, 1994. Send to F.X. Webster (address below).

Further information can be obtained from:

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