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ALARM PHEROMONE SYSTEM OF THE WESTERN CONIFER SEED BUG, *Leptoglossus occidentalis*

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Abstract—The alarm pheromones for adult and nymphal western conifer seed bugs, *Leptoglossus occidentalis*, were collected from the headspace volatiles of agitated bugs and from extracted adult thoraxes and nymphal abdomens. Adult bugs secreted a blend from the metathoracic glands that consisted of hexyl acetate, hexanal, hexanol, heptyl acetate, and octyl acetate (ratio of 152:103:8:1.5:1). Nymphal alarm pheromone produced by the dorsal abdominal glands consisted of (*E*)-2-hexenal. Agitated adults emitted ~24% of the pheromone contained within the glands, while nymphs released ~33% of their constitutive supply. The complete blend from both adults and nymphs, tested in a laboratory headspace bioassay, elicited a dispersal (or alarm) response in >70% of individuals tested. Nymphs in the field exposed to synthetic adult or nymphal pheromones, or a mixture of both, responded with >50% dispersing. When single components were tested on adults reared under summer conditions in a forced-air one-way bioassay, hexanal and hexyl acetate, the major components of the secretion, were responsible for eliciting the alarm response. Adults collected in the fall from the field were unresponsive to the tested blend, suggesting that adults seeking aggregation sites in the fall become refractory to alarm pheromone stimuli that would cause aggregations to disperse. The weak dispersal responses elicited in both adults and nymphs by either nymphal or adult pheromones are consistent with a trade-off in the advantage gained by avoiding predation and the disadvantage of leaving a food source. Because of these weak responses, use of alarm pheromones as pest management tools against *L. occidentalis* is unlikely.

Key Words—*Leptoglossus occidentalis*, western conifer seed bug, Hemiptera: Coreidae, alarm pheromone, metathoracic glands, hexyl acetate, hexanol, hexanal, octyl acetate and heptyl acetate.

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INTRODUCTION

Defensive secretions in some Homoptera and Heteroptera have been well documented. The secretions from aphids (Wohlers, 1981; Dawson et al., 1987); the coreids *Hotea gambiae* (Westwood) (Hamilton et al., 1985) and *Leptoglossus zonatus* (Dallas) (Leal et al., 1993); the alydids *Megalotomus quinquespinosus* (Say), *Alydus eurinus* (Say), and *Alydus pilosulus* Herrich-Schaeffer (Oetting and Yonke, 1978); the pentatomids *Eurydema rugosa* Motschulsky (Ishiwatari, 1974) and *Eurydema pulchra* Motschulsky (Ishiwatari, 1976); *Nezara viridula* (L.) (Lockwood and Story, 1987); and *Erthesino fullo* Thungberg (Kou et al., 1989) have been characterized as eliciting alarm behaviors (e.g., agitated walking, dropping from the host, flight attempt) among conspecifics.

These species share the following common characteristics: (1) they are easily disturbed and readily emit their offensive odor, (2) they form aggregations, and (3) most possess hexanal as a component of their defensive secretion. Only one *Leptoglossus* sp. has had its alarm pheromone system characterized (Leal et al., 1993). Given the variety of habitats and plant species used by members of this genus and the allopatric ranges of many species, we hypothesized that the alarm pheromone system would be fairly similar for related *Leptoglossus* species. The propensity toward gregariousness may be a prerequisite to the evolution of alarm pheromones (Nault and Phelan, 1984). Individuals in aggregations can benefit by perceiving volatile secretions used by other members of a group to repel predators and can disperse to avoid being targets for predation.

The western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae), is a common and potentially severe pest of conifer seed orchards in western North America (Hedlin et al., 1980; Schowalter and Sexton, 1990; Connelly and Schowalter, 1991). Throughout the summer, groups of adults and nymphs are easily disturbed and emit a defensive secretion. As hypothesized for other Hemiptera, this scent apparently elicits dispersal or alarm behavior in adults and nymphs. As defined by Starr (1990), alarm is the "communication of a shared danger. To show that it exists we need only find a correlation between the responses of an individual which perceives an intrusion and one which does not."

Our objectives were to capture, isolate, identify, and bioassay the alarm pheromones of adults and nymphs of *L. occidentalis*.

METHODS AND MATERIALS

Insects. Adult males, females, and nymphs of *L. occidentalis* for chemical analysis and most bioassay experiments were obtained from a laboratory colony

maintained at a 15L:9D photoperiod regime, 32°C and 20°C peak photophase and scotophase temperatures, respectively, and ~70% relative humidity. The colony was established in 1992 and revitalized using field-collected bugs each summer. Adults were segregated by sex for all but one phase of the study, but nymphs were not. One set of bioassay experiments was done with bugs collected in the field from Skimikin Seed Orchards, BC Forest Service, Tappen, British Columbia and Kalamalka Seed Orchards, Vernon, British Columbia, in mid-September 1994.

Headspace Bioassay for Adult and Nymphal Volatiles. Glass jars (130 ml) were washed and air dried prior to use and between replicates. Two sets each of five males or females were placed in separate jars covered with Parafilm. One set of insects, designated nonagitated, were not disturbed. The jar containing the second set of insects, designated agitated, was roughly handled and shaken until the putative alarm pheromone could be detected by the human nose.

For bioassays (Figure 1), a single nonagitated test insect was isolated in a Parafilm-covered jar and allowed to settle. Headspace air (10 cc), equal to approx. 0.4 bug equivalents, was drawn from the jar containing the nonagitated insects into a disposable syringe and injected into the jar containing the test insect. The test insect was then observed for any change in behavior, and then

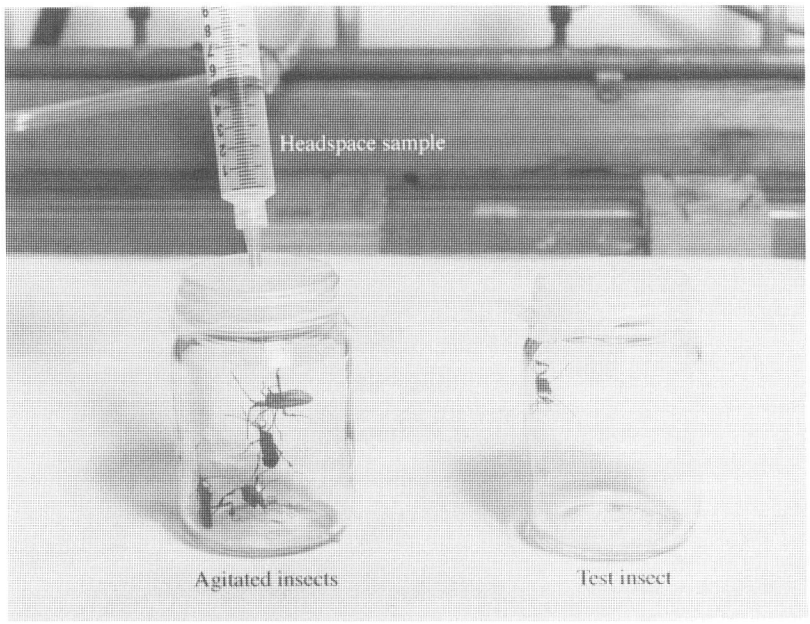

FIG. 1. Apparatus used in headspace bioassays testing for response by adult and nymphal *L. occidentalis* to headspace samples taken from agitated bugs.

was treated with either 10 cc of headspace air drawn from the jar containing the nonagitated insects (control) or the agitated insects (treatment). Again the test insects' behavior was observed. Any insect responding to air from nonagitated insects was discounted. An alarm response was recorded if the test insect exhibited agitated behavior, such as a sudden, rapid increase in movement or attempted flight. No change in behavior was deemed a negative response.

Fifteen individuals (replicates) of females, males, and nymphs were tested. Crossover experiments were then conducted with the same bioassay and 15 replicates obtained for all combinations of adults exposed to volatiles from nymphs and for nymphs exposed to volatiles from male or female adults. All results were analyzed by χ^2 analysis and Fisher's exact test (Zar, 1984).

In both laboratory and field bioassay, one bug equivalent was equal to the amount of volatiles given off by one agitated bug as determined by volatile capture.

Analysis of Volatiles. Volatiles from live adults or nymphs were collected on Porapak Q (Waters Associates Inc., Milford, Massachusetts). Twenty-five groups of 10 agitated males or females and five groups of 20 nymphs were placed in a glass chamber (6.5 cm high, radius 4.7 cm). Bugs were then agitated with a glass rod until the putative alarm pheromone was detected by the human nose. Then air was drawn by a water aspirator through a charcoal scrubber and over the insects for 10 min at 1.65 liters/min, allowing for ~36 exchanges of air in the aeration chamber. Volatiles were collected in a glass trap (6 mm OD × 30 mm) filled with Porapak Q (50–80 mesh). Trapped volatiles were extracted by eluting the Porapak with 2–3 ml of double-distilled pentane, and the volume was concentrated under a nitrogen stream to 1 ml. A 1-μl sample of the extract was analyzed with a Hewlett Packard 5830A gas chromatograph (GC) with an SP1000 nonbonded polar column (30 m × 0.32 mm ID) (Supelco, Oakville, Ontario) and flame ionization detector and with a Hewlett Packard 5890 gas chromatograph with a fused silica coated DB-5 nonpolar column (30 m × 0.25 mm ID) (J&W Scientific, Folsom, California).

Volatiles also were analyzed by coupled gas chromatographic–electroantennographic detection (GC-EAD) (Ärn et al., 1975, Gries et al., 1993), with a Hewlett Packard 5890A gas chromatograph and a custom built amplifier with a passive low-pass filter and a cutoff frequency of 10 kHz. An antenna was gently pulled out of the insect's head; the exposed nerve endings were suspended in a saline solution that contained the indifferent electrode and the distal end of the antenna was pierced with a recording electrode. Antennally active compounds were analyzed by coupled GC–mass spectroscopy (MS) with a Hewlett Packard 5985B GC equipped with a fused silica (30 m × 0.25 mm ID) DB-5 column in full-scan and selective ion monitoring (SIM) mode. Compounds were identified by comparison with published spectra (Jennings and Shibamoto, 1980) and identification was verified by GC with authentic standards. Quantities of

components present in the extracts were calculated by comparing area counts with those obtained from external standards of known concentration.

The contents of the metathoracic glands and reservoirs from 20 adult males and 20 females and the contents of the dorsal abdominal glands of 41 third and fourth instars were analyzed. Source insects were collected from the colony and held at −15°C for 20 min to facilitate handling and inhibit emission of volatiles or pheromones. Insects were processed in batches of 5–10. The thoraxes were excised, immersed in pentane chilled on dry ice, and pulverized with a glass rod. The extract was transferred to a volumetric vial, concentrated to 300 μl with a forced nitrogen stream, and placed in a glass tube containing glass wool attached to a Porapak Q trap (as described above). Volatiles were blown onto the trap by nitrogen gas for approximately 2 hr. Traps were then eluted with 1 ml of double-distilled pentane, and the extract was analyzed by GC and GC-MS and the amounts of components calculated as above.

Bioassay of Extracted and Synthetic Volatiles. A one-way forced-air bioassay was developed to quantify the response of adult bugs to single and blended volatiles (Figure 2). A 16/26 ground glass joint was welded to a glass tube resulting in a straight tube 48 cm long that was strapped horizontally to a board to prevent it from rolling. The ground glass joint fitted to a glass tube, 7 cm long, with a vertical open port, 3 cm long, which served as a receptacle for

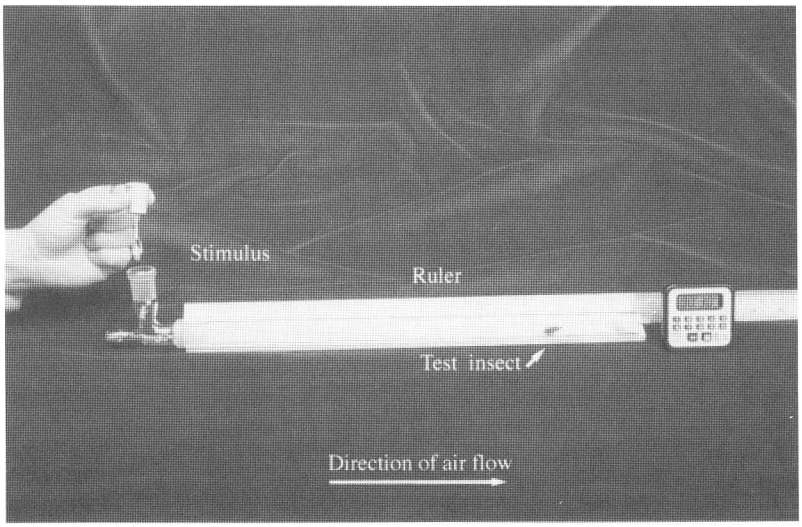

FIG. 2. Apparatus used in forced air bioassays during tests for response by adult *L. occidentalis* to synthetic and extracted alarm pheromone candidates.

volatile stimuli. A ruler was taped to the board with 0 cm positioned at the end of the ground glass joint. An adult was placed in the tube, near the 0 cm mark and its original position recorded. The component to be tested was applied to a glass ampoule, held by a cork, and inserted into the vertical port apparatus (Figure 2). Room air, humidified by passing through water, was blown at 1.5 liters/min into the horizontal tube, over the volatile stimulus and the test insect, and then out through the open end of the tube. Room temperature was maintained at ~25°C.

All stimuli were evaluated at one bug equivalent in 2 μl of pentane. Stimuli tested were an extract from excised adult thoraxes, each of five antenally active compounds found in the adult thoraxes, a five-component synthetic blend (hexyl acetate, hexanal, hexanol, heptyl acetate, and octyl acetate in the ratio 152:103:8:1.5:1) and (*E*)-2-hexenal, the putative nymphal alarm pheromone. Each insect had 30 sec to respond to the stimulus, after which its final position was recorded. Ten males and 10 females from the colony were tested in the spring and 10 field-collected individuals of each sex were tested in the fall. Ten control insects in each category were tested for their response to untreated air. Colony-reared "summer" females and males did not differ in their response to any of the stimuli ($F = 0.02$, $P = 0.89$) and were pooled for analysis. Mean distances moved in response to experimental stimuli were compared with distances moved in untreated air control tests by means of Dunnett's one-way test at $\alpha = 0.05$.

Field Bioassays. Adults and nymphs resting and feeding on cone clusters of western white pine, *Pinus monticola* Dougl. ex. D. Don, were located at Skimikin Seed Orchards. They were counted and their position identified with plastic flagging. Cone clusters were sprayed with a small (150-ml volume) atomizer containing 4 ml pentane spray with zero (control), two, or four bug equivalents of synthetic adult or nymphal alarm pheromone (as above), or both. The four-bug equivalent treatment of nymphal pheromone was lost by spillage in the field. Six cone clusters were sprayed for each treatment. Numbers of adults that responded by flying away from the cones and numbers of nymphs responding by either dropping or walking away from the cones were recorded. The numbers of nymphs were pooled for each treatment (heterogeneity chi-square values ranged from 3.14 to 14.82, P values ranged from 0.17 to 0.98), and percentage responses were compared with the response to control sprays (pentane study) by a test of difference between proportions with Z scores as described by Zar (1984).

Due to the low number of adults in the field bioassay ($N = 6$), a laboratory experiment for adults was designed. Potted Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, seedlings (approx. 25 cm high) were placed individually in a large mesh screened cage measuring 76 × 61 × 36 cm. Ten adults (mixed sex) were placed on the seedling, allowed to settle, and then sprayed as above with

two or four bug equivalents of synthetic (adult, nymphal, or a mixture) alarm pheromone. Ten replicates of 10 bugs each were tested for each treatment. Numbers of adults that left the seedling by flying and numbers that displayed agitated behavior were recorded. Mean percentage responses to experimental stimuli were compared with those to pentane control treatments by Dunnett's one-way test ($\alpha = 0.05$) following transformation with $\arcsin\sqrt{x}$ to stabilize the variances.

RESULTS

Headspace Bioassays. Males, females, and nymphs all showed a significant positive alarm response to the headspace volatiles from agitated males, females, and nymphs (and all combinations thereof) (Table 1). Males and females did not differ significantly in their responses to volatiles from their own or from the opposite sex, $\chi^2 = 0.36$, $P = 0.54$. Volatiles from nonagitated individuals elicted little or no response.

Analysis of Volatiles. GC, GC-MS, and GC-EAD analyses revealed that the antenally active volatiles emitted from agitated adults and those contained within the metathoracic gland and reservoir were hexanal, hexyl acetate, hexanol, heptyl acetate, and octyl acetate (Figure 3). The single antenally active volatile emitted by agitated nymphs was identified as (*E*)-2-hexenal (Figure 4), which is also produced by other hemipteran nymphs (Lockwood and Story, 1987). Male and female adults produced the same components in approximately the same quantity and ratio (Table 2). During the GC-EAD analysis at doses of ~4 bug equivalents, some antennae ceased to respond following exposure to the hexyl acetate or hexanal in the extract. To solve this problem, extracts were diluted 20-fold, enabling an antenna to respond for the duration of the analysis. A similar effect was observed in *Formica* spp. with *n*-undecane (Blum and Brand, 1972). This may be an adaptive mechanism that allows the emitter to hide from aggressive conspecifics. In our analysis with *L. occidentalis*, the concentration of hexyl acetate and hexanal were probably so high that there was a toxic inhibition of olfactory response.

Bioassay of Synthetic and Extracted Volatiles. A synthetic blend containing all five components of adult pheromone elicited an alarm response by males, females, and nymphs in the head space bioassay (Table 3). Synthetic nymphal alarm pheromone elicited a similarly ubiquitous response (Table 3). Hexanal and hexyl acetate, the most abundant components in both glands and headspace volatiles from agitated adults, and (*E*)-2-hexenal from nymphs elicited a significant response from colony-reared "summer" adults in the forced air bioassay (Figure 5).

Adults collected in the fall did not respond to the synthetic alarm pheromone

TABLE 1. ALARM BEHAVIOR RESPONSES OF ADULT AND NYMPHAL *L. occidentalis* IN HEADSPACE BIOASSAY TO HEADSPACE VOLATILES OF AGITATED AND NONAGITATED (CONTROL) BUGS[a]

Test insect and source of volatile stimulus	Postive response (%)	χ^2 probability experimental vs. control
Females		
Agitated females	100	
Nonagitated females	0	0.001
Females		
Agitated males	100	
Nonagitated males	0	0.001
Males		
Agitated females	87	
Nonagitated females	0	0.001
Males		
Agitated males	80	
Nonagitated males	0	0.001
Females		
Agitated nymphs	87	
Nonagitated nymphs	13	0.001
Males		
Agitated nymphs	93	
Nonagitated nymphs	0	0.001
Nymphs		
Agitated females	80	
Nonagitated females	7	0.001
Nymphs		
Agitated males	87	
Nonagitated males	7	0.001
Nymphs		
Agitated nymphs	93	
Nonagitated nymphs	0	0.001

[a] $N = 15$ bugs per test.

components ($F = 1.16$, $P = 0.27$ and $F = 1.10$, $P = 0.34$ for females and males, respectively) (Figure 5). In many cases, the insects moved towards the stimulus.

Field Experiments. Nymphs in the field showed a significant dispersal response to synthetic alarm pheromone sprays (Table 4). At a dose of 2 bug equivalents, synthetic nymphal pheromone and a mix of adult and nymphal pheromones were significantly more effective than the control (pentane only) at

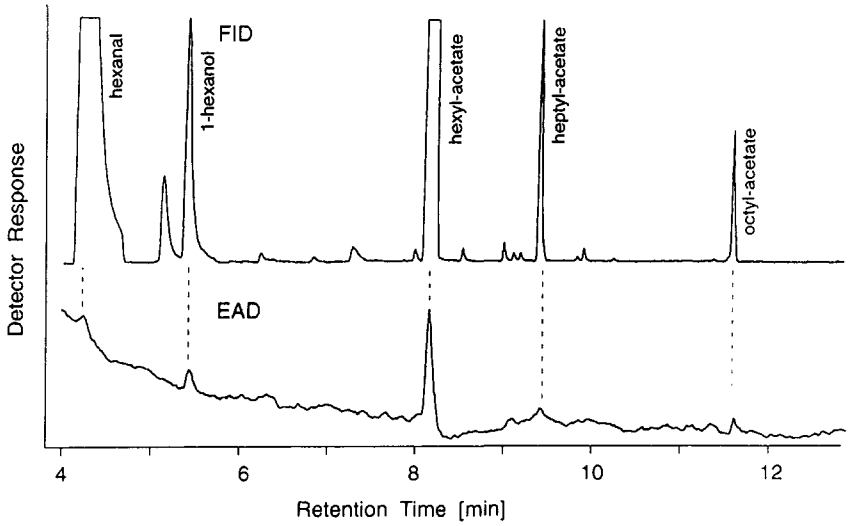

FIG. 3. Flame ionization detector (FID) and corresponding electroantennographic (EAD) trace from adult female antenna to alarm pheromone collected *in vivo* from adult females and analyzed on a DB-5 column with a program of 50°C for 1 min, 2°C/min to 65°C, 5°C/min to 120°C, and then 20°C/min to 240°C.

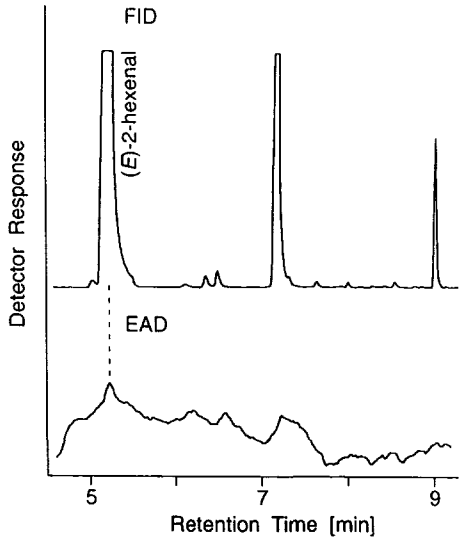

FIG. 4. Flame ionization detector (FID) and corresponding electroantennographic (EAD) trace from nymphal antenna to alarm pheromone collected *in vivo* from third and fourth instars and analyzed on a DB-5 column with a program of 50°C for 1 min, 2°C/min to 65°C, 5°C/min to 120°C, and then 20°C/min to 240°C.

TABLE 2. COMPARISON BETWEEN QUANTITIES OF ALARM PHEROMONE CONTAINED WITHIN METATHORACIC GLAND OF ADULTS AND DORSAL ABDOMINAL GLANDS OF NYMPHS AND QUANTITIES GIVEN OFF WHEN AGITATED

Pheromone source	Compound	Quantity (μg/bug) in gland (mean ± SE)[a]	Quantity (μg/bug) given off when agitated (mean ± SE)[b]	Percent of total content given off when agitated
Adult females	Hexanal	568.7 ± 170.40	17.1 ± 3.53	3.0
	Hexanol	12.2 ± 7.10	1.3 ± 0.93	10.6
	Hexyl acetate	390.7 ± 156.90	21.7 ± 2.69	5.6
	Heptyl acetate	0.3 ± 0.04	0.2 ± 0.10	92.3
	Octyl acetate	3.1 ± 1.18	0.2 ± 0.02	4.9
Adult males	Hexanal	339.7 ± 116.70	15.5 ± 4.46	4.6
	Hexanol	4.6 ± 1.04	1.4 ± 0.87	30.8
	Hexyl acetate	337.1 ± 96.70	27.2 ± 5.25	8.1
	Heptyl acetate	0.3 ± 0.14	0.2 ± 0.06	63.6
	Octyl acetate	1.5 ± 0.48	0.2 ± 0.01	11.6
Nymphs	(*E*)-2-Hexenal	3.6 ± 0.69	1.2 ± 0.35	32.7

[a] Data obtained from four replicates each consisting of five adult males or females (total = 20 of each sex), and four replicates of 10, and one replicate of 11 nymphs (total = 51).
[b] Data collected from 25 replicates each of 10 males or females (total = 250 of each sex), and five replicates of 20 nymphs (total = 100).

causing dispersal of nymphs from the cone clusters. Synthetic adult alarm pheromone at 4 bug equivalents caused a dispersal response similar to that caused by the nymphal–adult mix at the same dosage. At the 2 bug equivalent dose, the adult pheromone blend was no more effective than the control, suggesting that nymphs are more responsive to alarm pheromone from nymphs than from adults. While the pheromone treatments caused nymphs to walk away from the cones, the effect was short-lived, and they would return to the cones within 5 min. For all treatments tested, fewer than 20% of the nymphs present responded by dropping from the cone clusters (Table 4).

Adults tested on seedlings showed significant alarm response (Figure 6). The synthetic adult alarm pheromone at both 2 and 4 bug equivalents, either alone or in mixture with nymphal pheromone, caused significantly higher numbers of adults to become agitated than the pentane control treatment, but only the adult blend alone caused adults to leave the seedlings in significant numbers. Nymphal alarm pheromone had no significant effect.

TABLE 3. ALARM BEHAVIOR OF ADULT AND NYMPHAL *L. occidentalis* IN HEADSPACE BIOASSAY TO SYNTHETIC BLEND OF ADULT ALARM PHEROMONE CANDIDATES AND SYNTHETIC NYMPHAL ALARM PHEROMONE, (*E*)-2-Hexenal[a]

Test insect and source of stimulus	Percent positive response	χ^2 Probability experimental *vs.* control
Females		
Synthetic adult blend	80	
Pentane	13	0.005
Males		
Synthetic adult blend	87	
Pentane	6	0.001
Females		
Synthetic nymph	93	
Pentane	6	0.001
Males		
Synthetic nymph	87	
Pentane	6	0.001
Nymphs		
Synthetic adult blend	73	
Pentane	33	0.05
Nymphs		
Synthetic nymph	80	
Pentane	20	0.005

[a]Approximately 0.4 bug equivalents per stimulus. $N = 15$ bugs per test.

DISCUSSION

Hexanal, hexyl acetate, and hexanol, the most abundant semiochemicals found in *L. occidentalis* are also found in three other *Leptoglossus* species: *L. zonatus* (Dallas) (Leal et al., 1993), *L. oppositus* (Say), and *L. clypealis* Heidemann (Aldrich and Yonke, 1975). *Leptoglossus zonatus* produces hexanoic acid in small quantities (Leal et al., 1993), and both *L. oppositus* and *L. clypealis* produce small amounts of acetic acid (McCullough, 1968, 1969; Aldrich and Yonke, 1975), *n*-hexyl hexanoate, and (*E*)-2-octenyl acetate (Aldrich and Yonke, 1975). Hexaldehydes are a common component of defensive secretions in numerous other Hemiptera and Homoptera (Waterhouse and Gilby, 1964; McCullough, 1970, 1973a,b; Aldrich et al., 1978, 1979, 1984; Everton et al., 1979; Lockwood and Story, 1987). The frequent occurrence of hexanal in such secretions suggests that it is an effective, broad spectrum irritant easily

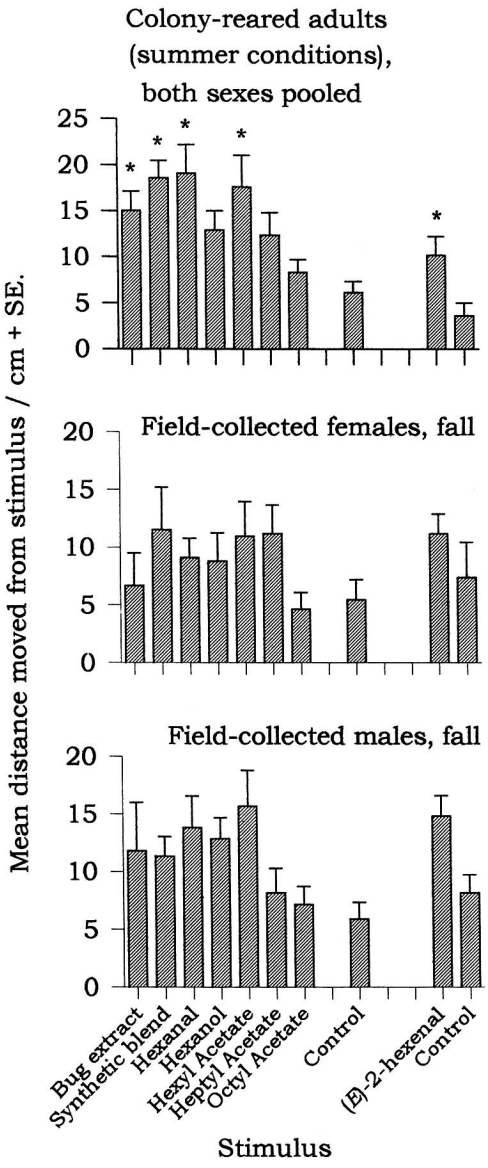

FIG 5. Mean distance moved away from adult and nymphal alarm pheromone and its components by *L. occidentalis* reared in colony under "summer" conditions or collected from the field in the fall. All components tested at a concentration of one bug equivalent. An asterisk indicates significant distance, Dunnett's one-way test, $P < 0.05$, moved from stimulus as compared with control (pentane).

TABLE 4. ALARM BEHAVIOR RESPONSE OF NYMPHAL *L. occidentalis* IN THE FIELD TO SYNTHETIC BLENDS OF ADULT ALARM PHEROMONE, NYMPHAL ALARM PHEROMONE, OR MIXTURE OF BOTH APPLIED AS ATOMIZED SPRAYS OF 2 AND 4 BUG EQUIVALENTS IN 4 ml OF PENTANE TO WESTERN WHITE PINE CONES IN THE FIELD

Source of pheromone	Bug equivalents	Number of insects tested	Percent dropping from cones	*P* value experimental *vs.* control	Percent dispersing away from cones	*P* value experimental *vs.* control
Control (pentane)	n/a	35	5.7		49	
Adult	2	39	10.3	0.348	54	0.334
	4	48	8.3	0.405	77	0.005
Nymph	2	37	13.5	0.255	95	0.0001
Mix	2	44	18.2	0.133	73	0.017
	4	49	14.3	0.218	82	0.001

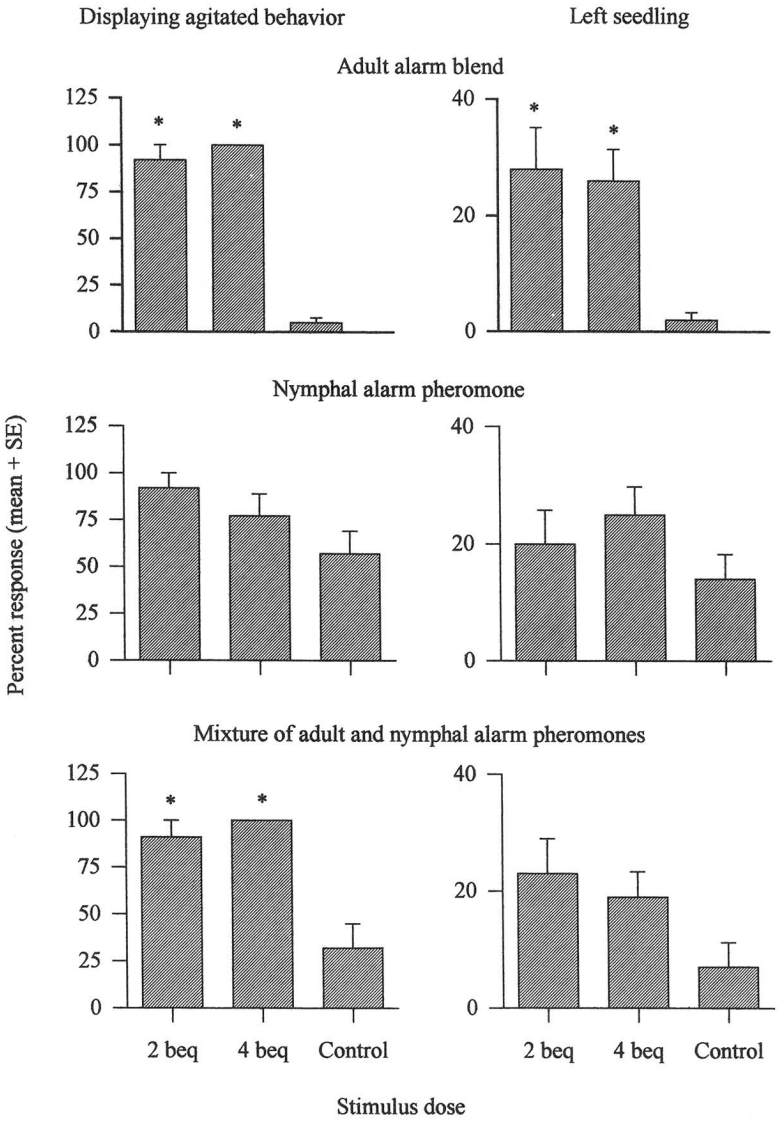

FIG. 6. Percentage of adults responding to synthetic alarm pheromone sprays in a laboratory test on caged seedlings. Significant response, Dunnett's one-way test, $P < 0.05$, compared with control (pentane) indicated by an asterisk.

produced or acquired by insects and useful for defense. It is a common green leaf volatile in herbaceous plants (Visser et al., 1979; and McCall et al., 1994), but its function may differ by insect species (Visser et al., 1979; Visser, 1986; Dickens et al., 1990, 1992).

Although hexanol, heptyl acetate, and octyl acetate were antenally active (Figure 3), they did not elicit alarm behavior in the bioassays, and there was no evidence of an additive or synergistic interaction of components in the synthetic blend. These minor components have been argued as inconsequential for conspecific recognition (Aldrich and Yonke, 1975). However, reanalysis of the volatiles from previously studied bugs by GC-EAD may reveal other minor compounds of potential behavioral importance. We hypothesize that these compounds, less volatile than hexanal and hexyl acetate, may serve as conspecific recognition signals.

The utilization of different pheromone components by adults and nymphs of *L. occidentalis* may indicate that pheromones with different properties are required to accommodate their differing release mechanisms. Adult *L. occidentalis* expel their pheromone through small openings in the thorax as a spray (personal observation) while nymphs release alarm pheromone through openings in their abdominal tergites. Hexanal, produced by adults, has a higher molecular weight than (*E*)-2-hexenal and is less volatile (bp 131°C). Hexanal may disperse into the air as an aerosol or may contact a predator prior to volatilization. (*E*)-2-Hexenal (bp 47°C), if released in a similar manner, will volatilize rapidly on exposure to air. As (*E*)-2-hexenal is probably released onto the surface of the tergites, rather than directly into the air, the large odor plume created would probably be as effective as a hexanal spray in warding off predators, particularly from a group of aggregated and alarmed nymphs. Aldrich and Yonke (1975) proposed that the difference in adult and nymphal alarm pheromones of *L. oppositus* and *L. clypealis* resulted from selection pressure by ants. This may be possible for species that are preyed upon by ants; however, ants have frequently been observed on conifer boles and branches, but have not been noted as predators of *L. occidentalis* or any other *Leptoglossus* species.

Metathoracic glands and the associated reservoir of male and female adults contain several hundred micrograms of pheromone, while dorsal abdominal glands of nymphs contain < 10 μg (Table 2). Because only a portion of this amount ($\sim 24\%$ of the total for adults and $\sim 33\%$ for nymphs) was emitted by agitated bugs, an individual insect should be capable of repeated emissions. However, approximately 15- and 45-min refractory periods occurred before adults and nymphs, respectively, could be provoked to reemit alarm pheromone. In the nymphs, the long refractory period may indicate that (*E*)-2-hexenal is more useful as a repellent against predation than as a conspecific alarm pheromone. Their aposematic coloring supports this hypothesis. Unlike adults, which can fly away from a host and return, nymphs dropping from cones onto lower

branches would escape the immediate threat of predation, but would potentially lose their food supply and increase the chances of desiccation and predation by spiders and rodents while on the forest floor. Pea aphids, *Acyrthosiphon pisum* (Harris), from Kamloops, British Columbia, were less likely than aphids from Vancouver to drop from their host plant in response to alarm pheromone (Roitberg and Myers, 1978). This was postulated as a means to avoid desiccation in areas where ground conditions are harsh.

Lockwood and Story (1987) hypothesized that alarming conspecifics evolved as a secondary function of the defensive secretion in *Nezara viridula* (L.). The reserve of pheromone remaining in the adults after one emission suggests that *L. occidentalis* could repeatedly defend itself against persistent attempts at predation, with alarm and dispersal by conspecifics being a secondary adaptation, as hypothesized for *N. viridula.*

Lockwood and Story (1985) demonstrated that in first-instar *N. viridula*, *n*-tridecane serves as an alarm pheromone at 1.0 bug equivalents and as an aggregation pheromone at 0.1 bug equivalents. While this behavior is dose dependent, the differential response of summer and fall adult *L. occidentalis* to their defensive secretion may be physiologically dependent. In the fall, both sexes of *L. occidentalis* seek cryptic overwintering sites, respond to an unknown male-produced aggregation pheromone (Blatt and Borden, 1996), and are commonly aggregated in large numbers (Blatt, 1994). In these sites they are not easily accessible to predators, such as birds. During this time, alarm responses followed by dispersal would be maladaptive. It is hypothesized that the lesser produced components, hexanol, heptyl acetate, and octyl acetate may be used as conspecific recognition cues.

Results obtained in the laboratory bioassays indicate that, like nymphs, adults are more responsive to their own pheromone than to the pheromone produced by bugs in a different life stage. The uniformly high responses to headspace volatiles from any life stage suggest that when stimuli are strong, there is an adaptive advantage to respond to any conspecific alarm signal. The low percentage of adults leaving the seedling suggests that other indications of danger, e.g., rapid movement and shadows, would be necessary to cause adults to depart (Clegg and Barlow, 1982). Of the six adults tested in the field, three flew away and the others dispersed from the cones by agitated walking. In both the field and the laboratory, adults that left the cones or seedlings returned within 10 min. Based on these results, we conclude that the alarm pheromone would not be operationally effective in reducing *L. occidentalis* populations or in preventing or reducing damage to seeds of conifers.

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CUTICULAR SURFACE HYDROCARBONS OF DESERT LOCUST NYMPHS, *Schistocerca gregaria*, AND THEIR EFFECT ON PHASE BEHAVIOR

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Abstract—The quantity of cuticular hydrocarbons is higher in solitarious nymphs of the desert locust, *Schistocerca gregaria*, compared to gregarious nymphs, but the total hydrocarbon fraction of solitarious nymphs does not significantly divert behavioral transition of isolated nymphs to the gregarious phase, while gregarious hydrocarbon extracts do. This suggests that qualitative differences in composition are responsible for the biological effect. The profile of cuticular hydrocarbon components is similar in the two phases, but some peak ratios differ. Crowding of solitarious nymphs leads to rapid changes in the profile of the hydrocarbon fraction, suggesting that specific hydrocarbons are produced and secreted as a consequence. Isolating previously crowded nymphs has an opposite effect. The composition of cuticular hydrocarbons from the migratory locust, *Locusta migratoria*, which differs considerably from that of *S. gregaria*, does not induce the gregarious behavioral phase in solitarious nymphs of the latter.

Key Words—*Schistocerca gregaria*, desert locust, phase transition, cuticular hydrocarbons, contact pheromones.

INTRODUCTION

Locusts undergo transition in response to population density from the solitary to the gregarious migratory phase and vice versa, characterized in part by mor-

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phometric and chromatic changes that are associated with the molting process. External changes gradually intensify, as the nymphs progress through additional molts. In some cases changes are fully expressed only after several generations (Uvarov, 1966). In contrast, physiological phase transition is characterized by rapid metabolic and behavioral changes, which may be detected within hours or days. Individuals of the gregarious phase are more active than are solitarious locusts, and socially interact. The relative intensity of these behavioral responses is dependent on the degree of crowding and its duration (Ellis, 1962, 1963; Rainey, 1962, 1989; Uvarov, 1966; Gillett, 1988; Roessingh and Simpson, 1994; Heifetz and Applebaum, 1995; Heifetz et al., 1996).

Long-range communication, which has been attributed to specific volatile aggregation and gregarization pheromones and to nonspecific volatile attractants, contributes to an increase in locust population density, essential for induction of phase transition (Gillett, 1968, 1975; Gillett and Phillips, 1977; Fuzeau-Braesch et al., 1988; Obeng-Ofori et al., 1993, 1994; Torto et al., 1994). Once they have aggregated, short-term communication in *Schistocerca gregaria* nymphs is mediated by cuticular lipids leading to the induction of behavioral phase transition (Heifetz et al., 1996). Of the total lipids, we have shown that hydrocarbons specifically interact with *S. gregaria* antennal receptors and induce gregarious behavior (Heifetz et al., 1997).

Long-chain linear and/or branched hydrocarbons are common components of many insect surface lipids and in some species comprise more than 90% of the total lipid fraction (Hadley, 1981). Hydrocarbons comprise more than half of all the cuticular lipids in *Locusta migratoria cinerascens* (Genin et al., 1986) and *S. gregaria* nymphs (Heifetz et al., 1997). It is increasingly evident that cuticular hydrocarbons function widely in chemical communication. Hydrocarbons may act as sex attractants or aphrodisiacs; antiaphrodisiacs; species-, caste-, and kin-recognition cues; aggregation pheromones; or kairomones (Nelson, 1993; Blomquist et al., 1993). Earlier work established that certain insect species, such as the housefly, tsetse fly, and German cockroach, exploited specific compounds or mixtures of a few compounds as either close-range or contact sex pheromones. In young *Tegenaria atrica* spiders the transition from gregarious to solitary life is accompanied by changes in agonistic behavior and in the composition of cuticular compounds (Trabalon et al., 1996). Females of several species of *Drosophila* produce cuticular hydrocarbons that function as contact pheromones in stimulating male courtship behavior (Cobb and Jallon, 1990).

Cuticular hydrocarbons in *S. gregaria* are reportedly produced by a subepidermal oenocyte-rich peripheral fat body (Diehl, 1975), then transported by hemolymph lipophorins (Pho et al., 1996), and excreted on the cuticle surface via pore canals (Strong, 1971). Cuticular hydrocarbons of locusts are extremely complex mixtures of highly branched molecules and of very high molecular

weight (Nelson and Sukkestad, 1975; Jackson, 1981; 1982; Soliday et al., 1974; Nelson et al., 1984; Genin et al., 1986). They are relatively nonvolatile and are perceived at close range only. Their low volatility at low temperatures precludes the use of coupled gas chromatography–electroantennographic detection (GC-EAD) for identification of active components. As an alternative, we have chosen to perform comparative analysis of phase-related cuticular hydrocarbons, which could contribute to the identification of hydrocarbon composition, relevant to the induction of behavioral phase transition.

METHODS AND MATERIALS

Insects. The culture of the desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae), used in these experiments was established from individuals field-collected in Niger in 1991. The African migratory locust (*Locusta migratoria migratorioides*) was from a laboratory strain originally obtained from the former COPR in London, UK. Stock cultures of each of the two locust species were maintained in separate culture rooms under crowded conditions at a temperature-controlled regimen of 28 ± 2°C within the cages, constant illumination, and density of about 200 locusts per cage (50 × 50 × 50 cm). They were fed fresh wheat plants supplemented with flaked oats. Isolated nymphs were reared in small aluminum rearing cages (20 × 20 × 10 cm), partitioned by aluminum into four chambers, in a temperature controlled room (28 ± 2°C) separate from the stock culture. They were kept under these conditions from the middle of the second instar until their use in experiments in mid-fourth instar and were fed fresh wheat sprouts and flaked oats.

Cuticular Surface Extracts. Groups of 10 or more fourth-instar males and females in equal proportions were immersed in a three- to fourfold excess of dichloromethane and gently shaken for 10 min. The extracts were collected, reduced in volume under nitrogen, and kept at −20°C until used. Separation into subfractions was performed by applying concentrated extract as a band to 10 × 20 cm TLC plates (Silica gel 60, F_{254}, Merck). Plates were first developed with hexane to a height 16 cm from the origin, air-dried, and developed a second time with hexane–diethyl ether–acetic acid (70:30:1; v/v/v) to a height 10 cm from the origin (Gilby, 1980). Separated bands were observed under short UV light, and marked, and the hydrocarbon fraction scraped into glass flasks and extracted with hexane (HPLC grade) for GC-MS.

Treatment of Solitarious Nymphs. Nymphal equivalents of cuticular extracts were applied to 10- × 15-cm polyethylene screens as solutions in organic solvents. The solvent was evaporated in a hood and one screen placed in each chamber. A nymphal equivalent is defined as the amount of lipids extracted

from the cuticle of one locust nymph. Solitarious nymphs were exposed to treated or control screens (solvent alone applied, then evaporated) for 5 hr. Ten exposed nymphs of each treatment were placed together in an arena and their behavior assayed.

Behavioral Assays. These were performed, as previously described, with developmentally synchronized mid-fourth solitarious nymphs, three to four days after the L_3 to L_4 molt. Briefly, 10 individually treated nymphs, or control nymphs, were placed in a 45-cm-diameter circular arena. Activity levels (the mean amount of time spent during 2 min in movement by each of the 10 nymphs in the arena) and associative indices were determined (Heifetz et al., 1996).

Gas Chromatography (GC). Hydrocarbon fractions were analyzed on a Tracor 540 GC system equipped with a DB-5 capillary column (25 m × 0.25 mm; J&W) and flame ionization detector (FID). An internal C_{19} standard was added to all chromatographic runs for purposes of quantification. Preliminary runs, conducted with a temperature program of 4°C/min from 50° to 300°C, indicated that no highly volatile hydrocarbons were present, and subsequent runs were performed under the same program from 150° to 300°C.

GC–Mass Spectrometry (GC-MS). Combined GC-MS was used to identify the hydrocarbons. Separation was performed as detailed above, with the same column and separation procedure, on a HP-5790 GC system coupled to a ZAB II F mass spectrometer. We used the electron impact (EM) mode with 70 ev, at an ion source temperature of 250°C. The GC-MS analysis was conducted with GC at 50°–300°C (4°C/min) and mass scanning from 50 to 500 *m/z* at 1.8 sec/dec, or GC at 150°–300°C (4°C/min) and mass scanning from 100 to 600 *m/z* at 1.8 sec/dec. The semiquantification of total distribution and relative ratios were derived from the GC-FID response based on the internal standard without normalization. For analysis of normal and some mixtures of the monomethyl-branched hydrocarbons we used the NSRDS-NBS mass spectral data base, as well as the GC-MS fragmentation patterns and chromatographic position as indicated by Genin et al. (1986), adopted from McCarthy et al. (1968) and Nelson et al. (1972). However, the dimethyl-branched mixtures were in some cases more difficult to assign as to positions of branching, due to complex fragmentation patterns. Since the majority of the branched compounds were substituted at positions higher than four, we used the 100–600 *m/z* scan mode for the identification of the fragmentation patterns and the 50–500 *m/z* for the general total ion current (TIC) profile. Because of the normalization of the mass fragmentograms to the highest peak (base peak), no attempt was made to group type analysis based on selective ion recording (SIR) to quantify the hydrocarbons.

Statistics. Statistical tests in SAS (1992) Version 6.04 included one-way ANOVA (tested with an F test) and proc GLM, as detailed in the specific experiments reported.

RESULTS

Effect of Cuticular Surface Extracts on Solitarious S. gregaria. When solitarious *S. gregaria* nymphs are exposed to cuticular extracts of conspecific gregarious nymphs, a statistically significant ($F = 10.47$; $P < 0.001$) dose-dependent increase in net activity levels (activity minus controls) is observed (Figure 1). Five nymphal equivalents increase activity 2.4-fold above the control levels. In comparison, exposure of solitarious *S. gregaria* nymphs to equivalent extracts of conspecific solitarious nymphs had no effect on activity levels ($F = 8.36$; $P < 0.008$).

The degree of species specificity of the nymphal cuticular lipid extract was examined next. The activity levels of solitarious *S. gregaria* nymphs were unaffected when exposed to cuticular surface lipids of gregarious *L. migratoria* (Figure 1). Briefly, only extracts of conspecific gregarious nymphs increase the activity of solitarious *S. gregaria* nymphs ($F = 3.92$; $P < 0.003$). These results indicate that the effect on activity level is species-specific and is dose- and phase-dependent.

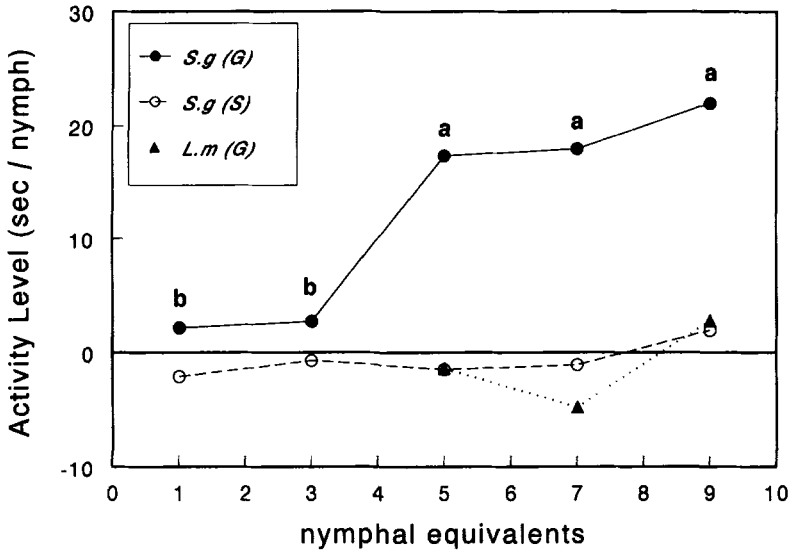

FIG. 1. Dose-response of *S. gregaria* nymphal activity elicited by exposure of solitarious L_4 nymphs to increasing nymphal equivalents of cuticular lipid extracts: *S.g. (G)* = *S. gregaria* gregarious nymphs; *S.g. (S)* = *S. gregaria* solitarious nymphs; *L.m. (G)* = *L. migratoria* gregarious nymphs. $N = 5$; different letters above the data points indicate significant differences by Duncan's multiple range test; $P < 0.001$.

A dose-dependent response of associative index is observed only when solitarious *S. gregaria* nymphs are exposed to gregarious cuticle extract. At 7 nymphal equivalents, the gregarious extracts increase the associative index fivefold, compared to the response to solitarious extracts or to *L. migratoria* extracts, but this difference is not significant (data not shown).

Gregarious behavior of *S. gregaria* solitarious nymphs is specifically induced by the hydrocarbon fraction of the gregarious nymphal cuticular extract. Antennal preparations from crowded *S. gregaria* nymphs generate a rapid and transient increase in the concentration of the second messenger inositol triphosphate when exposed to cuticular hydrocarbons of *S. gregaria*, while antennal preparations of *L. migratoria* do not respond correspondingly (Heifetz et al., 1997). This emphasizes the specific role of hydrocarbons in the process of behavioral phase transition. Clearly, the hydrocarbon composition of *L. migratoria* must differ from that of *S. gregaria* for such a differential response to occur, and a comparison of hydrocarbons from these two species should assist in narrowing down the search for the presumptive phase-transition pheromones. Accordingly, we next compared the composition of hydrocarbons from the cuticular surface of gregarious *S. gregaria* and *L. migratoria* nymphs by GC.

Comparative Hydrocarbon Composition of Gregarious Nymphal Cuticular Extracts of S. gregaria *and* L. migratoria. Comparative MS scans of gregarious nymphal cuticular hydrocarbons of *S. gregaria* and *L. migratoria* are presented in Table 1. The identification of the hydrocarbons is marked in Table 1 according to peak number and the same number is noted on the chromatogram (Figure 2). The GC and GC-MS analyses for all hydrocarbon fractions revealed the dominance of the odd straight chain hydrocarbons with *n*-C_{29} (peak 5) being the most abundant. Differences are apparent in the quantity of total hydrocarbons as assessed by the use of a C_{19} internal standard (see Methods and Materials) and in their specific composition as shown by the GC profiles (Figure 2). *S. gregaria* has a higher hydrocarbons content, more odd-numbered *n*-alkanes and more internally branched monomethylalkanes. *L. migratoria* hydrocarbons are composed of *n*-alkanes, terminally branched monomethylalkanes, and a low percentage of internally branched monomethylalkanes. Specific differences between the cuticular hydrocarbons of these two locust species are the presence of C_{28} (peak 3) in *L. migratoria* and in contrast, its lack of several C_{32} (peaks 12–13) and C_{34} (peak 15) branched hydrocarbons, as shown in the GC profile (Figure 2). However, these differences are seen better in Table 1. It is also obvious that compounds C_{34} (peak 15), (peaks 17–18), and C_{35} (peaks 19–20) are absent in *L. migratoria*.

The internal ratios between the compounds were calculated from GC quantification runs (Figure 3). Major differences between *L. migratoria* and *S. gregaria* are the greater abundance of C_{31} (peak 9) in *L. migratoria* (twofold more) and the ratio of the two branched-chain C_{32} (peaks 10–11) (5.7-fold more).

TABLE 1. COMPOSITION OF COMPONENTS ELUTING DURING GC-MS ANALYSIS OF CUTICULAR HYDROCARBONS FROM *S. gregaria* (S.g.) AND *L. migratoria* (L.m.) L_4 NYMPHS[a]

Peak (GC:GC-MS)	Scan No.	MS intensity (V.) S.g. S	S → G	G	G → S	L.m. G	Hydrocarbon	C_n
1	1227	0.2	0.3	0.3	1.3	0.05	*n*-pentacosane	C_{25}
2	1451	1	1	2.4	3.2	0.1	*n*-heptacosane	C_{27}
3	1529					0.6	3-methylheptacosane	C_{28}
4	1555	0.1	0.2	0.3	0.9	0.4	*n*-octacosane	C_{28}
5	1669	2.6	3.8	3.7	5.1	4.8	*n*-nonacosane	C_{29}
6	1692	0.2	0.25	0.1	0.3	0.2	11,19- and 13,17-dimethyloctacosane	C_{30}
7	1732	0.05	0.05	0.1	0.3	2	3-methylnonacosane	C_{30}
8	1756	0.1	0.3	0.4	0.2	0.7	*n*-tricontane	C_{30}
9	1858	0.9	1.8	2.1	1.9	2.3	*n*-hentriacontane	C_{31}
10	1883	0.1	3.7	2.4	4.6	0.5	11- and 13-methylhentriacontane	C_{32}
11	1913	0.4	1.1	0.4	1.4	0.05	9,11-dimethyltricontane and 11,13-dimethyltricontane	C_{32}
12	1974	0.05	0.3	0.26	0.4		11,22- and 13,19-dimethyltricontane	C_{32}
13	2001	0.05	0.15	0.1	0.2		11,14- and 20-methylhentriacontane	C_{32}
14	2038	0.2	0.4	0.2	0.45	0.25	*n*-tritriacontane	C_{33}
15	2070	1.7	4.7	4.3	4.9		11,13- and 15-methyltritriacontane	C_{34}
16	2092	1.8	3.8	1.7	3.6	0.1	9,14- and 11,17-dimethyldotricontane	C_{34}
17	2151			0.1	0.2		?	
18	2175			0.1	0.1		?	
19	2256	0.1	0.2	0.2	0.3		11- and 13-methyltetratricontane	C_{35}
20	2285	0.06	0.3	0.1	0.5		?	
21	2307		0.1		0.2		?	

[a]S = solitarious; G = gregarious; S → G = solitarious crowded for 24 hr; G → S = gregarious isolated for 24 hr.

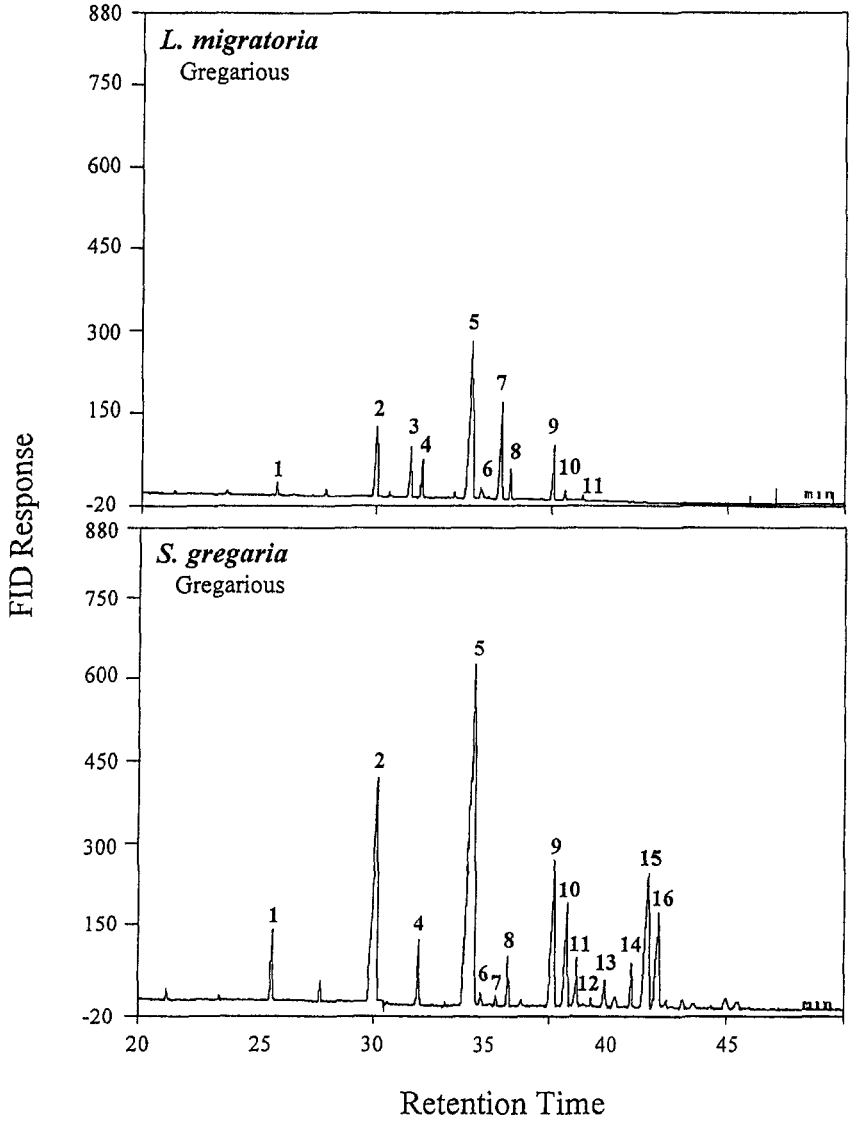

FIG. 2. GC profiles of cuticular hydrocarbons from gregarious nymphs of *L. migratoria* and *S. gregaria*. The numbers appearing above the individual peaks refer to the peak numbers analyzed and identified by GC-MS and listed in Table 1.

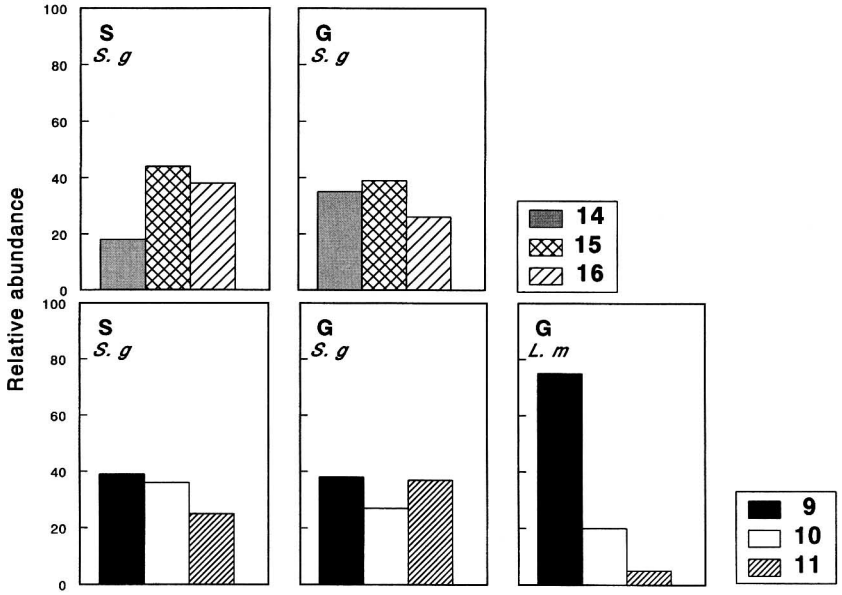

FIG. 3. Relative abundance of linear and branched-chain high-molecular-weight hydrocarbons in size groups of C_{31}–C_{32} hydrocarbons (peaks 9–11 in Table 1) and C_{33}–C_{34} hydrocarbons (peak 14–16 in Table 1), at different population densities. S = solitarious; G = gregarious; *S.g* = *S. gregaria*; *L.m* = *L. migratoria*.

Figure 4 shows the mass spectra of compounds marked as peaks 9–11. The ratio of C_{33} (peak 14) to C_{34} (peaks 15–16) for *L. migratoria* could not be determined, as C_{34} (peak 15) is absent. The differences in the hydrocarbon profiles between these two locust species may be associated with the specificity of response to the cuticular hydrocarbons in this present study and previously shown (Heifetz et al., 1997).

We see that exposure to cuticular hydrocarbons of solitarious *S. gregaria* does not induce gregarious-phase behavior in conspecific solitarious nymphs, whereas exposure to gregarious hydrocarbons does. These observations next led us to compare the effect of density on the composition of cuticular hydrocarbons in nymphs reared in isolation or crowded.

Comparative Hydrocarbon Composition of Gregarious and Solitarious S. gregaria *Nymphal Cuticular Extracts.* No qualitative differences between the profiles of linear and branched-chain high-molecular-weight hydrocarbons are evident in solitarious and gregarious *S. gregaria* nymphs. Neither can the total amount of cuticular hydrocarbons be regarded as responsible for the behavioral effect, since cuticular hydrocarbons are 1.75-fold greater in the extract of soli-

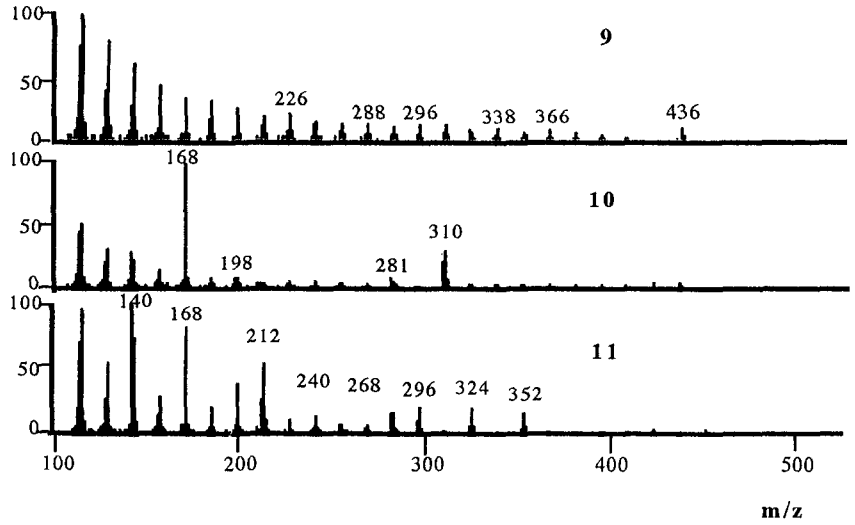

FIG. 4. Representative mass spectra for compounds marked as peaks 9–11, as seen in Table 1 and chromatograms in Figure 2. The identification of the branching position and the possible mixture is according to Genin et. al. (1986).

tarious nymphal cuticle. Differences exist between the two phases in the reversed ratio of branched-chain C_{32} (peaks 10–11) and in the different ratios of branched-chain C_{34} (peaks 15–16). In addition, the relative abundance of C_{33} (peak 14) is higher in gregarious nymphal extracts (Figure 3).

Are these relative differences evident during the initial stages of behavioral phase transition? We addressed this question by determining the initial effects of crowding or isolation on short-term changes in hydrocarbon composition.

Temporal Plasticity of Phase-Related Activity. Newly molted L_4 gregarious *S. gregaria* nymphs were isolated for three days and then crowded for 24 hr (S → G). Another group was crowded for three days at a density of 10 nymphs per chamber and then isolated for 24 hr (G → S). Their activity levels were compared to those of solitarized nymphs (isolated for four days) and gregarized (crowded for four days at a density of 10 nymphs per chamber) (Figure 5). The activity levels of recrowded nymphs (S → G) shifted towards the gregarious phase. Within 24 hr their activity levels did not significantly differ from conspecific gregarized nymphs. Conversely, activity levels of isolated gregarious nymphs (G → S) were reduced to solitarized levels.

Short-term Changes in Density-Induced Relative Hydrocarbon Composition. The relative amounts of hydrocarbons C_{32} (peak 10) and C_{34} (peak 16) increased after crowded nymphs were separated for 24 hr. This increase alters

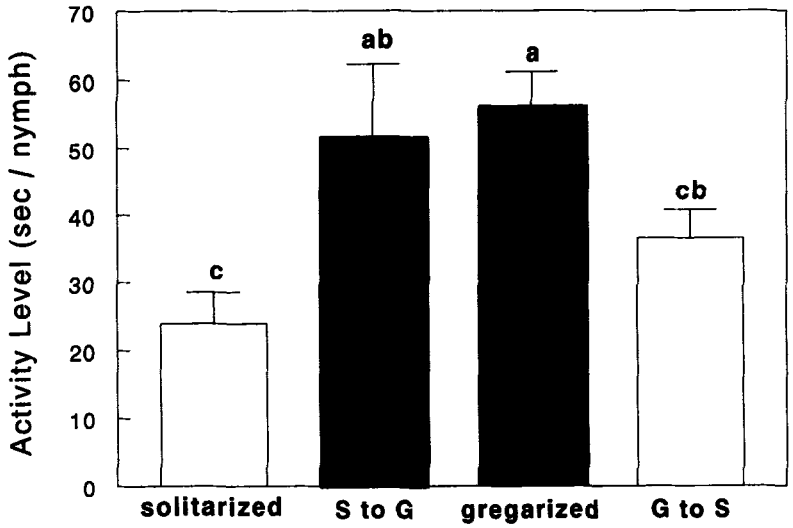

FIG. 5. The effect of changes in population density on activity levels of *S. gregaria* L_4 nymphs. Newly molted L_4 gregarious *S. gregaria* nymphs were: isolated for four days (solitarized); isolated for three days and then crowded for 24 hr (S → G); crowded for four days at a density of 10 nymphs per chamber (gregarized); or crowded for three days at a density of 10 nymphs per chamber and then isolated for 24 hr (G → S). Values are mean ± SE; $N = 5$; different letters above the data points indicate significant differences by Duncan's multiple range test; $P < 0.001$.

the ratio of C_{34} (peak 15): C_{34} (peak 16). The ratio of C_{32} (peak 10) to C_{32} (peak 11) appears similar to that of solitarious nymphs. Conversely, crowding of previously solitarious nymphs for 24 hr elicited a relative increase in C_{32} (peak 11) and C_{34} (peak 16), and these changes affected calculated ratios, too. In the transition states from gregarious to solitarious and vice versa, the ratios between each size group of hydrocarbons (Figure 6) differ from the starting state (compare to Figure 3).

DISCUSSION

The cuticular hydrocarbon profiles of solitarious and gregarious *S. gregaria* nymphs broadly contain the same compounds. Although the total quantity of nymphal cuticular hydrocarbons is higher in the solitarious phase, this extract does not induce gregarious phase behavior in solitarious nymphs. Conversely, comparable total hydrocarbons from gregarious nymphs do shift the phase status of treated solitarious nymphs to the gregarious state. We found that the relative

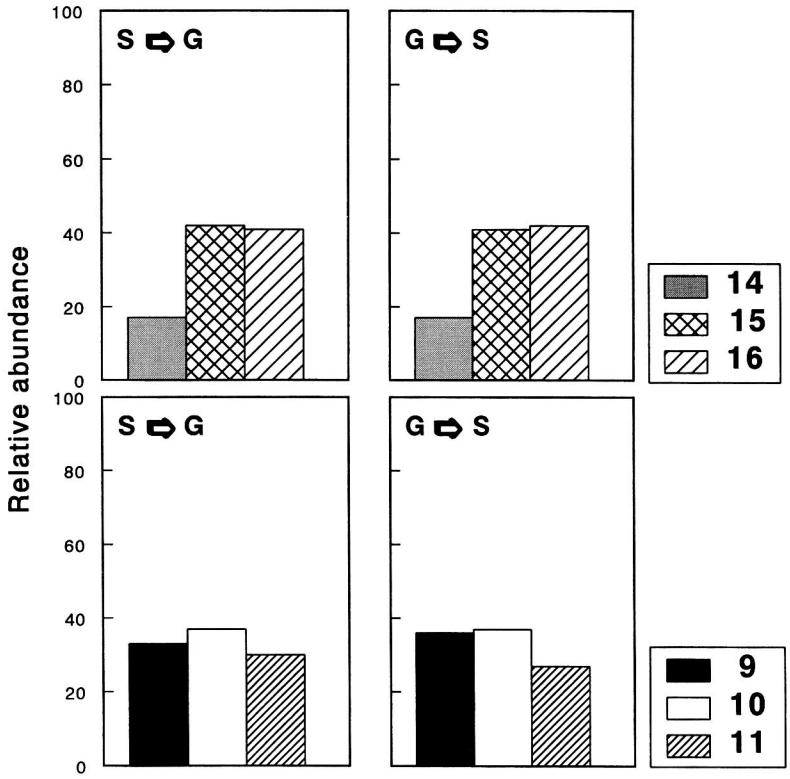

FIG 6. Relative abundance of linear and branched-chain high-molecular-weight hydrocarbons in size-groups of C_{31}–C_{32} hydrocarbons (peaks 9–11 in Table 1) and C_{33}–C_{34} hydrocarbons (peaks 14–16 in Table 1), affected by changes in population density, S → G = solitarious L_4 nymphs crowded at a density of 10 per chamber for 24 hr; G → S = gregarious L_4 nymphs isolated for 24 hr.

abundance of some of the *S. gregaria* hydrocarbons differs between the two phases, while hydrocarbons of *L. migratoria* differ even more markedly from those of *S. gregaria*, mainly in the composition of long-chain alkanes. *L. migratoria* hydrocarbons have no effect on the phase status of *S. gregaria* nymphs. This suggests that subtle differences in relative abundance of specific hydrocarbons may be associated with, and perhaps are responsible for, the phase transition elicited in solitarious *S. gregaria* nymphs by extracts from conspecific gregarious nymphs.

There are several examples from other insect systems supporting this possibility in principle. In the fruit fly, *Drosophila melanogaster*, the cuticular

hydrocarbons of males and receptive nonmated females are similar. Subsequently, mated females produce more of a specific antiaphrodisiac hydrocarbon component, which accumulates in higher amounts and depresses subsequent male courtship (Cobb and Jallon, 1990). In the related fly, *D. mojavensis*, the hydrocarbon profile is the same in both sexes, but it differs in the peak ratios of two diene compounds (Toolson et al., 1990). Different males produce different quantities of these two hydrocarbons, and a high C_{35}/C_{37} ratio increases their mating success. In the housefly, *Musca domestica*, the application of C_{28}–C_{30} branched alkanes to a decoy fly previously treated with (*Z*)-9-tricosene enhanced male mating activity with the decoy. Alone, neither of the alkanes elicited this effect (Uebel et al., 1976).

If hydrocarbons derived from the solitarious nymphs are not active in behavioral phase transition, how then is gregarious behavior induced after crowding of solitarious nymphs? The basis for answering this question is that hydrocarbon production is an ongoing process during the instar, and we hypothesize that rates of biosynthesis of the specific hydrocarbons are rapidly affected by interactions of nymphs when crowded. We might then expect that changes in the relative composition of cuticular hydrocarbons would start to be evident concurrent with the progress of gregarious behavior. Isolation of gregarious nymphs should lead to the reverse process in cuticular hydrocarbon production and such transitional states of hydrocarbon production should lead to behavioral phase transition. We found that changes in relative abundance of cuticular hydrocarbons do occur in the short time frame that solitarious or gregarious behavior is induced.

Newly produced and secreted hydrocarbons would not be expected to replace existing cuticular hydrocarbons, but rather to become diluted into the total existing pool. In fact, 24 hr after the density change of the nymphs, the hydrocarbon profiles and relations observed still differ from those of either phase. Over time, these changes would be expected to be more pronounced.

The sequence of events leading to gregarious phase transition is presumably initiated by convergence of solitarious individuals, which is assisted by the action of volatile aggregation pheromones (Obeng-Ofori et al., 1994). Populations are then maintained in proximity by cohesion pheromones (Fuzeau-Braesch et al., 1988). The production of specific hydrocarbon contact pheromones, found in certain ratios in the solitarious nymphs, changes subsequent to the initial contact between individual nymphs. It is this changing composition, perhaps in conjunction with other cuticular hydrocarbons, that is assumed to induce behavioral phase transition.

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PRIMARY ATTRACTION OF THE FIR ENGRAVER, *Scolytus ventralis*

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Abstract—In laboratory bioassays, Porapak Q-captured and steam-distilled volatiles from the bark of host trees, *Abies grandis*, particularly from root-rot-infected trees, attracted 50–70% of male and female fir engravers, *Scolytus ventralis*. Gas chromatographic–electroantennographic detection (GC-EAD) analyses of Porapak Q-captured bark volatiles revealed 19 EAD-active compounds of which 13 (mostly monoterpenes) were identified by GC–mass spectrometry (GC-MS). In separate field experiments, multiple-funnel traps baited with two blends of these 13 synthetic volatiles released at 280 and 340 mg/24 hr attracted 66 and 93% of the total *S. ventralis* captured, respectively. The clerid predator, *Thanasimus undulatus*, also responded strongly to the kairomonal volatiles. Additional experiments produced no evidence for aggregation pheromones in *S. ventralis*. These included laboratory bioassays and GC and GC-EAD analyses of Porapak Q-captured volatiles from male- and female-infested logs or trees undergoing mass attack in the field, GC analyses and/or bioassays of extracts from female accessory glands, extracted volatiles from emerged, attacking and juvenile hormone-treated beetles of both sexes, and videotape analysis of the behavior of attacking beetles on the bark surface. We argue against the hypothesis of pheromone-mediated secondary attraction in *S. ventralis* and conclude that the attack dynamics of this species can be explained solely by its sensitive primary attraction response to host volatiles.

Key Words—Semiochemicals, primary attraction, kairomones, *Scolytus ventralis*, *Thanasimus undatulus*, *Abies grandis*, monoterpenes, sesquiterpenes.

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INTRODUCTION

Bark beetles must locate and detect not only the right host species but also the most susceptible trees within the host population (Raffa and Berryman, 1987). There is conflicting evidence as to whether all bark beetles land on potential hosts at random, making a decision on host suitability at close range, or whether they orient toward host volatiles (primary attraction). It is widely accepted that after pioneer beetles have initiated attack the majority of the population orients to the host in response to secondary attractants, usually a blend of pheromones released by conspecifics and kairomones released by the tree (Wood, 1982; Birch, 1984; Borden, 1985).

Volatiles released by the host are attractive to subcortical scolytids in the genera *Dendroctonus*, *Hylastes*, *Hylurgops*, *Hylurgopinus*, *Ips*, *Pityogenes*, *Pseudohylesinus*, *Scolytus*, *Tomicus*, and *Trypodendron* (Goeden and Norris, 1964; Rudinsky, 1966a,b; Meyer and Norris, 1967; Moeck, 1970; Moeck et al., 1981; Byers et al., 1985, 1990; Miller et al., 1986; Lanne et al., 1987; Swedenborg et al., 1988; Voltz, 1988; Byers, 1989; Miller and Borden, 1990a; Moeck and Simmons, 1991; Lindelöw et al., 1992; Hobson et al., 1993; Tunset et al., 1993). Monoterpenes such as α-pinene, myrcene, terpinolene, β-pinene, β-phellandrene, and 3-carene, as well as sesquiterpenes like α-atlantone, α-cubebene, and cadinene are primary attractants when tested alone for bark beetles (Chararas, 1980; Byers et al., 1985; Millar et al., 1986; Philips et al., 1988; Schroeder, 1988; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989; Miller and Borden, 1990b; Phillips, 1980; Byers, 1992; Hobson et al., 1993). Synergistic effects on attraction often occur when terpenes are combined with the host kairomone ethanol or with insect-produced pheromones (Borden, 1985).

The fir engraver, *Scolytus ventralis* LeConte (Coleoptera: Scolytidae), is a major cause of mortality of true firs, especially white fir, *A. concolor* Hildebr. and grand fir, *A. grandis* (Dougl.) Lindl., in North America. Outbreaks of this bark beetle have occurred at least once a decade over the last 60 years (Ferrell, 1986; Wood and van Sickle, 1991). In the last decade, fir engravers have killed hundreds of thousands of grand and white fir in western North America (Wood and van Sickle, 1991; Campbell and Liegel, 1996; unpublished records from USDA, Forest Service, Northern Region). Mortality caused by the fir engraver depends on a strict mutualistic association with the plant–pathogenic fungus, *Trichosporium symbionticum* Wright, that is apparently essential for successful reproduction and colonization of trees (Wright, 1935; Livington, 1971; Wong and Berryman, 1977).

Some observations suggest that *S. ventralis* selects its host through random landing on both resistant and susceptible trees (Struble, 1957; Ashraf and Berryman, 1969; Berryman and Ashraf, 1970). However, a high correlation occurs

between root-rot infections and successful fir engraver attacks (Cobb et al., 1973; Hertert et al., 1975; Ferrell and Smith, 1976; Wright et al., 1984), which suggests that the insect can detect root-rot-infected trees. There is increasing evidence that diseased and healthy conifers can be detected by released volatiles. White fir trees that survived fir engraver attacks had a different monoterpene composition than trees that were killed (G. T. Ferrell, USDA Forest Service, Redding, California, personal communication). Concentrations of five monoterpenes, tricyclene, α-pinene, camphene, γ-terpinene, and bornyl acetate were significantly higher in lodgepole pine, *Pinus contorta* Dougl., attacked by one or more diseases (dwarf mistletoe, comandra blister rust, and root rot) than in healthy ones (Nebeker et al., 1995). Similarly, spruce, *Picea excelsa* Lk., infested with *Armillaria* root rot, contained increased amounts of oils (Madziara-Borusiewicz and Strzelecka, 1977). Moreover, needles of drought-stressed Norway spruce, *Picea abies* (L.), had a higher total monoterpene content and greater amounts of tricyclene, α-pinene, and camphene than control trees (Kainulainen et al., 1993).

Several attempts have been made to find evidence for primary and secondary attraction of the fir engraver. Vité and Pitman (1967) reported that *S. ventralis* and *S. unispinosus* LeConte respond to host odors in field trials and suggested that an insect-produced attractant was not indicated. Ferrell's (1969, 1971) field experiments showed that the fir engraver can land on different species but will land preferentially on its host, white fir. Fir engravers were trapped twice as frequently on girdled or severed-standing trees as on ungirdled controls. However, these experiments could not differentiate between primary and secondary attraction, because test trees were not protected from insect attacks, and thus secondary attraction was not prevented. In laboratory bioassays, both male and female *S. ventralis* were highly attracted to aged host phloem and less so to frass produced by virgin females (Ferrell, 1969).

Fir engravers exposed to constitutive grand fir oleoresin or to its volatile monoterpenes (individually presented) died at significant rates within 4–12 hr after exposure (Ferrell, 1969; Raffa et al., 1985). The monoterpenes tricyclene, α-pinene, β-pinene, camphene, myrcene, sabinene, limonene, β-phellandrene, bornyl acetate, and terpinolene are present in the constitutive resin. The composition of traumatic resin induced by wounding is similar, except for the addition of Δ^3-carene, the absence of bornyl acetate, and a significant increase in the quantities of β-pinene and myrcene (Russell and Berryman, 1976; Raffa and Berryman, 1987; Lewinsohn et al., 1990). Each of these compounds was repellent to walking beetles in laboratory bioassays (Bordash and Berryman, 1977). Growth of *T. symbionticum* was inhibited by camphene, β-pinene, myrcene, Δ^3-carene, and limonene (Wong and Berryman, 1977; Raffa et al., 1993).

Possible evidence for secondary attraction in *S. ventralis* was found by Ashraf and Berryman (1969). They observed that grand fir logs attacked by the

fir engraver attracted more flying conspecific beetles than uninfested control logs. Ethanolic extracts of *S. ventralis* frass were strongly attractive in the field. However, there were only two replicates, the frass-producing sex was not reported, and there was no control for ethanol, a known semiochemical for other bark beetles (Pitman et al., 1975; Moeck, 1970). However, Ferrell (1969) found that *S. ventralis* were not arrested by ethanol in laboratory bioassays. As the season progressed, attacks by *S. ventralis* became increasingly aggregated, but because attack density is directly related to gallery elongation (Ashraf and Berryman, 1969), an attractant could either be released by the beetle or by the host tree.

In a 1968 study by Ferrell and Borden (unpublished), laboratory bioassays revealed that virgin female frass and fresh grand fir phloem sawdust arrested equal numbers of *S. ventralis* at high doses, but at progressively lower doses the response to the frass disappeared before the response to sawdust. Virgin male- and female-produced frass was equally attractive. Grand fir phloem disks containing a mining female remained highly attractive for hours, while disks lacking a beetle rapidly lost potency. Fecal pellets separated from virgin female frass proved no more attractive on an equal weight basis than whole frass. The above results support the hypothesis of primary attraction for *S. ventralis*, but do not rule out the possibility of a pheromone.

Secondary attraction does occur in the genus *Scolytus*. The smaller European elm bark beetle, *S. multistriatus* Marsham, produces and responds to the pheromones 4-methyl-3-heptanol and multistriatin in combination with the sesquiterpene α-cubebene; the large elm bark beetle, *S. scolytus* F. utilizes only 4-methyl-3-heptanol and α-cubebene (Lanier et al., 1977; Blight et al., 1978). Field tests with these three components have also caught *S. pygmaeus* F. and *S. laevis* Chapuis (Minks and Van Deventer, 1978; Bejer, 1979), suggesting that the same compounds are involved in secondary attraction for these beetles. For *S. quadrispinosus* Say (Goeden and Norris, 1964), *S. numidicus* Brisout (Chararas, 1980), and *S. rugulosus* Ratzeburg (Kovach and Gorsuch, 1985), there is evidence only for primary attraction.

We report the results of laboratory and field experiments supporting the hypothesis that primary attraction occurs for *S. ventralis* and elucidating the kairomones involved. We argue against the hypothesis that *S. ventralis* requires secondary attraction for successful host selection.

METHODS AND MATERIALS

Collection of Insects and Host Material. Bolts of grand fir from healthy and root-rot-infected trees (Hagle et al., 1987), as well as from trees infested with *S. ventralis*, were collected in August and September 1993–1995 from

felled trees near Coeur d'Alene, Idaho. All logs were kept at 2°C until used. Infested logs were transferred to mesh screen cages at 24–30°C, and water was sprayed on them every five to six days to prevent desiccation. Emerged beetles were collected daily and sexed by comparing morphological characteristics of the abdominal sternites and the frons (Blackman, 1934; Edson, 1967).

Collection and Analysis of Beetle Host Volatiles. Volatiles from logs were obtained by drilling entrance holes (1.5 mm diam.) approximately 3 cm apart in the bark of fresh grand fir bolts ca. 21 cm long × 12 cm diam. These bolts were set inside separate glass aeration chambers (28 cm long × 15 cm diam.), and either 130 males or 130 females were allowed to bore into the bark, or the log remained without beetles as an uninfested control. Air was drawn through the chamber at 1.7 liters/min, and then through glass tubing (14 mm OD × 20 cm long) containing Porapak-Q (Byrne et al., 1975). Volatiles were eluted from the trap with 150 ml of distilled pentane and the effluent was concentrated to 5 ml by distillation in a 30-cm Dufton column.

Differential diagnosis (Vité and Renwick, 1970) of male- and female-produced volatiles was used to search for sex-specific compounds. GC analyses employed Hewlett Packard 5830A, 5880A, and 5890A instruments equipped with capillary inlet systems and FID. Capillary columns (30 m × 0.25 or 0.32 mm ID) coated with SP-1000 (Supelco, Bellefonte, Pennsylvania) or DB-1 (J & W Scientific Inc., Folsom, California) were used. Coupled GC–mass spectrometry (GC-MS) employed a DB-23 column and a Varian Saturn ion trap. Helium was the carrier gas for GC and GC-MS.

Isolation of Bark Oil. Bark tissue (cortex plus phloem) was peeled from fresh logs of either healthy or root-rot-infected grand fir and cut into small chips (approx. 1 cm^2). Bark oil was obtained by steam distillation. A concurrent steam distillation–continuous extraction still head (Flath and Forrey, 1977) was employed for the isolation of volatile oil from bark chips. The steam distillation was conducted for 4 hr after boil-up, and pentane was used as the extraction solvent. After evaporation of most of the pentane under a stream of nitrogen, residual solvent was removed by brief vacuum pumping.

Fractionation of Bark Oil. A Varian 1200 gas chromatograph (GC) equipped with a 10:1 effluent splitter and thermal gradient collector (Brownlee and Silverstein, 1968) was employed for micropreparative fractionation of *A. grandis* bark oil. The column was a stainless steel tube (3.05 m × 3.18 mm OD) packed with 10% SP-1000 on Supelcoport (100/120 mesh) (Supelco). The temperature program was 70°C for 2 min, then 4°C/min to 180°C and held for 20 min. The injection port and flame-ionization detector (FID) temperatures were 260°C and 270°C, respectively, and helium was the carrier gas. Typically, 1.5-μl aliquots of oil were used per run, and fractions were rinsed from the collection tubes with pentane into 1-ml volumetric tubes that were made up to volume. A Hewlett Packard 5830 GC fitted with a glass column (30 m × 0.50 mm ID) coated with

SP-1000 and FID was employed for determination of components in the fractions by the external standard method. The temperatures and carrier gas were as above. The FID was calibrated by analyzing a solution containing a known concentration of bark oil. Fraction 1 contained monoterpenes (slightly beyond the retention time of phellandrene), and fraction 2 contained the remaining compounds, mostly sesquiterpenes.

GC-EAD Analysis. Extracts and oils obtained by steam distillation and by laboratory and field aerations were subjected to coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Arn et al., 1975) adapted for an intact bark beetle (Gries, 1995) or an excised bark beetle antenna. A Hewlett Packard 5890 A instrument equipped with a DB-23-coated fused silica column (30 m × 0.32 mm ID; J & W Scientific) was used. Responses of excised antennae were amplified by utilizing a custom-built amplifier with a passive low pass filter and a cutoff frequency of 10 kHz. Compound identities were confirmed by comparison of their mass spectra with those of authentic samples.

Analysis of the chirality of α- and β-pinene, camphene, and limonene was performed as follows: ca. 10 μg of grand fir steam-distilled oil was injected twice under split conditions into a Varian 3400 GC remodified according to Brownlee and Silverstein (1968) to a preparative GC. The GC was equipped with a DB-23 column (30 m × 0.32 mm ID; J & W Scientific; GC conditions: 40°C hold for 5 min, then program 5°C/min up to 200°C; the injector was set to 240°C and the auxiliary heater for the preparative unit at 250°C). At these conditions α-pinene eluted at 3.64 min retention time, camphene at 4.66, β-pinene at 5.70, and limonene at 7.83, respectively. They were condensed in glass tubes (25 cm long × 1 mm ID), which were then rinsed with 25 μl of hexane into a 1.5 ml vial. The two collections were combined to give a total of 50 μl, and the samples of camphene and limonene were concentrated to ca. 10 μl. Each singly collected monoterpene was then injected (1 μl) into another Varian 3400 GC equipped with a Cyclodex-B-column (30 m × 0.25 mm ID, J & W Scientific; GC conditions: split injection, 80°C isothermal, injector and detector at 200°C). Chiral monoterpenes coincided with authentic standards of (+)-α-pinene and (−)-α-pinene, (−)-camphene, (−)-β-pinene, and (−)-limonene.

Preparation of Test Stimuli for Laboratory and Field Bioassays. Bark and sapwood sawdust from fresh logs of grand fir were obtained by drilling with a 1.5-mm-diam. bit. Frass produced by the insects was obtained by confining male or female beetles in gelatin capsules attached to a fresh bolt of grand fir. Frass deposited into the capsules was collected on days 2–8 after the insects began to bore into the bark. Frass was stored in air-tight vials and kept at −15°C until used.

All chemicals used in preparation of test stimuli, their purity, and their

sources are listed in Table 1. Two synthetic blends (SB1 and SB2) (Table 2) were prepared to mimic as closely as possible the spectrum of antenally active volatiles in the bark. SB1 was prepared with crude β-phellandrene, which had limonene as an impurity in a 2:1 proportion (limonene–β-phellandrene), much higher than the 1:10 ratio in the grand fir bark oil. SB2, containing synthetic β-phellandrene of greater purity (53%) and without limonene, was prepared as follows. A solution of dimsyl anion was prepared by adding, after washing, 29.6 g (0.77 mol) of 60% sodium hydride dispersion to 400 ml dimethyl sulfoxide (DMSO). The mixture was slowly warmed, and stirred for 3 hr until H_2 evolution had ceased. To this was added methyltriphenylphosphonium bromide, 289 g (0.71 mol), to produce a yellow mixture that was difficult to stir until more DMSO was added. A solution of 100 g (0.68 mol) 4-isopropyl-2-cyclohexenone (Aldrich Chemical Co.) in 100 ml DMSO was added via a dropping funnel, and the mixture was stirred overnight. The reddish mixture was quenched with 50% aqueous methanol and extracted with hexane. The combined hexanes were filtered, washed with more 50% methanol, then with saturated salt solution,

TABLE 1. CHEMICAL PURITY AND SOURCES OF COMPOUNDS USED IN THIS STUDY

Chemical	Purity (%)	Source
(±)-α-Pinene	98	Sigma Chemical Co.
Camphene	81	Matheson, Coleman & Bell
(−)-β-Pinene	99	Aldrich Chemical Co.
(+)-β-Pinene	98	Aldrich Chemical Co.
Myrcene	90	Aldrich Chemical Co.
(*S*)-(−)-Limonene	96	Aldrich Chemical Co.
(*R*)-(+)-Limonene	97	Aldrich Chemical Co.
β-Phellandrene (synthetic)	53	Synthesized
β-Phellandrene (comm.)	30	*Glidco Organics*
α-Terpinolene	29	Givaudan Lab.
p-Cymene	99	Aldrich Chemical Co.
(−)-α-Cubebene	98	Fluka Chemical Corp.
(+)-Longifolene	90	Sigma Chemical Co.
(*E*)-Pinocarveol	90	Phero Tech Inc.
Bornyl acetate	98	Matheson, Coleman & Bell
α-Terpineol	95	Aesar
(−)-Borneol	99	Aldrich Chemical Co.
Cadinene	72	Phero Tech Inc.
Verbenone	93	Phero Tech Inc.
(*E*)-Nerolidol	95	Aldrich Chemical Co.
Nerolidol	98	Aldrich Chemical Co.
Methyl-isoeugenol	99	Aldrich Chemical Co.

TABLE 2. CHEMICAL COMPONENTS AND THEIR PERCENTAGES IN SYNTHETIC BLENDS SB1 AND SB2[a]

Chemical	Percent composition in blend	
	SB1	SB2
(±)-α-Pinene	13.4	15.6
Camphene	3.0	3.2
β-Pinene	41.7	45.7
Myrcene	1.5	1.5
(±)-Limonene	17.5	1.3
β-Phellandrene (synthetic)		18.8
β-Phellandrene (comm.)	9.2	
α-Terpinolene	0.8	0.8
α-Cubebene	0.03	0.03
(±)-Longifolene	0.5	0.5
Bornyl acetate	7.0	7.3
(−)-Borneol	2.5	2.5
Nerolidol	2.6	2.6
Methyl-isoeugenol	0.2	0.2

[a]The commerical β-phellandrene used in SB1 includes limonene as an impurity. β-Pinene was deployed in a 1:50 ratio of (−) and (+) enantiomers, and nerolidol was deployed in a 2:1 ratio of *E* and *Z* isomers. α-Cubebene and methyl-isoeugenol were present in the bark oil in 3.53 and 0.49%, respectively. However, due to short supply they were deployed in the low percentages that appear in this table.

and dried over sodium sulfate. The crude product was distilled at 120°C, 20 torr, to yield 22.7 g of β-phellandrene, which was identical to authentic β-phellandrene by GC and GC-MS analysis.

Laboratory Experiments. The bioactivity of captured volatiles was tested in an arena olfactometer in which beetles made a choice between responding to a photic and an olfactory stimulus (Moeck, 1970). A light source (microscope lamp, low power) was located 49 cm from the insect release point (which received a light intensity of 76.6 lux), and the air carrying test stimuli was delivered perpendicular to the light beam 6.5 cm from the release point. The arena surface, a filter paper strip (Whatman chromatographic 3 MM) 30 cm long × 15 cm wide, was replaced every time a different sex or stimulus was tested. Prior to bioassays, beetles were held in groups of five (sexes kept separately) in Petri dishes with moistened paper at 21°C and 69 lux for 2 hr. Bulk stimuli (frass or sawdust) were placed in weighing boats directly below the air outlet and even with the arena surface. Extracted or captured volatiles were released from a glass tube (9 mm ID) lined with filter paper (10 cm diam.)

impregnated with volatile extract in pentane. Medical-quality air was passed continuously through the tube at 1200 ml/min. A positive response was recorded if a beetle entered and stayed inside a rectangular area (3 × 15 cm) transverse to the runway just in front of the air outlet.

Attractiveness of stimuli was tested in eight bioassay experiments. Experiments 1–5 and 7 used male and female beetles; experiments 6 and 8 employed only females, the most responsive sex. Experiment 1 tested volatiles emanating from 250 mg of freshly ground grand fir sapwood, bark, or frass produced by males or females; medical-quality air was the control stimulus. Experiment 2 tested Porapak Q-trapped volatiles from female-infested grand fir logs at doses of 0.03, 0.3, 3, 30, and 90 beetle-hours (bh) (1 bh = volatiles released by 1 female in 1 hr). Porapak Q-trapped volatiles from an uninfested grand fir log were used as the control stimulus. Experiment 3 tested Porapak Q-trapped volatiles from male-infested grand fir logs at doses of 0.3 and 3 bh. Trapped volatiles from an uninfested grand fir log were used as the control stimulus. Experiment 4 tested steam-distilled bark oil from a healthy tree at doses of 0.009, 0.097, 0.975, 9.75, and 97.5 mg equivalents, with pentane as a control stimulus (1 mg equiv = amount of oil distilled from 1 mg of starting material). Experiment 5 tested Porapak Q-trapped volatiles from an uninfested grand fir log at doses of 0.001, 0.01, 0.1, 1, and 10 mg/μl, with pentane as a control stimulus. Experiment 6 compared female responses towards steam-distilled bark oil from healthy and root-rot-infected grand fir at doses of 0.0006, 0.006, 0.06, 0.6, 6, and 60 mg equiv, with pentane as a control stimulus. Experiment 7 tested two fractions of steam distilled bark oil from root-rot-infected grand fir at 1 μg equiv, with pentane as a control stimulus. Experiment 8 compared the activity of the two fractions tested in experiment 7 with the activity of two tentative synthetic fractions[3] without complete confirmation of the bioactivity of all components.

Field Experiments. Synthetic blends of compounds that were antennally active, attractive in the laboratory, and available in sufficient quantity were field tested in a mature *Abies grandis/Acer rubrum* forest with moderately abundant Douglas fir (Steel and Gier-Hayes, 1992), located 10 km north of Coeur d'Alene, Idaho. Twelve-unit, multiple-funnel traps (Lindgren, 1983) (Phero Tech, Inc.) baited with candidate kairomonal blends were deployed in 10 randomized complete blocks, with $\geq$ 15 m between traps and 15 m between trap lines. Captured beetles were bagged and frozen until they could be counted and sexed. Experiments 9 and 10, respectively, tested attraction to two different synthetic blends

[3]Composition (μg) of synthetic fraction 1: α-pinene (6.9), camphene (1.2), β-pinene (21.4), myrcene (0.2), limonene (0.5), and crude β-phellandrene (3). Composition of synthetic fraction 2: α-terpinolene (0.3), *p*-cymene (0.001), longifolene (0.02), (*E*)-pinocarveol (0.04), bornyl acetate (1), α-terpineol (0.3), borneol (0.4), cadinene (0.8), verbenone (0.06), (*E*)-nerolidol (0.04), (*Z*)-methyl isoeugenol (0.04), and (*E*)-methyl isoeugenol (0.08).

(SB1 and SB2) of 13 components each (Table 2), as well as steam-distilled bark oil, and an unbaited control. The synthetic blends used in these experiments differ from those employed in experiment 8 only by the presence of α-cubebene and the lack of *p*-cymene, (*E*)-pinocarveol, and verbenone. α-Cubebene was present in the bark oil and was erroneously identified as an antennally active peak. It was incorporated as 0.03% of the synthetic blends SB1 and SB2. Release rates of SB1, SB2, and the bark oil, determined under laboratory conditions at 32°C, were 340, 280, and 50 mg/24 hr, respectively.

Statistical Analysis. Percentages of male and female beetles responding in laboratory bioassays were transformed by $\arcsin\sqrt{x}$ to normalize the data and stabilize the variances between replicates (Zar, 1984), except for experiment 6, and were analyzed by ANOVA followed by the Ryan-Einot-Gabriel-Welsh (REGW) multiple range test (Day and Quinn, 1989). For experiment 6, responses to the volatiles from healthy or root-rot-infected trees at each dose were compared by *t* tests. The REGW test was also used for data from field experiments, but with a $\log_{10}(x + 1)$ transformation (Zar, 1984). All analyses employed SAS computer software (SAS Institute, 1994) with $\alpha = 0.05$.

RESULTS

In most laboratory bioassays, females were more responsive and less variable in their responses than males. All stimuli in experiment 1 (Figure 1) were significantly more attractive to males and females than the air control. Host bark, sapwood sawdust, and male or female frass were equally attractive. Captured volatiles from female-infested logs were no more attractive at any of five doses than those from uninfested logs in experiment 2 (Figure 1). In experiment 3, volatiles from male-infested logs were less attractive to females than were volatiles from uninfested logs, but for males there was no difference in response to treatments (Figure 1).

In experiment 4 (Figure 2), responses by females to steam-distilled bark extract were significantly higher than those to pentane at doses of 0.975 and 9.75 mg equiv; males responded significantly to stimuli at these doses and also at a dose of 0.975 mg equiv. Experiment 5 (Figure 2) showed a similar trend for captured volatiles; females responded significantly at doses of 0.1, 1.0, and 10.0 mg/μl, while males responded only at the two highest doses. Steam-distilled bark volatiles from root-rot-infected trees were more attractive to walking beetles at most doses than volatiles from uninfected trees, and significantly at two doses (Figure 2, experiment 6). At doses >0.0006 mg equiv and <60 mg equiv, 61% of all responses were to the volatiles from infected trees.

In experiment 7, neither fraction of bark beetle oil distillate alone was more attractive to walking beetles than the pentane control stimulus, but there was a

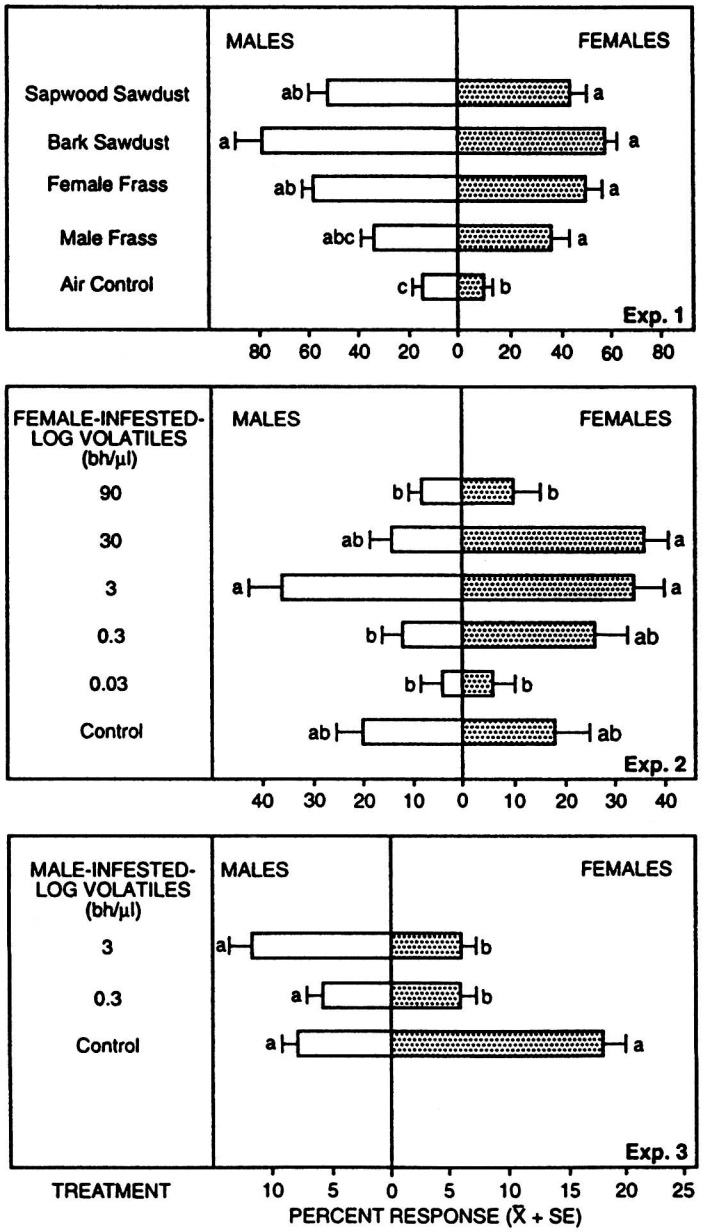
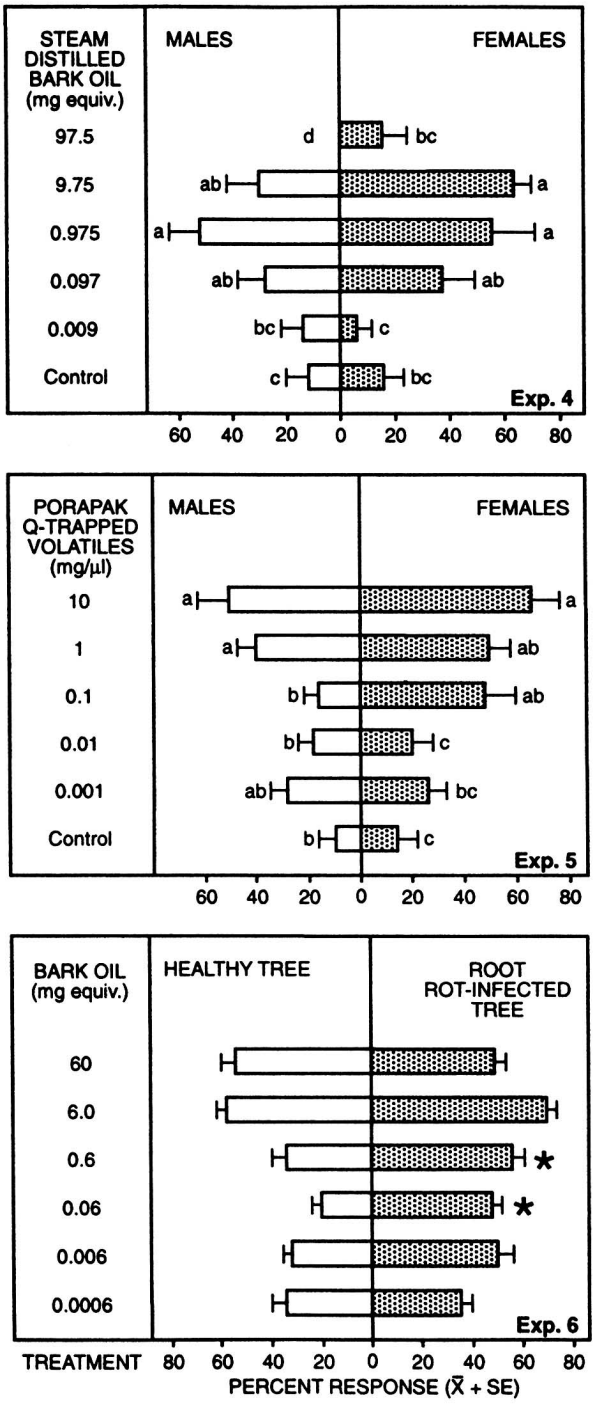


FIG. 1. Results of laboratory bioassays showing the percent responses of walking male and/or female *S. ventralis* tested in 10 groups of five insects (males or females) to sawdust or frass presented in 250 mg doses (experiment 1), Porapak Q-trapped volatiles from logs infested with female *S. ventralis* (experiment 2) or with males (experiment 3). Bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$.


STEAM DISTILLED BARK OIL (mg equiv.)
MALES
FEMALES
97.5
9.75
0.975
0.097
0.009
Control
d
bc
ab
a
a
a
ab
ab
bc
c
c
bc
Exp. 4
60 40 20 0 20 40 60 80
PORAPAK Q-TRAPPED VOLATILES (mg/µl)
MALES
FEMALES
10
1
0.1
0.01
0.001
Control
a
a
a
ab
b
ab
b
c
ab
bc
b
c
Exp. 5
60 40 20 0 20 40 60 80
BARK OIL (mg equiv.)
HEALTHY TREE
ROOT ROT-INFECTED TREE
60
6.0
0.6
0.06
0.006
0.0006
*
*
Exp. 6
TREATMENT
80 60 40 20 0 20 40 60 80
PERCENT RESPONSE (X̄ + SE)

very clear synergistic effect of combining the two fractions, especially for females (Figure 3). This effect was reproduced in experiment 8 by combining the defined synthetic fractions (Figure 3).

In GC-EAD analyses of *A. grandis* volatiles, female *S. ventralis* antennae responded to many compounds, including (±)-α-pinene, (−)-camphene, (−)-β-pinene, myrcene, (−)-limonene, β-phellandrene, α-terpinolene, longifolene, bornyl acetate, borneol, (*E*)-nerolidol, and methyl-isoeugenol (Figure 4). When the synthetic blends SB1 and SB2 (the latter with a correct ratio of limonene–β-phellandrene) were tested in the field in experiments 9 and 10, both *S. ventralis* and the clerid predator, *Thanasimus undatulus* Say, were captured in significant numbers in traps baited with the synthetic blends (Figure 5). Neither species responded to the bark oil distillate.

A summary of negative results from experiments searching for evidence of secondary attraction in *S. ventralis* is given in Table 3. These experiments included laboratory and field aerations, hormone treatments, GC analysis of gland extracts, and videotaping of behavior.

DISCUSSION

When examined in detail our results consistently support the primary attraction aggregation hypothesis. Both sexes of *S. ventralis* displayed the same general trend of response to any material tested, with females attracted in slightly higher numbers to a broader dose range of stimuli than males. This finding is concordant with the role of females as the pioneer sex that must perceive and select the most suitable host trees. The superior attraction of volatiles from female-infested logs over those from male-infested logs can be accounted for by the higher rate of boring by females, which would release more host volatiles than boring by males. The results from experiment 3 suggest that boring males produced a repellent pheromone, but this hypothesis was not followed further.

The results from GC-EAD analyses, bioassays that indicate a requirement for a blend of host volatiles to achieve attraction, and the finding that attraction can be reproduced by substituting synthetic blends for natural ones are all indicative of a species highly adapted to respond to host kairomones. Further evidence

FIG. 2. (Opposite) Results of laboratory bioassays showing the percent responses of walking male and/or female *S. ventralis* tested in 10 groups of five insects (males or females) to steam distilled bark oil (experiment 4), Porapak Q-captured volatiles from grand fir bark chips (experiment 5), and steam-distilled bark oil from root rot-infected or healthy grand firs (experiment 6). For experiments 4–5, bars for each sex with the same letter are not significantly different, REGW test, $P < 0.05$. Asterisks in experiment 6 indicate significant difference in paired responses within a dose, t test, $P < 0.05$.

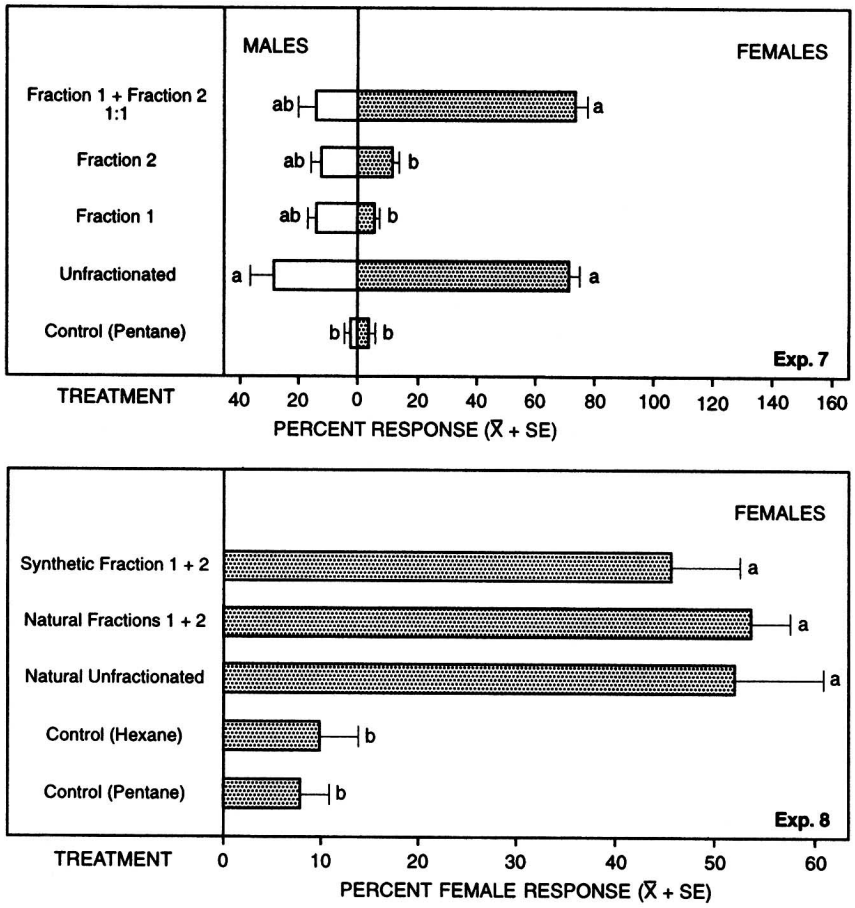


FIG. 3. Results of laboratory bioassays showing the percent responses of walking male and/or female *S. ventralis* tested in 10 groups of five insects (males or females) to fractionated and unfractionated steam-distilled bark oil from root-rot-infected grand fir presented in 1-μg doses (experiment 7), and combinations of fractions of natural and synthetic bark oil from grand fir presented in 100-ng doses (experiment 8). Within each experiment and sex of beetles, bars with the same letter are not significantly different, REGW test, $P < 0.05$.

of the sensitivity of *S. ventralis* to specific volatiles from its host is that it is repelled in laboratory bioassays at equivalent doses by bark oil from subalpine fir (unpublished results). Although both *A. grandis* and *A. lasiocarpa* are similar in their major volatile components, some minor components are not shared by both (Zavarin, 1968; von Rudolf, 1975).

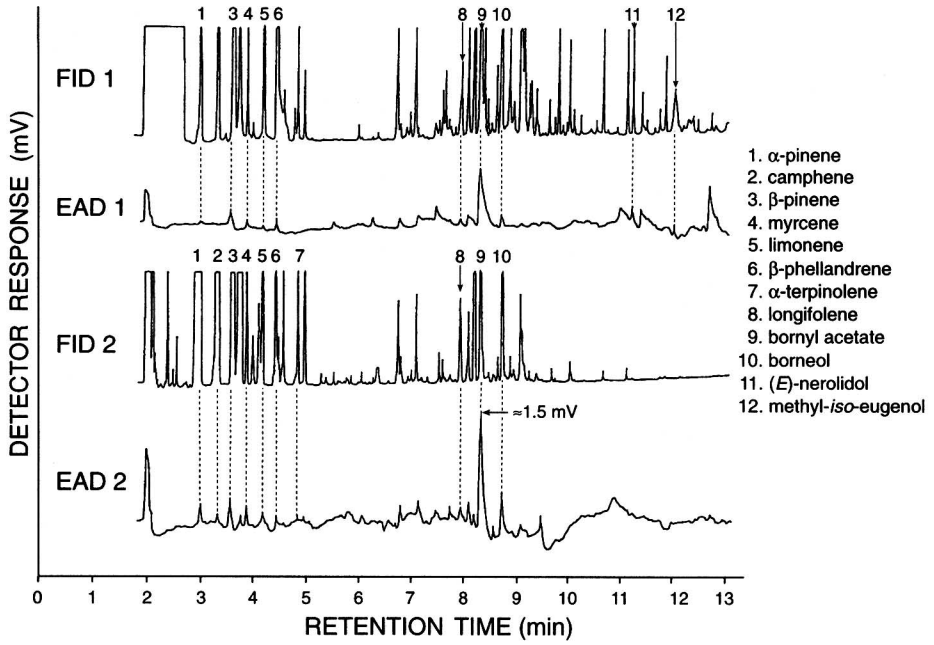


FIG. 4. Representative paired recordings of FID and EAD with female *S. ventralis* antenna to steam-distilled grand fir bark volatiles. Chromatography: DB-23 column (30 m × 0.32 mm ID) splitless injection; injector and FID temperature 240°C. Temperature program: 50°C for 1 min, then 10°C/min to 200°C, FID 1 = Porapak Q-trapped volatiles from female-infested logs; FID 2 = Porapak Q-trapped volatiles from field aeration of a 1-m section of a root-rot-infected, uninfested standing grand fir.

The repellent effect of individual terpenoid components found by Bordash and Berryman (1977) was apparently overturned by offering a blend of the same materials in a ratio of components similar to that found in the naturally occurring bark volatiles. An equivalent situation was found by Visser and Avé (1978), in which the Colorado potato beetle, *Leptinotarsa decemlineata* Say, was attracted to a specific blend of green leaf volatiles, but when these compounds were tested individually or incorporated into the blend at different ratios, the attraction ceased or decreased, respectively. Similarly, Anderson et al. (1993) reported that oviposition by the cotton leafworm, *Spodoptera littoralis* (Boisd.), was strongly deterred by a mixture of six compounds from conspecific larval frass. If one of the compounds was excluded from the mixture, the deterrent effect was lost.

The response of the predator *T. undatulus* to the same blend that attracts its prey is analogous to similar predator–host interactions in which *T. undatulus* responded to the pheromone frontalin produced by the Douglas fir beetle,

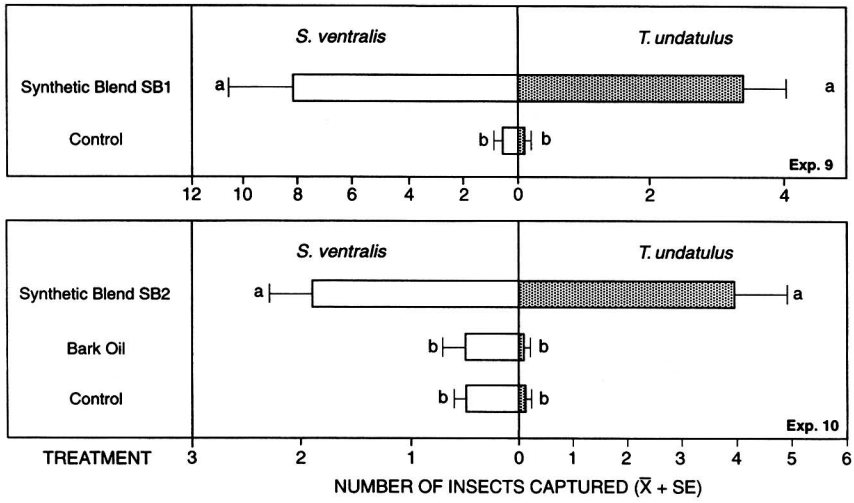


FIG 5. Numbers of *S. ventralis* and *T. undatulus* captured in multiple-funnel traps in experiment 9 (July 7–16, 1996) and experiment 10 (July 2–9, 1996), Coeur d'Alene, Idaho; $N = 10$. Release rate for SB1 was 340 mg/24 hr; for SB2, 280 mg/24 hr; and for the bark oil, 50 mg/24 hr. For each experiment and insect, bars with the same letter are not significantly different, REGW test, $P < 0.05$.

TABLE 3. SUMMARY OF ADDITIONAL EXPERIMENTS (MACÍAS-SÁMANO, 1997) PERFORMED TO TEST HYPOTHESIS OF SECONDARY ATTRACTION IN *S. ventralis*

Experiments	Results
Laboratory aerations Volatiles collected on Porapak-Q from groups of 20–500 male, female, or mixed-sex *S. ventralis* held in glass tubes (Rudinsky et al., 1973). Beetles were virgin-unfed, virgin-fed, or mated-fed. Aerations also made of grand fir bolts infested with males, females or both.	GC and GC-EAD analyses of captured volatiles revealed no sex-specific compounds. No preferential response by beetles of either sex to volatiles from either sex or type of treatment.
Field aerations Adapting the methodology of Browne et al. (1979), 1-m-long sections of bole of standing grand fir trees under attack by *S. ventralis* were wrapped in a plastic sheet open at the top with the bottom attached to a Porapak Q trap under vacuum from a portable pump. Aerations continued for 50 hr. Volatiles from unattacked control trees captured in identical manner.	GC analysis of the captured volatiles revealed no volatiles specific to attacked trees and no differences in ratios of components. This was confirmed by comparative GC-EAD analysis of extracts from one infested and one uninfested tree.

TABLE 3. CONTINUED

Experiments	Results
Hormone treatment	
With Harring's (1978) technique, 120 *S. ventralis* of each sex were treated topically with 1 and 10 μg of methoprene in 1 and 10 μl of pentane. After 24 hr, treated and control beetles were extracted in pentane. Extracts also made of male and female fir engravers allowed to bore into phloem pieces treated with 1 and 10 μg of methoprene.	No sex-specific volatiles revealed by GC analysis of extracted beetles.
Gland extracts	
Exploring the hypothesis of Gore et al. (1977) that accessory glands at the base of vaginal palpi of *S. multistriatus* were associated with pheromone production, we excised 276 abdominal tips (containing the palpi and gland) from unfed female *S. ventralis* as well as from females that had been fed for two days. Excised tips were macerated in ice-cold pentane.	GC analysis of abdominal tip extracts revealed trace amounts of *exo*-brevicomin as confirmed by GC-MS. No attraction to *exo*-brevicomin in laboratory bioassays or field experiments was observed.
Videotaping	
Over 20 hr of videotaping of male and female *S. ventralis* walking on surface of grand fir logs, females initiating attack, and males courting females were evaluated.	Several females were observed rubbing the tip of the abdomen on bark in apparent marking behavior, and a few others possibly calling while running with protruded, swollen abdominal tips.[a] There was no observed response by males to either marking or calling females, and no sex-specific volatiles were revealed by GC analysis of aerated or extracted beetles (above). Gallery initiation and courtship similar to that by *S. multistriatus* (Svihra and Clark, 1980) was observed, but no behavior observed that would suggest pheromone release.

[a] J. E. Macías-Sámano and J. H. Borden: Host finding and mating behavior of the fir engraver. Paper presented at the annual meeting of the Entomological Society of America, Indianapolis, Indiana, December 13–15, 1993.

Dendroctonus pseudotsugae Hopkins (Ross and Daterman, 1995), and ipsdienol produced by *Ips* spp. (Miller et al., 1991). In each instance, the predators and their prey responded to identical stimuli, suggesting that *T. undatulus* may have distinct semiochemical-based, prey-adapted races. *Thanasimus* spp. are characteristically attracted to the pheromones of several host scolytid species (Vité

and Williamson, 1970; Bakke and Kvamme, 1981; Mizell et al., 1982; Payne et al., 1984; Raffa and Klepzig, 1989; Herms et al., 1991; Miller et al., 1991). Another predator, the blackbellied clerid, *Enoclerus lecontei* (Wolcot), reported as the most abundant predator of the fir engraver (Struble, 1957; Ashraf and Berryman, 1969; Berryman and Ferrell, 1988), was not trapped in response to the synthetic blends, but at the same field site was attracted (Macías-Sámano, 1997) by the aggregation pheromone (Macías-Sámano et al., 1997) of *Pityokteines elegans* Swaine, which implies that this clerid is following *P. elegans* and not *S. ventralis*.

The thresholds for perception of and response to bark oil and the blend of synthetic host kairomones are similar to those for pheromones in the genera *Dendroctonus* and *Ips* (Borden, 1985), and much lower than for kairomones in other genera (Dickens, 1979). In particular, the threshold for response near 0.1 mg equiv of bark oil distillate is easily equivalent to the response of male *S. multistriatus* in laboratory bioassays to 10 mg of pheromone-laden frass from virgin females (Peacock et al., 1973). The ability of the synthetic blends released at 340 and 280 mg/24 hr for SB1 and SB2, respectively, to attract *S. ventralis* in the field is also remarkable, considering the competition from natural odor sources in the forest, and the fact that these are the summed release rates for 13 components (Table 2). In comparison, red turpentine beetles, *Dendroctonus valens* (LeConte, were attracted in the field to specific enantiomers of α- and β-pinene released at 0.8–70 ml/24 hr (Hobson et al., 1993), doses ranging from 2 to 200 times those at which SB1 and SB2 were released.

Although grand fir bark oil was highly attractive to *S. ventralis* in laboratory bioassays, its failure to attract *S. ventralis* in the field when released at 50 mg/24 hr suggests that the release rates of 340 and 280 mg/24 hr for SB1 and SB2, respectively, were just above the threshold for attraction. Additional experimentation has shown bark oil to be attractive to fir engravers when released at 386 mg/24 hr (Macías-Sámano, 1997).

Because of the intolerance of *S. ventralis* to resin and the inhibition of growth by *T. symbionticum* in the presence of monoterpenes (Raffa et al., 1985), there is a low likelihood that fir engravers could overcome the induced defense system of healthy trees (Raffa, 1991), even by mass attack. Therefore, avoidance of stimuli associated with host resistance would be adaptive (Raffa and Berryman, 1987), as would orientation toward stimuli associated with susceptible weakened hosts. Mass attack behavior would be adaptive only to the extent that slightly resistant trees could be included as suitable hosts. A preference for weakened hosts is supported by the high correlation of root-rot infections and fir engraver attacks (Cobb et al., 1973; Hertert et al., 1975; Ferrell and Smith, 1976; Wright et al., 1984) and by our findings that fir engravers are more attracted to oil from the bark of root-rot-infected trees than from the bark of healthy trees. Other bark beetles can apparently also detect fungus-infested hosts.

For example, seven times more pine engravers, *Ips pini* (Say), bored into trees infected by *Leptographium terebrantis* Barras and Perry, than into healthy ones (Raffa and Klepzig, 1996). Nebeker et al. (1995) showed that lodgepole pines infested with *Armillaria* root rot had a 20 times higher bornyl acetate content in the resin than healthy trees. Conversely, traumatic resin from grand fir contains no bornyl acetate (Russell and Berryman, 1976; Raffa and Berryman, 1987; Lewinsohn et al., 1990) and may not be attractive to host-seeking fir engravers. In our experiments bornyl acetate elicited a very clear and strong EAD response in fir engraver antennae and was attractive to walking *S. ventralis* when tested alone at doses ranging from 10 to 100 ng in laboratory bioassays (results not shown).

In a kairomone-driven system, it would be adaptive for both sexes to respond at high levels to a host kairomonal signal, and to rely on close-range recognition factors for mate selection. Accordingly, one would not expect the sex ratio of responding beetles to be altered if the host were under attack by conspecifics. Field investigations by Ferrell (1969) support this hypothesis; the sex ratios of *S. ventralis* caught on girdled unattacked and girdled attacked trees did not differ, nor did they differ from the sex ratio at emergence. Results from experiment 1 and from the female–male sex ratios of 12:8 and 47:35 in experiments 9 and 10, respectively, are consistent with Ferrell's (1969) observations.

There are other bark beetles that are attracted to host resin and/or some of its components and that also do not seem to have aggregation pheromones. The red turpentine beetle is attracted to mixtures of α-pinene, myrcene, and 3-carene (Hobson et al., 1993) and demonstrates a remarkable chiral specificity toward (*S*)-(−)-α-pinene (White and Hobson, 1993). The native elm bark beetle, *Hylurgopinus rufipes* (Eichhoff), is attracted to cut elm wood (Martin, 1936), wounded elms (Landwehr et al., 1981), and to naturally and artificially moribund elms (Gardiner, 1979; Millar et al., 1986). Several sesquiterpenes were attractive to *H. rufipes* when deployed in traps (Millar et al., 1986), but no pheromone could be demonstrated in this species (Swedenborg et al., 1988).

Historically, research on the chemical ecology of scolytids has disclosed occasions when species were considered to be kairomone-driven but were later shown to employ aggregation pheromones. For example, Meyer and Norris (1967) attributed the higher attraction of *S. multistriatus* to female- than male-infested logs to the greater release of host volatiles by the actively boring females. However, Peacock et al. (1971) demonstrated the presence of a female-produced aggregation pheromone by showing much higher attraction to logs infested with 40 females than to logs infested by 400 males. The contention by Byers et al. (1985) and Vité et al. (1986) that the pine shoot beetle, *Tomicus piniperda* (L.), relies solely on primary attraction in host selection has recently been countered by Teale (1996), who used GC-EAD analysis as the basis for demonstrating that this species also uses an aggregation pheromone. Therefore, we pursued an

exhaustive series of experiments using GC and GC-EAD techniques as well as other approaches to test the hypothesis of secondary attraction in *S. ventralis*, with consistently negative results. Moreover, a review of 50 years of published information on the fir engraver failed to yield compelling evidence for a pheromone-driven system and supported the hypothesis that from an evolutionary perspective the fir engraver has become well adapted to rely on host kairomones to mediate host selection.

One significant adaptation is the transverse orientation of *S. ventralis* galleries, which allows *T. symbionticum* to be inoculated into the greatest possible amount of vascular tissue (Wong and Berryman, 1977). This in turn results in a large area invaded by the fungus and a correspondingly large area in which the insect can breed. Because *S. ventralis* can avoid encountering large amounts of constitutive resin, which is localized primarily in cortical pitch blisters (Bannan, 1936; Littelfield, 1973; Ferrell, 1969, 1983), there is little selective pressure to develop a resin detoxification system (Raffa and Berryman, 1987), a process by which other bark beetles produce pheromones (Renwick, 1988).

Because exposure to resin can be avoided, fir engravers produce single galleries in living trees that can be as successful, or more so, as those in mass-attacked trees (personal observation). Several reports (Struble, 1957; Johnson and Shea, 1963; Berryman, 1969; Berryman and Ashraf, 1970; Felix et al., 1971; Ferrell, 1973) describe the scars of old isolated attacks, both successful and unsuccessful, embedded in the xylem of living firs. The ability to kill a patch of bark and potentially reproduce in it (Ferrell, 1973; personal observation) is found in very few other bark beetles. Among them is *Dendroctonus micans* Kugelann, which like *S. ventralis* is not known to orient by pheromonal communication (Grégoire, 1985).

Scolytus ventralis flies over an eight-week period (Struble, 1957; Ashraf and Berryman, 1969) and can respond to stressed trees throughout the growing season as they become available (Raffa and Berryman, 1987). Linking the long period of flight with the absence of strong mass attack behavior (Ashraf and Berryman, 1969), and great variability in attack density when trees are mass attacked (Berryman, 1968a,b), there is presumably less advantage to synchronous flight and attack than in other species of tree-killing bark beetles (Raffa and Berryman, 1987) that are driven by pheromones. This hypothesis is supported by the fact that grand fir is incapable of induced responses to wounding under conditions of intense water stress (Lewinsohn et al., 1993).

As hypothesized for pheromone-mediated mass attack (Alcock, 1982), mass attack by *S. ventralis*, when it does occur, would simply be a consequence of each beetle attempting to maximize its fitness by responding to the volatiles emitted by a potentially suitable host. In support of this hypothesis, Berryman and Ashraf (1970) found that aggregation on a host by the fir engraver is directly associated with the degree of gallery elongation.

Based on our findings and the above discussion, we hypothesize that both sexes of *S. ventralis* are attracted to and aggregate on a tree, first because of odors emitted by the tree, and subsequently because the pioneer boring insects (females) liberate host kairomones, not insect-produced pheromones, by exposing vascular tissue to the air. These compounds would signal other insects of the presence of a suitable host. Close-range phagostimulatory signals might stimulate boring, and mating might be regulated by a combination of stridulatory signals (Ferrell, 1969; Rudinsky et al., 1978) and close-range pheromonal stimulation. Such close-range signals and their regulation have been suggested by observations of apparent calling and marking behavior revealed by videotape analysis.

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FORMATION OF COLONY ODOR IN PONERINE ANT *Pachycondyla apicalis*

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Abstract—The mechanisms operating in the formation and maintenance of colony odor in the ponerine ant *Pachycondyla apicalis* were studied using radioactive tracers. Using [1-^{14}C]acetate as a precursor, the de novo biosynthesis and distribution of pentane-extractable lipids within the ant's body were followed. Twenty-four hours after injection, newly synthesized alkanes, alkenes, as well as more polar lipids were found in the postpharyngeal gland, the epicuticle, and internally. Ants that had their mouths blocked with beeswax possessed significantly less radiolabeled lipids (all classes measured) in the postpharyngeal gland but had augmented levels in the internal pool and the epicuticle. Both hydrocarbons and more polar lipids were exchanged between the respective postpharyngeal glands and the epicuticle. The transfer to postpharyngeal glands was highest when both ants had an open mouth or when the recipient ant had an open mouth but the donor's mouth was blocked. This suggests that the transfer to the postpharyngeal gland in this species is by allogrooming and not by trophallaxis. Transfer to the cuticle was low and comparable in all treatments. Behavioral observations during the first 6 hr of the dyadic encounters and in intact colonies confirmed that the ants did not engage in trophallaxis. The level of transfer to the postpharyngeal gland in *P. apicalis* was significantly lower than in representatives of other Formicidae subfamilies studied so far. We attribute this difference to the evolution of trophallaxis in the higher Formicidae.

Key Words—Postpharyngeal glands, epicuticle, hydrocarbons, nestmate recognition, ants, *Pachycondyla apicalis*.

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INTRODUCTION

It is generally accepted that in social insects the segregation of populations into colonies is usually accompanied by the formation of a specific label and a nestmate recognition ability (Hölldobler and Michener, 1980). Consequently alien ants are recognized and excluded from the colony, thus protecting the colony resources from exploitation by conspecifics. In the majority of ant species studied so far, members of the colony can be characterized by a complex mixture of cuticular hydrocarbons having a nest specific pattern that is distinguishable from that of other nests (Bonavita-Cougourdan et al., 1987, 1989, 1993; Morel et al., 1988; Nowbahari et al., 1990; Hendersen et al., 1990; Vander Meer et al., 1989). Hydrocarbon composition in the postpharyngeal gland is likewise species specific and for each species is largely congruent with the pattern found on the epicuticle (Bagnères and Morgan, 1991; Do Nascimento et al., 1993; Hefetz et al., 1992; Soroker et al., 1995b). The role of the postpharyngeal gland content in nestmate recognition was recently established for at least two ant species (Soroker et al., 1994; Hefetz et al., 1996). All this evidence, although circumstantial, suggests that the hydrocarbon constituents of the postpharyngeal gland play a role in nestmate recognition. Our experiments with *Cataglyphis niger* indicate that the hydrocarbon constituents of the postpharyngeal gland are sequestered by internal transport as well as from the body surface by self-grooming (Soroker et al., 1994, 1995a,b). The link between the postpharyngeal gland and body surface enables the ants continuously to refresh, and thus update, their epicuticular hydrocarbons.

Recognition cues can be derived from the environment, carried into the nest by foragers (Obin, 1986; Obin and Vander Meer, 1988, 1989), or from genetically determined odors, contributed by nestmates, the queen (Carlin and Hölldobler, 1983, 1986, 1987; Provost, 1989) or other workers (Crosland, 1990; Errard and Jallon, 1987; Vienne, 1993). Whatever the source, for large colonies it is predicted that each colony will display a uniform odor that constitutes a gestalt (Crozier and Dix, 1979; Crozier, 1987). According to this model, members of a colony share and exchange the recognition cues, and the resulting odor is an approximate average of the individual odors of the ants in the colony. Such an exchange among workers has been demonstrated in *C. niger* (Soroker et al., 1994, 1995a), with artificially mixed species groups of *Formica selysi* and *Manica rubida* (Hefetz et al., 1992; Vienne et al., 1995), and in *Camponotus vagus* (Meskali et al., 1995). The exchange between individuals can occur actively via allogrooming and trophallaxis or passively through physical contact. It was further demonstrated that the postpharyngeal gland is involved in this active exchange and acts as a gestalt organ (Soroker et al., 1994, 1995a; Vienne et al., 1995). At least in *C. niger*, trophallaxis is the primary means for distributing recognition chemicals among colony members (Soroker et al., 1994, 1995a).

In species of the primitive ant subfamily Ponerinae, the occurrence of trophallaxis has been controversial for many years. In *Hypoponera eduardi*, Le Masne (1952) described trophallactic exchanges between adults, and this was confirmed with radioisotopes by Gösswald and Kloft (1960), but not by Hölldobler (1985). Recent reports described trophallaxis in *Hypoponera* sp. (Hashimoto et al., 1995) as well as exchange of small droplets during social interactions in *Ponera coarctata* (Liebig et al., 1994, 1997). It is further postulated that trophallaxis appeared relatively late in ant evolution and therefore characterizes the higher order subfamilies (Wilson, 1971). Comparison of hydrocarbon profiles between and within colonies of *Pachycondyla apicalis* confirmed that a unified colony odor is maintained in this species and is expressed both on the cuticular surface and in the postpharyngeal gland (Hefetz et al., unpublished observations). The objective of this study was to assess, using hydrocarbons as a model system for nestmate recognition cues, the means by which colony odor formation is accomplished in *P. apicalis* and to examine the role of the postpharyngeal gland in this process.

METHODS AND MATERIALS

Collection and Maintenance of Ant Colonies. Nineteen colonies of *Pachycondyla apicalis* were collected at the biological reserve of Los Tuxtlas (rain forest) in Mexico, and from cocoa plantations of the research institute of Ceplac in Itabuna, State of Bahia, Brazil, during 1995–1996. In the laboratory the colonies were transferred to artificial nests and reared under a controlled temperature of 24 ± 4°C. For observation of the ants' behavior in intact colonies, all members of the colony were individually marked either by color dots or numbered tags. The ants were fed three times a week with an identical diet of sugar water and a mixture of minced insects and apple in honey.

De Novo Lipid Biosynthesis. Workers were each injected through the intersegmental membrane of the abdomen with 0.5 μl of medium prepared according to Katase and Chino (1984) and containing 1 μCi (56 mCi/mmol) [1-^{14}C]sodium acetate (NEN, Boston, Massachusetts). The incubation was stopped after 24 hr by freezing the ants. For extraction, dissected postpharyngeal glands were immersed in 100 μl of pentane. Epicuticular lipids were extracted by immersing the thorax alone or the thorax together with the abdomen, from which Dufour's gland was removed (cuticular wash), in 400 μl of pentane for 5 min. Following this short-term extraction, the lipids were subjected to a long-term extraction (10 days) in a new vial containing pentane at −20°C to obtain internal lipids. The extracts were separated by TLC using silica gel (polygram Sil G) impregnated with 10% silver nitrate in double distilled water (Vienne et al., 1995). Radioactivity of the fractions containing alkanes, alkenes, and more polar lipids

(the identity of which was not determined) was detected by exposure on an image plate for 22 hr using a Phosphor-Imager Autoradiography System (Fuji BAS 100 analyzer).

To determine the effect of mouth blocking on the distribution of newly synthesized lipids, the ants had their mouths blocked with beeswax prior to injection. Treatment of the ants and lipid analysis were performed as described above.

Hydrocarbon Transfer Between Nestmates. To assess the rate of hydrocarbons transfer between nestmates, ants were injected with 1 μCi [1-^{14}C]sodium acetate as described previously (donor ants) and after 24 hr of incubation (25 $\pm$ 3°C) each was presented with one prestarved nestmate (recipient) in a Petri dish (9 cm diameter). All encounters were stopped after 24 hr by freezing the ants. The occurrence of labeled hydrocarbons and more polar lipids in the postpharyngeal gland and on the thoracic epicuticle was monitored for both donor and recipient. The degree of transfer of labeled lipids from donor to recipient was calculated for each pair and for each tissue (postpharyngeal gland and epicuticle) separately as the percent label found in the recipient out of the total label found in the donor and recipient combined.

In another set of experiments, either the donors or recipients or both had their mouths blocked with beeswax. Encounters included all possible combinations of prelabeled and blocked or nonblocked ants, and nonlabeled and blocked or nonblocked ants. Analyses of hydrocarbons and assessment of transfer between the ants were completed as described above.

Behavioral Observations. During the first 6 hr of each dyadic encounter, the behavior of both ants was recorded simultaneously every 5 min. Behavioral items were classified in five categories: trophallaxis, self-grooming, allogrooming, physical contact (antennal and body contacts), and no contact.

Similar observations were also performed on two small but intact colonies maintained in the laboratory (colony 1 comprising four individuals; colony 2 comprising 12 individuals). The nests were videotaped for 24 hr, from which sessions of 6 hr for individual ants were selected. Behaviour was recorded every 5 min as described above.

RESULTS

The occurrence of newly synthesized lipids in the postpharyngeal gland and the epicuticle following an injection of radiolabeled sodium acetate to workers of *P. apicalis* is presented in Table 1. Labeled hydrocarbons, alkanes and alkenes, as well as more polar lipids were detected both in the thoracic epicuticle and the postpharyngeal gland. Separation of the various extracts by TLC also revealed the presence of three radiolabeled lipid fractions that were more polar

TABLE 1. BIOSYNTHESIS AND DISTRIBUTION OF RADIOLABELED LIPIDS IN POSTPHARYNGEAL GLAND AND ON THORACIC EPICUTICLE OF *Pachycondyla apicalis* WORKERS ($N = 9$)[a]

Organ	Alkanes (R_f 5.6) (dpm/ant)	Alkenes (R_f 3.8) (dpm/ant)	Alkane/alkene ratio	Polar lipids (dpm/ant)[b]
PPG	1982 ± 391	1242 ± 215	1.7 ± 0.3	1525 ± 324
Epicuticle	1483 ± 144	874 ± 112	1.8 ± 0.2	946.6 ± 121

[a] Values are mean ±SEM.
[b] Three lipid fractions that migrated in the TLC to R_f 0.5, 1.1, and 2.1, respectively.

than the alkenes. The specific identity of these was not investigated further, and they are considered together as polar lipids. The total amount of newly synthesized hydrocarbons was about twice that of the polar lipids.

To investigate whether a link exists between cuticular and glandular hydrocarbons, experiments using ants that had their mouths blocked with beeswax (thus preventing any pick up of cuticular hydrocarbons through self-grooming) were conducted. Blocking of the mouthparts did not alter the ants' ability to synthesize alkanes, alkenes, or the more polar lipids de novo (Table 2), but their distribution among the postpharyngeal gland, the epicuticle, and the internal pool changed (Figure 1). The amount of hydrocarbons (alkanes and alkenes alike) in the postpharyngeal gland of the blocked ants was about half that of the control, nonblocked ants (1046 ± 99 vs. 592 ± 16, and 1354 ± 151 vs. 632 ± 22 dpm/ant for alkenes and alkanes, respectively), whereas the amount of polar lipids in the blocked ants reached only 20% that of the nonblocked ants

TABLE 2. TOTAL BIOSYNTHESIS OF RADIOLABELED LIPIDS BY MOUTH-BLOCKED AND CONTROL (NONBLOCKED) *Pachycondyla apicalis* WORKERS[a]

Compound	Radioactivity (dpm/ant)[a]	
	Control ($N = 17$)	Mouth blocked ($N = 15$)
Alkanes	3919 ± 322	4508 ± 456[b]
Alkenes	2844 ± 178	3690 ± 342[b]
Polar lipids	7708 ± 886	8668 ± 636[b]

[a] Values are mean ± SEM.
[b] NS; Mann-Whitney U test.

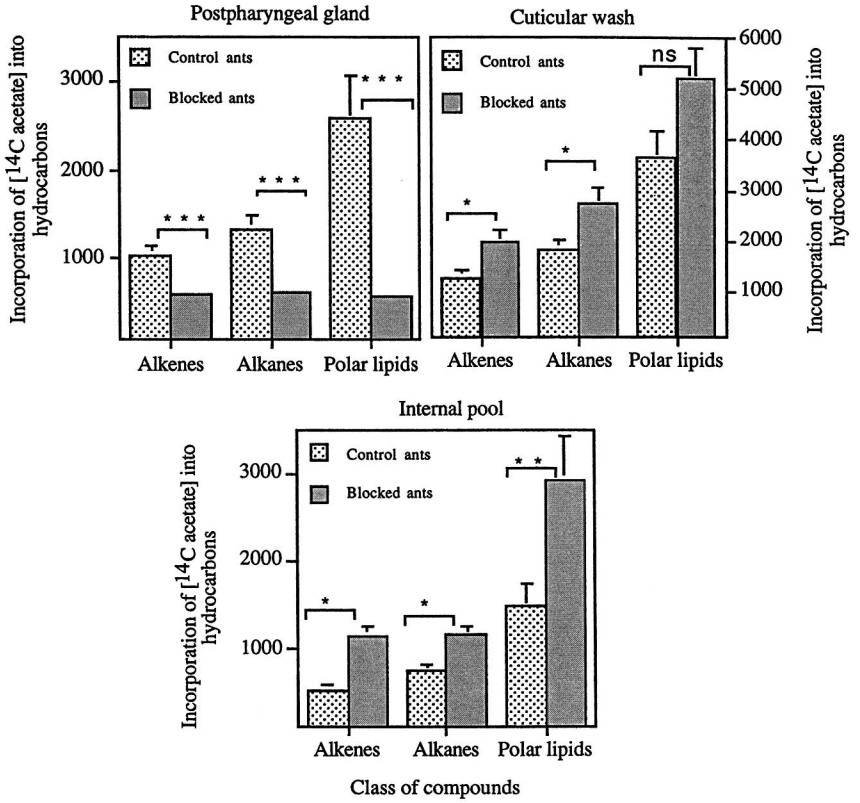

FIG. 1. The effect of mouth blocking on the distribution of newly synthesized lipids. Data are mean ± SEM of a minimum of 15 replicates. Different letters indicate the groups that are statistically different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Mann-Whitney U test).

(2587 ± 471 vs. 571 ± 25 dpm/ant). This indicated that although some of the lipids found in the postpharyngeal gland of the nonblocked ants have an internal origin, the majority of the lipid constituents reached the gland externally. In the cuticular wash, on the other hand, the amount of hydrocarbons was higher in the blocked ant, but there were no differences in the polar lipids between the two treatments. In the internal pool, the amount of newly synthesized alkanes, alkenes, or the more polar lipids in the blocked ants was consistently higher than in the control ants.

The next set of experiments was aimed at investigating whether there is an exchange of substances between nestmates and the behavioral modality by which

it proceeds. First, dyadic encounters between a prelabeled ant and a nonlabeled nestmate were conducted, revealing that all TLC fractions monitored (alkanes, alkenes and the more polar lipids) are transferred between workers. These experiments were done with queenright and queenless colonies, but since there was no difference in lipid transfer between the groups [percent of hydrocarbons transferred to the postpharyngeal gland: 5.7 ± 3.11, 3.5 ± 1.29; to the epicuticle 1.1 ± 0.37, 3.8 ± 1.23; percent of polar lipids transferred to the postpharyngeal gland: 2.5 ± 1.47, 1.5 ± 0.62; to the epicuticle 0.9 ± 0.41, 2.8 ± 1.90; mean % ± SEM for queenless (N = 8) and queenright (N = 11) respectively, $P > 0.05$, Mann-Whitney U test], the data were pooled. The total level of lipid transfer to the postpharyngeal gland was slightly higher than to the epicuticle (3.1 ± 0.85 vs. 1.7 ± 0.39), but this difference was not significant (Wilcoxon signed rank test, $P = 0.15$). Observation of the ants' behavior during the first 6 hr of each encounter revealed that there was no trophallaxis between the ants (Table 3).

Further dyadic encounters consisted of one ant (donor or recipient) or both ants with blocked mouthparts, therefore preventing any trophallaxis and enabling only unidirectional allogrooming or none at all (Figure 2). While the level of transfer to the epicuticle was the same in all groups, the transfer to the postpharyngeal gland was dependent on the treatment. When the recipient ant had an open mouth, but the donor ant had a blocked mouth (RODB in Figure 2), the amount of newly synthesized hydrocarbons transferred to the postpharyngeal gland of the recipient ants did not differ from cases in which both ants had open mouth parts. Since trophallaxis was impossible in the first case, the accumulation of radioactive hydrocarbons in the postpharyngeal gland of the recipient must also have been the consequence of allogrooming. By inference, allogrooming must also have been the major route by which newly synthesized hydrocarbons

TABLE 3. FREQUENCY OF BEHAVIORS PERTAINING TO TRANSFER OF HYDROCARBONS IN DYADIC ENCOUNTERS BETWEEN WORKERS AND IN INTACT COLONIES OF *Pachycondyla apicalis*

	Percent (mean ± SEM)		
Behavioral item	Donor	Recipient	Intact colonies
Trophallaxis	0	0	0
Self-grooming	8.5 ± 1.3	7.3 ± 1.1	15.7 ± 1.4
Allogrooming	0.8 ± 0.4	0.5 ± 0.3	3.0 ± 0.7
Physical contact	42.4 ± 2.8	42.2 ± 2.8	8.5 ± 1.2
No contact	48.3 ± 3.6	50.1 ± 3.0	72.8 ± 2.7
Replicates (N)	15	15	9

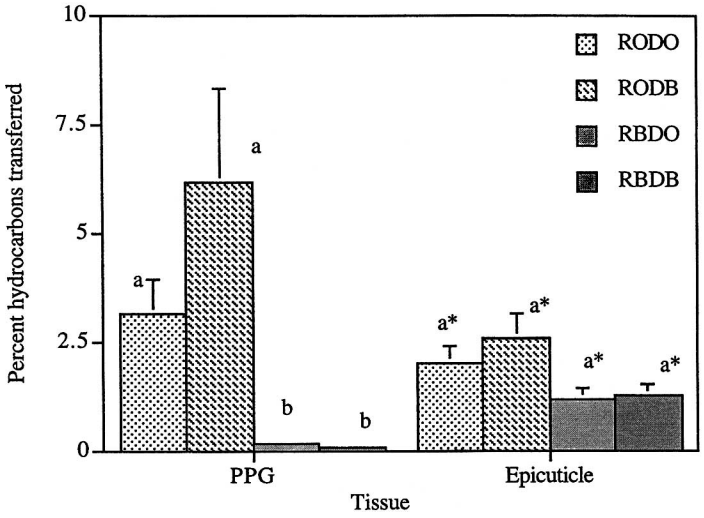

FIG. 2. The effect of mouth blocking on hydrocarbon transfer between nestmate workers of *Pachycondyla apicalis*. Data are relative amount of labeling in recipients' tissue, mean percent ± SEM of a minimum of 10 replicates. RODO = recipient open–donor open; RODB = recipient open–donor blocked; RBDO = recipient blocked–donor open; RBDB = recipient blocked–donor blocked. Different letters indicate the groups that are statistically different ($P < 0.05$; Mann-Whitney U test).

reached the postpharyngeal gland in cases in which both ants had open mouths. Thus, when the recipient ant had its mouthparts blocked, irrespective of the treatment the donor ants received, radiolabeled hydrocarbons were found in the postpharyngeal gland only in minute quantities, if at all.

Behavioral observations conducted during the first 6 hr of each encounter revealed that the ants were engaged mostly in physical contact, with self-grooming being the second most common behavior. There were no incidences of trophallaxis during this observation period, but allogrooming was apparent (Table 3). Observation of workers in intact colonies gave comparable results. Self-grooming was recorded 15% of the time, whereas allogrooming occupied about 3% of the observation time. There were no incidences of trophallaxis. It is worth noting that in intact colonies the ants interacted less frequently; over 70% of the time there was no contact between the particular ant under observation and the nestmates.

DISCUSSION

Maintenance of a gestalt colony odor requires the continuous production of innate recognition cues accompanied by an efficient mechanism for their

distribution on the individual body surface, as well as a means for their exchange between colony members. The results obtained with *P. apicalis* reveal the significance of the postpharyngeal gland in this process. At the individual level, the comparison between control ants and ants with blocked mouthparts revealed that less than half the hydrocarbons present in the postpharyngeal gland had originated internally. The majority of hydrocarbons apparently reach the gland from the cuticular surface by self-grooming. Although we can not exclude the possibility that some of the hydrocarbons in the postpharyngeal gland are synthesized *in situ*, we suggest, based on our studies with *Cataglyphis niger*, that they are synthesized elsewhere and transported to the gland internally through the hemolymph. In any case, the exchange of material between the postpharyngeal gland and the epicuticle provides a mechanism for spreading the putative recognition cues throughout the body surface and is responsible for the chemical congruency between these two body parts. This finding is consistent with the mechanism described for other Formicidae (Bagnères and Morgan, 1991; Do Nascimento et al., 1993; Hefetz et al., 1992, and unpublished observations; Soroker et al., 1995b). The similarity between the results obtained with the ponerine *P. apicalis* and the system found in the formicine *C. niger* (Soroker et al., 1994, 1995a,b), representatives of two phylogenetically remote ant subfamilies, suggests that the ability of the postpharyngeal gland to sequester lipids may have arisen early in ant evolution.

The postpharyngeal gland in *P. apicalis* is also involved in the distribution and exchange of hydrocarbons among colony members, albeit in an indirect way. The results of the dyadic encounters indicate that the major mode of transfer between ants is by allogrooming. In the encounter in which the donor's mouthparts were blocked but the recipient had open mouthparts (RODB), trophallaxis was impossible; nonetheless there was an appreciable amount of radiolabel in the postpharyngeal gland. The fact that the amount of transfer to the postpharyngeal gland was not significantly different from the encounter in which both participants had open mouthparts (RODO) further supports the idea that in the latter case trophallaxis was not responsible for the occurrence of radiolabeled hydrocarbons in the recipients' postpharyngeal gland.

The transfer data are consistent with our behavioral observations both during the dyadic encounters and for the whole colony. We never observed any incidence of trophallaxis, but allogrooming and particularly self-grooming were rather common. During the dyadic encounters physical contact seemed to be higher than observed in intact colonies, probably reflecting the different social environments. In intact colonies the ants attend to many duties in the nest such as caring for the brood, cleaning the nest, etc. In the dyadic encounters, on the other hand, the ants are isolated from all other duties in the colony and are therefore more engaged in mutual investigation and physical contact. Although the amount of hydrocarbons transferred to the postpharyngeal gland was relatively low, it might still be sufficient to update the gestalt colony odor in rela-

tively small size colonies, such as is characteristic for *P. apicalis* (77.9 ± 6.7 individuals, mean ± SEM, $N = 113$) (Fresneau, 1994). Thus the involvement of the postpharyngeal gland in achieving a uniform colony odor in this species may still be significant, and it can still be considered as a gestalt organ.

The chemical identity of nestmate recognition cues in ants is still unclear. Although multiple circumstantial evidence suggests that hydrocarbons act as nestmate recognition cues in ants (Howard, 1993), experiments with the cuckoo ant *Leptothorax kutteri* and its host *L. acervorum* indicated that fatty acids might also be involved, at least in some ant species (Franks et al., 1990). In *P. apicalis* we have shown that the postpharyngeal gland processes polar lipids in the same manner as hydrocarbons. Thus, irrespective of the identity of the lipid substances that operate in nestmate recognition, the involvement of the postpharyngeal gland in their distribution among nestmates in *P. apicalis* is compatible with its function as a gestalt organ.

Although the number of ant species investigated to date is still limited, based on a comparison of the extent of hydrocarbon exchange between nestmates (Figure 3) (Soroker et al., 1994, 1995a; Vienne et al., 1995), we can hypothesize on the evolution of the postpharyngeal gland as a gestalt organ in ants. The level of hydrocarbon transfer in *P. apicalis* is the lowest of all the species depicted in Figure 3 (roughly 3% vs. 13–25%). We attribute this low level to the lack of trophallaxis in this species and a complete reliance on allogrooming for chemical exchange. Nonetheless, the fact that the postpharyngeal gland in *P. apicalis*, a member of such a relatively primitive subfamily as Ponerinae, is involved in the gestalt formation, indicates that this mechanism appeared early in the evolution of Formicidae. We suggest that once recognition systems evolved from individual odors to a gestalt odor, the need for an efficient distribution of recognition cues became a major selective pressure directing the evolution of many social behaviors. In the primitive ponerine ants, colonies are rather small (Table 3-2 in Hölldobler and Wilson, 1990) and may rely on individual recognition as a mechanism for nestmate recognition. In evolutionary perspective, as the number of individuals in the colony rose (e.g., *P. apicalis*) individual recognition became impossible, resulting in the evolution of gestalt colony odor. The gestalt was achieved by acquiring the colony odor through friction between individuals and passive transfer of chemicals between the cuticles. Concomitantly, the postpharyngeal gland evolved as a storage organ, facilitating refreshment of the individual odor through self-grooming. We argue that once this glandular function was established, allogrooming evolved to facilitate the mixing of individual odors to a unified colony odor. As populations became larger, selective pressure on the development of an efficient means of cue transfer may have directed the evolution of trophallaxis. Early signs for the evolution of trophallaxis in the Formicidae have been detected already in a few ponerine species (Hashimoto et al., 1995; Liebig et al., 1997). Observations that the

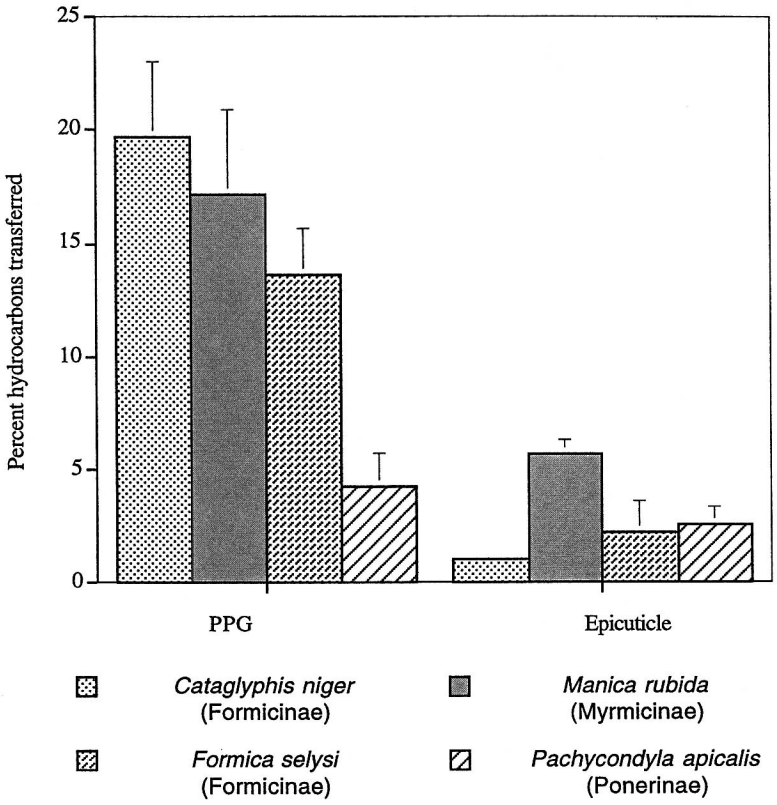

FIG. 3. Comparative analysis of hydrocarbon transfer between control ant workers belonging to different subfamilies. Data are relative amount of labeling in recipients' tissue, mean percent ± SEM of a minimum of seven replicates. Results on transfer in *Manica rubida* and *Formica selysi* are taken from Vienne et al. (1995). Results on transfer in *Cataglyphis niger* are from Soroker et al. (1995a).

liquid transferred consists of small droplets in *P. coarctata* (Liebig et al., 1997) may imply that the droplets are comprised of postpharyngeal gland secretion rather then food. Reports that some trophallactic interactions do not always involve a significant food exchange (Heinze, 1996), indicate that this behavior has evolved for improving cue transfer. Trophallaxis also evolved as an act of appeasement for settling aggressive encounters between nestmates and aliens (reviewed by Hölldobler and Wilson, 1990; Liebig et al., 1997) and for food exchange. Although all these functions are evidently not mutually exclusive, we suggest that the demand for efficient colony closure in large colonies necessitates the evolution of mechanisms for exchange of recognition cues. We believe

that this requirement was the principal selective force for the evolution of trophallaxis and that food exchange was a later adaptation.

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SPECIES-SPECIFIC VOLATILE SUBSTANCES IN THE VENOM SAC OF HOVER WASPS

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Abstract—Analyses of the volatile compounds in the venom sacs of seven species of stenogastrine wasps, belonging to three different genera, have revealed a mixture of linear alkanes and alkenes, with a chain length ranging from C_{11} to C_{17} in all the species. Among conspecifics, the composition of the mixture was consistent, while clear differences have been found between different species. Venom glands of some species also contained oxygenated compounds and, in one species, some pyrazines. Most of these compounds were found to be species-specific but were not always found in every individual of a species. In the genus *Parischnogaster*, *P jacobsoni* and *P. mellyi* showed the presence of some spiroacetals that were not found in *P. alternata* and *P. striatula*. The possible functions of the venom sac volatiles in the biology of the Stenogastrinae are discussed.

Key Words—Stenogastrinae, Vespidae, venom, pheromones, hydrocarbons, spiroacetals.

INTRODUCTION

Social wasps are widely known for deterring enemies through the use of their venom, and efforts have been made to analyze the proteinaceous components

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of the venom of several species (Edery et al., 1987; Nakajama, 1986; Schmidt, 1982, 1990). [For an anatomical description of the venom apparatus see Landolt and Akre (1979), Billen (1987), and Downing (1991).] In some Vespidae, as in several species of Apidae and Formicidae, volatiles have been found in the venom together with proteins, and in some species they have been shown to have a pheromonal function.

Results of chemical analyses of volatile components in the venom have been reported for *Vespa orientalis* L. (Saslavasky et al., 1973), *Vespula vulgaris* (L.) (Aldiss, 1983, quoted by Landolt et al., 1995), *Vespa crabro* L. (Veith et al., 1984), *Vespula squamosa* (Drury) (Heath and Landolt, 1988), and *Vespula maculifrons* (Buysson) (Landolt et al., 1995), all species for which the presence of an alarm pheromone in the venom has been demonstrated in bioassays [See also Maschwitz (1964) and Moritz and Bürgin (1987) for *Vespula vulgaris*; Landolt and Heath (1987) for *V. squamosa*; and Moritz and Bürgin (1987) for *Vespa crabro*).]

Methylbutanols, methylbutenols, and their corresponding acetic esters have been found in the venom sac of *Vespa crabro* and one of the identified alcohols, 2-methyl-3-buten-2-ol, has been shown to elicit alarm during behavioral experiments (Veith et al., 1984).

The amide, *N*-3-methylbutylacetamide, structurally related to 3-methylbutyl acetate, has been found in *Vespula vulgaris* and *V. squamosa* venom sacs and in the sting apparatus of *V. maculifrons*. This substance was tested in the last two species and shown to elicit alarm behavior (Heath and Landolt, 1988; Landolt et al., 1995). However as unnaturally high doses are required to elicit alarm in *V. maculifrons*, it has been suggested that other volatiles from the sting apparatus may also act as alarm pheromones (Landolt et al., 1995).

For *Vespa orientalis*, the presence of a series of alkanes with chain length varying from C_{10} to C_{34} has been reported in the venom, but neither the precise compounds nor their proportions have been given. Behavioral experiments have shown that linear alkanes varying from C_{11} to C_{14} have a weak alarm-eliciting effect (Saslavasky et al., 1973).

It appears that results have never been reported for chemical analyses of volatiles from the venom of species belonging to the subfamily Polistinae or Stenogastrinae. However, behavioral experiments have shown the presence of an alarm pheromone in the venom of three New World *Polistes* species, *P. canadensis* (L.) (Jeanne, 1982), *P. exclamans* Viereck, and *P. fuscatus* (F.) (Post et al., 1984) and in a swarming species, *Polybia occidentalis* (Olivier) (Jeanne, 1981). In *P. fuscatus*, *P. exclamans*, and in *Belonogaster petiolata* (Degeer), venom has also been shown to have a male-attracting effect (Post and Jeanne, 1983, 1984; Keeping et al., 1986); but, at least for the *Polistes*, the effect is not species-specific.

Compared with the Polistinae and Vespinae, the colonies of Stenogastrinae contain only a few individuals (see Turillazzi, 1996). All the species of this subfamily, which shows the most primitive social organization among the social wasps (Turillazzi, 1989, 1991, 1996), live in the South East Asian rain forests.

Even though they have an effective sting and an apparently functional venom apparatus (Turillazzi, 1989), Stenogastrinae normally show little aggression towards predators (Turillazzi, 1991) and, at least for man, their sting causes much less pain than that of the other social wasps (Turillazzi, 1990). Nothing is known about the chemical composition of venom proteins in the Stenogastrinae.

When disturbed on the nests, these wasps drop passively from it, except when the disturbance is caused by conspecifics or ants (Turillazzi, 1991). As pointed out by Turillazzi (1991), this behavior may make it difficult for a predator to detect the exact position of the nest and, together with the mimesis of the nests, may contribute to the defense against predators.

The first aim of the present work was to examine whether volatile compounds were present in Stenogastrinae wasps; seven different species were analyzed and the compositions of their mixtures of volatiles were compared to verify if they were species-specific.

METHODS AND MATERIALS

Specimens Collection. The venom sacs of the following stenogastrine species were analyzed for volatile chemicals: *Eustenogaster fraterna* (Bingham), *Liostenogaster vechti* Turillazzi, *L. flavolineata* (Cameron), *Parischnogaster striatula* (Du Buysson), *P. alternata* Sakagami, *P. jacobsoni* (Du Buysson) and *P. mellyi* (Saussure). All samples were collected in peninsular Malaysia, in Pahang State, during September and/or October of 1993, 1995, and 1996 (Table 1). Some of the specimens were collected at various points on Fraser's Hill, others at various localities along the old road that joins Kuala Lumpur to Genting Highland, at about 30 km (locality A), 40 km (locality B), and 50 km (locality C) from Kuala Lumpur.

All insects were killed by freezing soon after capture and then dissected in distilled water under a binocular microscope. For each individual either the venom sac or the whole sting apparatus was placed in a glass capillary (2 mm diameter $\times$ 20 mm long), which was then sealed in a flame. Capillaries were stored at $-20°C$ until analysis. During the dissections, almost all the specimens of *P. striatula*, *P. alternata*, and *L. vechti* collected in 1996 and those of *L. flavolineata* collected in 1995 (Table 1) were classified into small, medium, and large classes, according to their ovarian development.

TABLE 1. NUMBER OF INDIVIDUALS, NUMBER OF NESTS FROM WHICH THEY ORIGINATED, NUMBER OF INDIVIDUALS OF KNOWN OVARIAN DEVELOPMENT (OvD) AND LOCALITY WITH YEAR OF COLLECTION FOR THE SEVEN SPECIES INVESTIGATED

Species	Number			Locality and year
	Individuals	Nests	OvD	
Eustenogaster fraterna				Fraser's Hill
	5	2	0	September and October 1995
Liostenogaster flavolineta				Locality B
	5	unknown		September and October 1993
	13	4	8	September and October 1995
Liostenogaster vechti				Fraser's Hill
	6	more than 1	0	September and October 1995
	7	4	7	October 1996
Parischnogaster striatula				Fraser's Hill
	5	3	0	September and October 1996
	7	4	7	October 1996
				Locality B
	1	1	1	October 1996
Parischnogaster alternata				Locality A
	3	1	0	September and October 1995
	6	2	5	October 1996
				Locality B
	1	1	1	October 1996
Parischnogaster jacobsoni				Fraser's Hill
	5	3	0	September and October 1995
Parischnogaster mellyi				Locality C
	4	1	0	September and October 1995
				Locality A
	3	2	0	October 1996

GC-MS Analyses. Samples were analyzed by gas chromatography–mass spectrometry (GC-MS), using the solventless analysis technique described by Morgan and Wadhams (1972) and Morgan (1990). Analyses were carried out on a Hewlett Packard 5890 chromatograph connected to a Hewlett Packard 5970 mass spectrometer (quadrupole mass spectrometer using 70 eV electron impact ionization). Both instruments were controlled by a HP59970C Chemstation. The gas chromatograph was equipped with a fused silica capillary column (12 m × 0.32 mm) coated with a film of dimethylsiloxane (thickness of 0.33 μm) and provided with a precolumn (1.5 m long) and a postcolumn (1.5 m long) of deactivated silica tubing (0.25 mm ID). Helium was used as carrier gas. The

mass spectrometer was set to scan at *m/z* 30–500. Each glass capillary was placed in the injector, heated, and crushed after 2 min. The chromatography started simultaneously.

In the analyses the injector port was set at 160°C. The temperature program of the oven was maintained at 30°C for 3 mins, then raised at 7°C/min to 250°C and held there for 10 min. The transfer line was at 250°C. All analyses were carried out in a splitless mode.

Identification of compounds was based on comparison of mass spectra of natural compounds with those reported in the literature (Wiley library) and on comparison of the retention times and of the mass spectra of standard compounds. Mass spectrometric fragmentation patterns of spiroacetals have been described by Francke et al. (1979a, 1980).

Methylthiolation of Double Bonds. P. alternata and *P. striatula* contained pentadecene as the major compound of their secretion. For the two species, an extract in hexane (about 40 μl) from the venom sac of a few individuals was prepared in a Wheaton-Keele Microreactor (Wheaton Scientific, Millville, New Jersey). Dimethyldisulfide adducts were prepared as described by Billen et al. (1986), except that the reactions were carried out at 80°C overnight and under a nitrogen atmosphere, with complete exclusion of oxygen, which gave an almost quantitative yield of the adducts. The products were analyzed by GC-MS under the condition listed above, except that the oven temperature was programmed from 100°C to 250°C at 5°C/min and the injector port was set at 200°C. The positions of the double bonds were deduced from the prominent fragment ions in the mass spectra of the adducts.

Data Analyses. All the chromatograms were integrated, except those of the five *L. flavolineata* specimens collected in 1993, and, for each sample, the percentage of each hydrocarbon was calculated.

Because the compositions of the secretions of *P. alternata* and *P. striatula* seemed to be similar, the percentages of each compound were transformed by arcsin [arcsin $\sqrt{(\text{percentage}/100)}$] and the values obtained for the two groups ($N = 10$ and $N = 11$, respectively) were compared using the t test.

The quantity of hydrocarbons in the samples was estimated by comparison of peak area with that of a known amount of a standard solution of hexadecane in hexane (100 ng/μl).

RESULTS

The venom sacs of all seven species contained a mixture of linear hydrocarbons, the chain length of which varied from 11 to 17 carbon atoms (Table 2). The amount varied from about 187 ng in *E. fraterna* to about 1500 ng in *P. jacobsoni* (Table 2). However, the standard deviation of the amount is very

high for all species. The composition of the mixture of hydrocarbons was rather constant among individuals belonging to the same species. With the exception of *P. striatula* and *P. alternata*, which looked similar at first sight, each species had a recognizably different composition.

In *Eustenogaster fraterna*, the venom sac mainly contained C_{13}–C_{17} alkenes, with pentadecene clearly being the major compound (Table 2). Traces of hydrocarbons with a higher molecular weight have been found in all samples, but these compounds are likely to be due to contamination from the sting cuticle or from the Dufour's gland. Hydrocarbons ranging from C_{21} to C_{33} have, in fact, been found in the Dufour's gland (Keegans et al., 1992b, 1993) of three stenogastrinae species.

Three samples of *E. fraterna* also showed traces of ethyl oleate (Table 3), which was identified by its retention time and mass spectrum. In all samples we found an unidentified sesquiterpene with a molecular weight of 236, eluting shortly after heptadecane. In all samples, the area given by this terpene was similar to that of heptadecene.

In both the *Liostenogaster* species, the saturated hydrocarbons were clearly predominant over the unsaturated ones (Table 2).

The venom sacs of *L. flavolineata* showed a mixture of C_{11}–C_{17} hydrocarbons, mainly alkanes. In 16 of 17 samples (no secretion was found in one of the 18 sacs), tridecane was the most abundant compound (Table 2), followed by pentadecane, which was the compound in greatest quantity in this last sample. Again, traces of some hydrocarbons with a higher molecular weight were found in all samples, and traces of ethyl stearate, ethyl oleate, and ethyl palmitoleate (these last two identified by their retention times and mass spectra) were found in a few samples (Table 3).

In the samples of *L. vechti* ($N = 13$) hydrocarbons ranged from tridecane to heptadecane (Table 2). In all samples, the main compound was pentadecane, which constituted, on average, 86% of the total. Some samples also contained traces of higher-molecular-weight hydrocarbons, and the quantity of these compounds was higher in the samples containing the whole sting apparatus ($N = 3$). Two unknown isomeric monoterpene aldehydes (molecular weight 152), eluting with a retention time close to that of decane were found, sometimes only in traces, in seven samples (Table 3). In the three samples in which the quantity of these unidentified compounds was highest, traces of some monoterpene hydrocarbons were also found. Three of them were identified as *cis*- and *trans*-ocimene and limonene.

Parischnogaster mellyi venom sacs also contained C_{11}–C_{17} hydrocarbons (Table 2). In five of seven samples, the main compound was tridecane, followed by pentadecane and pentadecene in almost equal amounts. In the other two samples, pentadecene and pentadecane occurred in higher amounts than tride-

TABLE 2. AVERAGE PERCENTAGE (± SD) OF EACH HYDROCARBON AND AVERAGE TOTAL AMOUNT (± SD) OF HYDROCARBONS IN VENOM SAC OF SEVEN SPECIES INVESTIGATED[a]

Hydrocarbon	*E. fraterna* (*N* = 5)	*L. flavolineata* (*N* = 13)	*L. vechti* (*N* = 13)	*P. mellyi* (*N* = 7)	*P. jacobsoni* (*N* = 5)	*P. striatula* (*N* = 11)	*P. alternata* (*N* = 10)
n-C_{11}		4.6 ± 2.5		3.2 ± 2.6	**19.1 ± 2.8**	7.8 ± 3.0	6.3 ± 3.6
$C_{12:1}$						traces	traces
n-C_{12}		2.5 ± 0.9		0.8 ± 0.7	2.8 ± 0.8	1.2 ± 0.5	0.8 ± 0.5
$C_{13:1}$	**13.0 ± 2.8**	0.7 ± **0.3**		3.6 ± 2.7	1.9 ± 0.9	**14.4 ± 4.3**	**16.4 ± 5.1**
n-C_{13}	8.7 ± 2.0	**66.7 ± 13.3**	2.9 ± 2.1	**34.9 ± 17.5**	**60.1 ± 7.3**	**26.2 ± 3.4**	**17.4 ± 7.1**
$C_{14:1}$	1.9 ± 1.1			traces		2.2 ± 0.7	2.4 ± 1.1
n-C_{14}		1.09 ± 0.5	4.3 ± 2.3	1.0 ± 0.5	1.1 ± 0.3	traces	traces
$C_{15:1}$	**70.8 ± 8.7**	traces	2.8 ± 2.0	**24.2 ± 6.5**	1.7 ± 1.4	**43.0 ± 7.7**	**47.5 ± 12.3**
n-C_{15}	2.2 ± 0.6	**23.1 ± 13.7**	**86.0 ± 6.3**	**29.6 ± 14.1**	**13.2 ± 4**	4.0 ± 1.7	7.2 ± 3.1
n-C_{16}			traces				
$C_{17:2}$				traces		traces	traces
$C_{17:1}$	3.3 ± 3.9	traces	1.3 ± 2.0	1.3 ± 1.4	traces	0.6 ± 0.8	1.4 ± 1.1
n-C_{17}		traces	2.5 ± 2.1	traces	traces		
Total quantity (ng)	187 ± 240	381 ± 316	566 ± 624	432 ± 592	1504 ± 1129	694 ± 832	774 ± 905

[a] Percentages above 10 are reported in bold; percentages lower than 0.5 are reported as traces.

TABLE 3. NONHYDROCARBON COMPOUNDS AND NUMBER OF SAMPLES CONTAINING THESE COMPOUNDS FOR EACH SPECIES

Compounds	No. of samples/ No. analysed	Species
Unidentified terpenoid	5/5	*E. fraterna*
Unidentified compound I	7/13	*L. vechti*
Unidentified compound II	7/13	*L. vechti*
1-Hexanol	2/7	*P. mellyi*
2,5-Dimethylpyrazine	2/7	*P. mellyi*
Trimethylpyrazine	2/7	*P. mellyi*
(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**1**)[a]	5/7	*P. mellyi*
	5/5	*P. jacobsoni*
(*E*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**2**)[a]	1/7	*P. mellyi*
	4/5	*P. jacobsoni*
(*Z*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**5**)[a]	4/5	*P. jacobsoni*
(*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**)[a]	1/7	*P. mellyi*
	4/5	*P. jacobsoni*
(*E*,*E*)-7-Ethyl-2-methyl-1,6-dioxaspiro[4.5]decane (**4**)[a]	1/7	*P. mellyi*
	4/5	*P. jacobsoni*
(*E*,*Z*)-7-Ethyl-2-methyl-1,6-dioxaspiro[4.5]decane (**6**)[a]	4/5	*P. jacobsoni*
Ethyl palmitoleate	1/13	*L. flavolineata*
	2/7	*P. mellyi*
Ethyl oleate	3/5	*E. fraterna*
	4/13	*L. flavolineata*
	2/7	*P. mellyi*
Ethyl stearate	1/13	*L. flavolineata*
Hexadecyl acetate	4/7	*P. mellyi*
(*Z*)-9-Octadecenyl acetate	4/7	*P. mellyi*

[a]Each spiroacetal corresponds with the numbered structure in Figure 1 and the text.

cane. 1-Hexanol, 2,5-dimethylpyrazine, and trimethylpyrazine were found in two samples as minor components (Table 3). Four samples contained hexadecyl acetate and (*Z*)-9-octadecenyl acetate (identified by comparison with the mass spectra of the standard) in quite large amounts and other acetates in traces. Two of these samples also contained ethyl oleate and ethyl palmitoleate. Five of the samples contained, at least in traces, a spiroacetal, identified as (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**1**) (Figure 1). In one of the samples containing the highest amount of secretion, we also found traces of the *E*,*Z* isomer of this compound (**2**) and traces of (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**) coeluting with (*E*,*E*)-7-ethyl-2-methyl-1,6-dioxaspiro-[4.5]decane (**4**) (Figure 1).

The major compound of *P. jacobsoni* was tridecane, followed by undecane

and pentadecane (Table 2). Spiroacetal (**1**) was found in all samples in similar amounts to tetradecane. Four of five samples also contained traces of spiroacetals (**2**, **3**, and **4**) plus (*Z*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**5**) and (*E*,*Z*)-7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane (**6**) (Figure 1).

In 10 of the 11 samples of *P. striatula* (no volatiles were found in two of the 13), pentadecene was the main compound, followed by tridecane (Table 2). Most of the samples also showed traces of hydrocarbons with a higher molecular weight; their amount was higher in the samples containing the whole sting apparatus ($N = 3$). From the mass spectra of the dimethyldisulfide adducts, we could identify the major alkenes as 7-pentadecene and 5-tridecene.

The samples of *P. alternata* seemed to be very similar to those of *P. striatula*, including the presence of 7-pentadecene and 5-tridecene (Table 2).

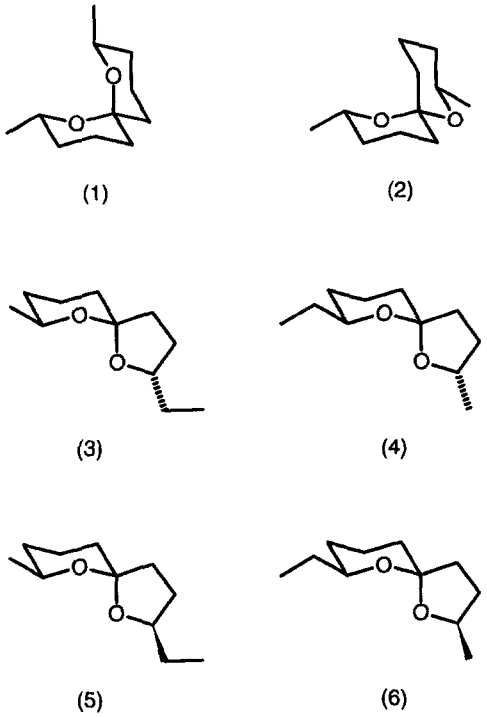

FIG. 1. Chemical structure of the spiroacetals from the venom sac of *Parischnogaster mellyi* and *P. jacobsoni*; (**1**) (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane; (**2**) (*E*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane; (**3**) (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro-[4.5]decane; (**4**) (*E*,*E*)-7-ethyl-2-methyl-1,6-dioxaspiro[4.5]; (**5**) (*Z*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane; and (**6**) (*E*,*Z*)-7-ethyl-2-methyl-1,6-dioxaspiro[4.5]decane.

However, the t test on the transformed percentages of the compounds [arcsin $\sqrt{(\text{percentage}/100)}$] gave significant differences between the percentages of three compounds in the two species ($df = 19$: for n-C_{13}, $t = 3.65$, $P < 0.01$; for n-C_{15}, $t = 2.95$, $P < 0.01$; and for $C_{17:1}$, $t = 2.1$, $P < 0.05$).

Spearman tests between the total amount of hydrocarbons and the class of ovarian development were performed for *P. striatula*, *P. alternata*, *L. vechti*, and *L. flavolineata* (Methods and Materials; Table 1), and none gave a statistically significant result. Moreover, no relationship between ovarian state and the presence of nonhydrocarbon compounds was observed for these species.

DISCUSSION

The venom sacs of the seven species studied contain hydrocarbon mixtures in species-specific compositions. The presence of other compounds seems also to be species-specific. This is the case for the two unidentified compounds found in *Liostenogaster vechti*, for the unidentified compound found in *Eustenogaster fraterna*, and for the acetates found in *Parischnogaster mellyi*. Two pyrazines and hexanol have been found only in this last species, but, since only two of seven samples contained these compounds, their presence needs to be confirmed.

(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane and three isomeric compounds present in lower amounts have been found both in *P. jacobsoni* and *P. mellyi*. None of these spiroacetals or other nonhydrocarbon compounds were found in *P. alternata* and *P. striatula*.

Despite being characteristic of only one species, with the exception of the spiroacetals, the presence of all these nonhydrocarbon compounds is quite variable among conspecifics and some indiviuals lacked these compounds completely.

Spiroacetals are widespread in insects and have been found in Hymenoptera, Coleoptera, Diptera and Hemiptera; (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**1**) seems to be the most common. This compound was first identified in the mandibular gland secretion of solitary bees belonging to the genus *Andrena* (Francke et al., 1980; Bergström et al., 1982). Later it was also found in the cephalic secretion of the anthophoride bees *Epeolus cruciger* Pz. and *E. variegatus* (L.) (Tengö et al., 1982), in the mandibular glands of the parasitoid wasp *Megarhyssa nortoni nortoni* Cresson (Davis and Madden, 1985), in the head extracts of other ichneumonids such as *Meringopus titillator* (L.) and *Banchus compressus* F. (Francke and Tengö, unpublished), as well as in the abdominal extracts of the paper wasps *Polistes dominulus* (Christ) and *P. gallicus* L. (Lübke, 1990). In addition compound **1** was identified in the mandibular glands of two ponerine ant species of the genus *Diacamma* (Keegans, 1992). Apart from Hymenoptera, compound **1** is a component of the

defensive secretion of rove beetle *Ontholestes murinus* (L.) (Dettner and Schwinger, 1986) and of the rectal glands of male Australian fruit flies *Dacus cucumis* (French) and *D. halfordiae* (Tryon) (Kitching et al., 1986) and of *Bactrocera* spp. fruit flies (Fletcher and Kitching, 1995). In addition, compound **1** was found to be present in the aposematic shield bug *Cantao parentum* (White) (Moore et al., 1994). In many cases, compound **1** was accompanied by small amounts of its *E,Z* diastereomer (2).

Some spiroacetals, including the ones found in *Parischnogaster mellyi* and *P. jacobsoni* and indicated as compounds **3–6**, have been found in the abdominal extracts of *Vespula* (quoted as *Paravespula vulgaris*), *V. germanica* (quoted as *P. germanica*), and *Dolichovespula saxonica* (F.) by Francke et al. (1978, 1979b). These authors reported that dead wasps treated with these compounds were less attacked by conspecifics than untreated ones. Dependent on concentrations, a repellent or attractive effect of these spiroacetals has been reported by Weston et al. (1997). Spiroacetals **3** and **6** were also found to be present in solitary bees of the genus *Andrena* (Bergström et al., 1982).

Among the social wasps, Stenogastrinae is the least well known subfamily and, as pointed out by Turillazzi (1991), the systematics of this group, especially that of the genera *Eustenogaster*, *Liostenogaster*, and *Parischnogaster*, needs revision. Differences in some morphometric, morphological, and ethological characters have sometimes raised doubts about the existence of undescribed species or of subspecific taxa. An example has been recently reported by Coster-Longman and Turillazzi (1995) for *P. alternata*. The analysis of volatile compounds from exocrine glands may be useful as a taxonomic clue in Stenogastrinae, as already reported for other social insects (see, for example, Keegans et al., 1992a; Morgan, 1992).

The biological function of the volatiles found in the venom sac is unclear. Preliminary experiments carried out in nature on *L. flavolineata* and *P. striatula* did not resolve the issue of whether venom sac volatiles induce alarm behavior or dropping behavior from the nest, which has been mentioned earlier. Further experiments on *P. mellyi* and *P. alternata* kept in captivity seem to exclude the existence of a form of alarm communication in these wasps (Landi et al., 1998).

For some species of ants, *n*-decane and *n*-undecane have been shown to act as alarm pheromones (see Hölldobler and Wilson, 1990), and for the subfamily Formicinae, where *n*-undecane is often the major component of the Dufour's gland secretion, it has also been hypothesised that this compound may disrupt the olfactory acuity of enemies (Blum and Brand, 1972; Blum, 1981). A behavior in which the extremity of the abdomen is pointed against the enemies in aggressive encounters with conspecifics or with ants trying to enter the nest has been noted in many species of Stenogastrinae (Turillazzi, 1991). However, spraying from the abdomen has never been reported.

As already mentioned, the venom of some Polistinae has an unspecific

male-attracting effect (Post and Jeanne, 1983, 1984; Keeping et al., 1986). If the volatiles we found in the venom of the Stenogastrinae had a male-attracting function, one would not expect to find volatiles in females engaged in egg-laying, which are probably already inseminated. In contrast, volatiles, at least hydrocarbons, have been found in almost all the specimens analyzed.

So far none of the functions hypothesized for these volatile compounds seems to be supported either by experimental data or by observation of the behavior; further behavioral experiments are needed.

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SEX PHEROMONES OF *Thysanoplusia intermixta* and *T. orichalcea*: IDENTIFICATION AND FIELD TESTS

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Abstract—*Thysanoplusia intermixta*, which inhabits the temperate zone in Japan, is a defoliator of plants in the family Compositae, Apiaceae, and others. By GC-MS analysis, (*Z*)-7-dodecenyl acetate, (*Z*)-7-dodecen-1-ol, (5*E*, 7*Z*)-5,7-dodecadienyl acetate and (5*E*,7*Z*)-5,7-dodecadien-1-ol were identified from a pheromone gland extract of the virgin females in a ratio of 18:3:100:13. A lure baited only with the major diene acetate could attract male moths of *T. intermixta* in the field. Other minor components had a synergistic effect on the attraction of the diene acetate. The same four components were included in a very different ratio of 100:6:11:1 in the gland extract of *Thysanoplusia orichalcea*, a closely related subtropical species. The monoene and diene acetates are essential for field attraction, and a 100:11 mixture is an optimum lure for *T. orichalcea* males. However, this mixture, including the diene acetate as a minor component, captured very few *T. intermixta* males, suggesting the possibility that these two *Thysanoplusia* species are reproductively isolated from each other by pheromonal communication.

Key Words—Sex pheromone, attractant, *Thysanoplusia intermixta*, *Thysanoplusia orichalcea*, Lepidoptera, Noctuidae, Plusiinae, (5*E*,7*Z*)-5,7-dodecadienyl acetate, (*Z*)-7-dodecenyl acetate, reproductive isolation.

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INTRODUCTION

Thysanoplusia intermixta (Warren) and *Thysanoplusia orichalcea* (F.) (Lepidoptera, Noctuidae, Plusiinae) are defoliators of the plants in the family Compositae, Apiaceae, and others. In Japan, the former, a temperate-zone species, is distributed in the northern areas from Yakushima Island, and the latter subtropical species is found on Okinawa Island. However, it is still unknown whether the distribution areas of the two species are separated or overlapping. Although their outward appearances are very similar, some physiological and genetic differences are known (Nomura, unpublished). We are now studying their sex pheromones in order to define their sexual communication, one of their most important physiological and ecological aspects. In addition identification of the pheromone may lead to a monitoring tool for pest management. This paper deals with the structural identification of pheromone components and field evaluation with synthetic pheromones.

METHODS AND MATERIALS

Insects and Pheromone Extracts. Larvae of *T. intermixta* and *T. orichalcea* were collected in an edible burdock field in Chiba Prefecture and Okinawa Island, respectively. They were reared singly on an artificial diet prepared mainly from kidney beans for *Acanthoplusia agnata* (Kawasaki et al., 1987) at 20°C and on a 16L:8D cycle. Abdominal tips containing the pheromone glands were removed from 2 to 3 day-old female moths during scotophase and soaked in *n*-hexane for 15 min to extract pheromone components.

GC-MS Analysis. Electron impact (EI) GC-MS was performed with a Jeol JMS-SX 102A mass spectrometer with DB-23 and DB-1 capillary columns (0.25 mm ID × 30 m, J&W Scientific). The temperature program of the DB-23 column, which was used for analysis of natural pheromones, was 80°C for 1 min, programmed to rise at 8°C/min to 220°C. The program for the DB-1 column used for ozonolysis products was 50°C for 1 min, then rising at 50°C/min to 150°C, and then rising 8°C/min to 300°C. The ionization voltage was 70 eV, and the ion source temperature was 240°C. The spectra (*m/z* 50–800) were measured twice per second in a cyclic scan mode lasting from 5 to 25 min after sample injection.

Ozonolysis of a Pheromone Component. A crude extract of 100 abdominal tips of *T. intermixta* was injected into an HPLC column (ODS-5, 8 mm ID × 25 cm, Senshukagaku, Tokyo, Japan) with acetonitrile–water (90:10 v/v) as the mobile phase at a flow rate of 1.5 ml/min to purify the major pheromone component (diene acetate, R_t 7.5 min), which was detected by a UV detector operated at 235 nm. This purified component was dissolved in *n*-hexane (50 μl)

and cooled in a Dry Ice–acetone bath. Ozone was added for 2 min, and an aliquot of this solution was analyzed by GC-MS to determine the thermal decomposition products derived from the ozonide of the diene acetate.

Chemicals. Syntheses of pheromone components and other authentic compounds included monoene acetates and alcohols (Ando et al., 1977) and diene compounds (Ando et al., 1982, 1985a). For field tests, we used monoenes and dienes with a purity greater than 98% and 95%, respectively.

Field Evaluation. Attractive activity of synthetic pheromone was examined in a temperature zone (Matsudo-shi in Chiba Prefecture) for *T. intermixta* and in a subtropical zone for *T. orichalcea* (Ishigaki Island in Okinawa Prefecture). Cone traps (15 cm ID × 30 cm), hand-made with a cylindrical plastic container having a hole at the bottom (15 cm ID × 9 cm), plastic sheet and funnel-shaped net (Kawasaki and Sugie, 1990), were set at a height of 1.2 m in fields where larvae were collected. Synthetic chemicals were applied to natural rubber septa (8 mm OD, white rubber, Aldrich) as dispensers, and one lure was placed on the net which was inserted into the plastic container through the hole for capturing the attracted moths, and used without renewal during the experiments. The sex, species, and number of moths captured were recorded every 10 days.

RESULTS

Mass Spectra of Compounds in Pheromone Extracts. Female sex pheromone extract from *T. intermixta* was analyzed by GC-MS with a polar capillary column (DB-23) under a programmed temperature that covered the volatility range of C_{10} to C_{16}-chain alcohols and their derivatives (acetates and aldehydes). An informative part of the total ion chromatogram (TIC) of one female equivalent injection is shown in Figure 1A. Acetates and alcohols with a straight chain of the same length produced the characteristic fragment ions ($[M\text{-}AcOH]^+$ and $[M\text{-}H_2O]^+$) with the same mass number after the elimination of acetic acid and water, respectively, from molecular (M^+) ions. While the monoene compounds scarcely showed M^+ ions, the diene compounds produced remarkable M^+ ions. Figure 1B–E shows mass chromatograms from C_{12} compounds. We monitored M^+ ions at *m/z* 224 for diene acetates and at *m/z* 182 for diene alcohols and fragment ions at *m/z* 166 for monoene acetates and alcohols and at *m/z* 164 for diene acetates and alcohols. Monitoring of an ion at *m/z* 61 (Figure 1F) was performed to detect acetates. As shown in these figures, the pheromone extract included four C_{12}-chain compounds, monoene acetate (component I, R_t 10.35 min), monoene alcohol (component II, R_t 10.59 min), diene acetate (component III, R_t 11.95 min), and diene alcohol (component IV, R_t 12.28 min), in a ratio of 18:3:100:13. The peak height of component III in the TIC (Figure 1A) indicates that one female possessed ca. 15 ng of this major

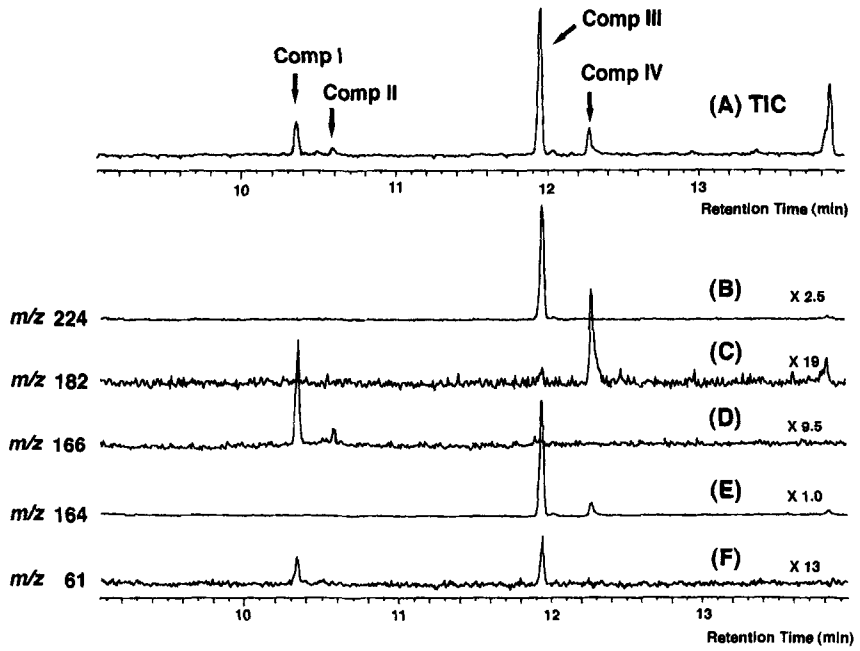

FIG. 1. GC-MS analysis of the pheromone gland extract (one female equivalent) of *Thysanoplusia intermixta*. (A) Total ion chromatogram (TIC); (B–F) mass chromatograms monitoring the fragment ions at *m/z* 224, 182, 166, 164, and 61. Component I = Z7-12:OAc, II = Z7-12:OH, III = E5,Z7-12:OAc, and IV = E5,Z7-12:OH.

component. The pheromone extract of *T. orichalcea* was analyzed under the same GC-MS conditions. Four components with the same mass chromatographic behavior as components I–IV were detected, but in a different ratio: 100:6:11:1, as shown in the TIC of Figure 2. This extract contained ca. 20 ng/female of component I. Even with the injection of 10 female equivalents of extract of each species, no other pheromone candidates with a different chain length or with an aldehyde functional group were detected with the mass chromatograms.

Identification of Monoene Components I and II. Figure 3A and B shows the mass spectra of the monoene acetate and alcohol of *T. orichalcea*, respectively. While an analyzable mass spectrum of the smallest component of *T. intermixta*, the monoene alcohol (component II), was not recorded, a well-defined spectrum of monoene acetate (component I) of *T. intermixta* was obtained, and it was very similar to that of *T. orichalcea*. The spectra of components I and II are in good agreement with those of synthetic (Z)-7-dodecenyl acetate (Z7-12:OAc) and (Z)-7-dodecen-1-ol (Z7-12:OH), respectively, as

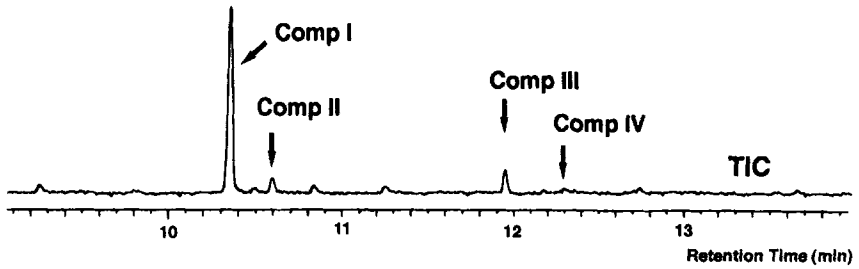

FIG. 2. GC-MS analysis (TIC) of the pheromone gland extract (one female equivalent) of *Thysanoplusia orichalcea*. Component I = *Z*7-12:OAc, II = *Z*7-12:OH, III = *E*5, *Z*7-12:OAc, and IV = *E*5,*Z*7-12:OH.

part of a series of synthetic monoenes with double bond at a higher position than position 5. To confirm the double-bond position quantitatively, the fitness indexes proposed by Kuwahara et al. (1986) were examined for these natural monoene components. This index was calculated by dividing the absolute difference between the intensity ratio of selected fragmentation pairs of a synthetic isomer and the corresponding ratio of the natural pheromone by the latter. Table 1 shows the average values of the indexes computed for each natural component with the relative intensities of 13 fragment ions of the seven synthetic positional isomers. For each of the two components, the smallest value of the average fitness index was obtained with the chemical unsaturated at position 7 indicating the best fit to the natural pheromone, even though spectral similarity of monoenes unsaturated at positions 7 and 8 was observed. Furthermore, comparison with the R_ts of the synthetic compounds in Table 1 indicated the *Z* configuration of the natural components.

Identification of Diene Components III and IV. Figure 3C and D shows the mass spectra of the diene acetate (component III) and alcohol (component IV) of *T. intermixta*. The mass spectrum of component III in the extract of *T. orichalcea* was quite similar to that in Figure 3C, while the amount of component IV in this extract was too small to yield an analyzable full mass spectrum. The rather large relative intensity of M^+ in these spectra and late elution from a polar capillary column suggested that the pheromone components included a conjugated diene system (Ando et al., 1982). We have reported the mass spectra of a series of conjugated dienes with a C_{12} straight chain (Ando et al., 1985b). Among them, the spectrum of component III fits that of 5,7-dodecadienyl acetate with abundant ions at *m/z* 136, 93, and 79, and the spectrum of component IV fits that of 5,7-dodecadien-1-ol with abundant ions at *m/z* 93, 79, and 67, although the spectra of synthetic dienes were measured with a different mass spectrometer and ionization voltage. Average fitness indexes

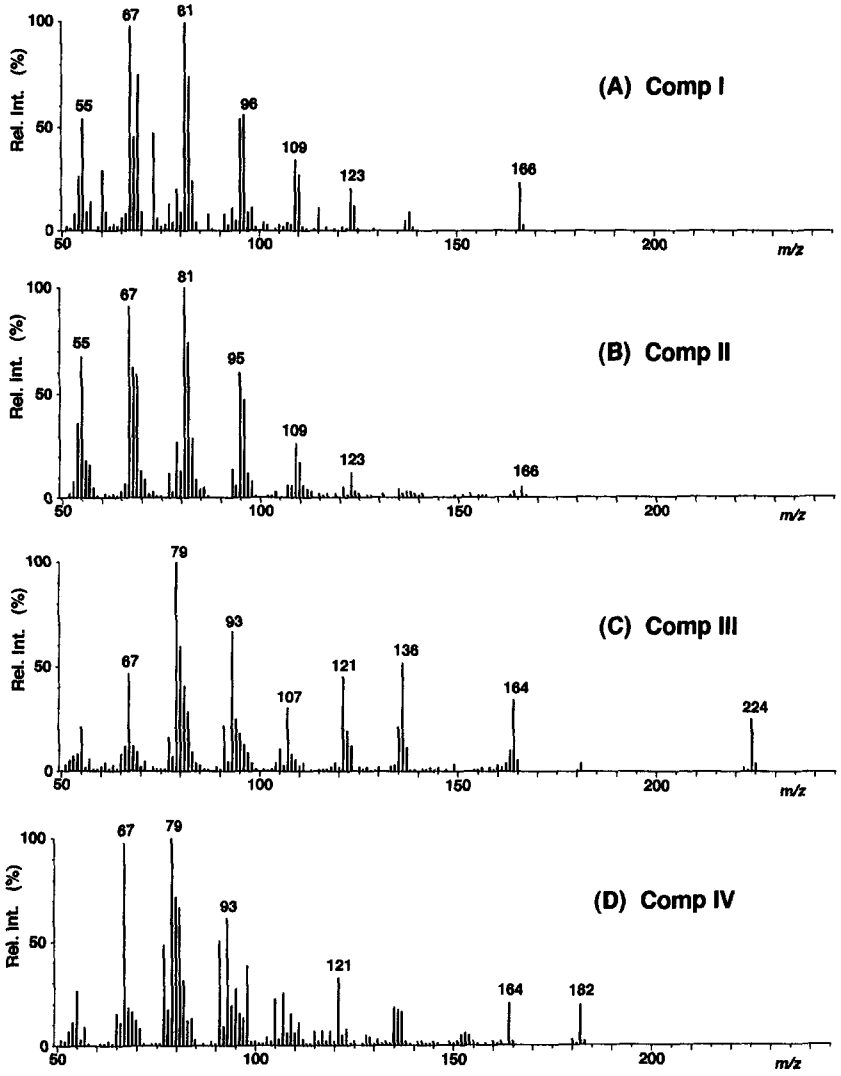

FIG. 3. Mass spectra of *Thysanoplusia* pheromone components. (A) component I (Z7-12:OAc) of *T. orichalcea*; (B) component II (Z7-12:OH) of *T. orichalcea*; (C) component III (*E*5,Z7-12:OAc) of *T. intermixta*; (D) component IV (*E*5,Z7-12:OH) of *T. intermixta*.

TABLE 1. AVERAGE FITNESS INDEXES OF MASS SPECTRA OF COMPONENT I (C_{12} MONOENE ACETATE) AND COMPONENT II (C_{12} MONOENE ALCOHOL) TO THOSE OF SYNTHETIC COMPOUNDS,[a] AND THEIR RETENTION TIMES (R_ts)

	Position of (Z)-double bond						
	5-ene	6-ene	7-ene	8-ene	9-ene	10-ene	11-ene
Average fitness indexes to synthetic C_{12} monoenes							
Comp I of *T. intermixta*[b]	38.8	42.5	18.2	21.9	42.0	40.0	40.1
Comp I of *T. orichalcea*[c]	50.1	29.1	10.2	12.8	31.2	39.5	33.5
Comp II of *T. orichalcea*[d]	58.5	36.3	25.2	30.3	46.7	47.1	51.9
R_t (min) of synthetic standard							
C_{12} monoene acetate	10.18	10.24	10.34	10.41	10.49	10.82	10.47
C_{12} monoene alcohol	10.39	10.42	10.59	10.63	10.72	11.02	10.66

[a]The smallest index values are underlined, indicating the position of the double bond in the pheromone compounds. The indexes (Kuwahara et al., 1986) were calculated with the relative intensities of 13 fragment ions (*m/z* 54, 55, 67, 68, 69, 81, 82, 83, 95, 96, 109, 110, and 138). Mass spectra of the natural pheromone components and synthetic standards were measured with the same Jeol JMS-SX 102A mass spectrometer operated at 70 eV ionization voltage.

[b]The R_t of this acetate in the pheromone extract was 10.35 min, and the R_t of synthetic *E*7-12:OAc was 10.16 min.

[c]The R_t of this acetate in the pheromone extract was 10.34 min.

[d]The R_t of this alcohol in the pheromone extract was 10.59 min, and the R_t of synthetic *E*7-12:OH was 10.41 min.

were also computed for two natural dienes from the relative intensities of twelve fragment ions of the synthetic compounds (Table 2), and the smallest values were observed in compounds with a 5,7-diene system. Finally, the double bond at position 5 of the diene acetate was confirmed by an ozonolysis experiment with component III of *T. intermixta* after its purification by HPLC. GC-MS analysis of the crude product showed only 5-acetoxypentanal (R_t 4.66 min) with diagnostic fragment ions at *m/z* 101, 87, and 73. Four geometrical isomers of each 5,7-diene compound were separable on a DB-23 capillary GC column. The synthetic standards showed the following R_t values: (5*E*,7*Z*)-5,7-dodecadienyl acetate (*E*5,*Z*7-12:OAc) 11.95 min, 5*Z*,7*E* isomer 11.83 min, 5*Z*,7*Z* isomer, 12.04 min; and 5*E*,7*E* isomer 12.16 min; (5*E*,7*Z*)-5,7-dodecadien-1-ol (*E*5,*Z*7-12:OH) 12.27 min, 5*Z*,7*E* isomer 12.13 min, 5*Z*,7*Z* isomer 12.36 min, and 5*E*,7*E* isomer 12.49 min. Therefore the 5*E*,7*Z* configuration was proposed for components III and IV.

Field Attraction. Results of the field tests with synthetic pheromone components are shown in Tables 3 and 4. The preliminary evaluation of synthetic components in Chiba Prefecture in 1995 (Table 3) revealed that male moths of *T. intermixta* were attracted to the lure baited only with *E*5,*Z*7-12:OAc, a

TABLE 2. AVERAGE FITNESS INDEXES OF MASS SPECTRA OF COMPONENT III (C_{12} DIENE ACETATE) AND COMPONENT IV (C_{12} DIENE ALCOHOL) TO THOSE OF SYNTHETIC COMPOUNDS[a]

Pheromone component	Position of (*E*)-double bonds				
	5,7-diene	6,8-diene	7,9-diene	8,10-diene	9,11-diene
Comp III of *T. intermixta*	63.1[b]	123.9	171.1	176.6	110.6
Comp III of *T. orichalcea*	78.3[b]	164.7	232.9	276.5	143.9
Comp IV of *T. intermixta*	40.3[c]	107.5	167.1	183.3	96.3

[a]The smallest index values are underlined, indicating the positions of the double bond in the pheromone compounds. The indexes (Kuwahara et al., 1986) were calculated using the relative intensities of 12 fragment ions (*m/z* 54, 67, 68, 79, 80, 81, 82, 95, 96, 109, 110, and 123). Mass spectra of the natural pheromone components were measured by a Jeol JMS-SX 102A mass spectrometer operated at 70 eV ionization voltage, and those of synthetic dienes were measured by a Hitachi RMU-6MG spectrometer at 20 eV ionization voltage (Ando et al., 1985b).
[b]Significant at the 1% level by the *t* test.
[c]Significant at the 0.5% level by the *t* test.

TABLE 3. FIELD TESTS WITH SYNTHETIC PHEROMONE COMPONENTS IN VEGETABLE FIELDS IN CHIBA PREFECTURE (MATSUDO-SHI) FOR *T. intermixta* AND IN OKINAWA PREFECTURE (ISHIGAKI ISLAND) FOR *T. orichalcea*

Lure components (μg/rubber septum)[a]				Males captured/trap[b]			
				T. intermixta		*T. orichalcea*	
I	II	III	IV	1995[c]	1996[d]	1995[e]	1996[f]
0	0	1000	0	37.3 a	3.0 d	0.0 b	0.3 c
180	0	1000	0		8.0 c		11.3 b
0	0	1000	130		17.3 b		
180	0	1000	130		32.7 a		
180	30	1000	130		41.0 a		
1000	0	0	0	0.0 b	0.0 d	0.0 b	0.0 c
1000	0	110	0	0.3 b	1.3 d	10.5 a	54.0 a
1000	60	0	0				0.0 c
1000	60	110	0				48.0 a
0	0	0	0	0.0 b	0.0 d	0.0 b	0.0 c

[a]Component: I = *Z*7-12:OAc, II = *Z*7-12:OH, III = *E*5,*Z*7-12:OAc, and IV = *E*5,*Z*7-12:OH.
[b]Numbers within each column followed by a different letter are significantly different at $P < 0.05$ by Duncan's multiple-range test.
[c]Tested with three traps for each lure from October 12 to November 5.
[d]Tested with three traps for each lure from August 24 to October 23.
[e]Tested with two traps for each lure from May 1 to May 13.
[f]Tested with three traps for each lure from May 28 to July 11.

TABLE 4. *T. intermixta* MALES CAPTURED BY FOUR SYNTHETIC ISOMERS OF 5,7-DODECADIENYL ACETATE IN A VEGETABLE FIELD IN CHIBA PREFECTURE (MATSUDO-SHI)[a]

	Treatment (1000 μg/rubber septum)			
	*Z*5,*E*7-12:OAc	*E*5,*Z*7-12:OAc	*Z*5,*Z*7-12:OAc	*E*5,*E*7-12:OAc
Males captured/trap[b]	2.7 b	48.0 a	3.7 b	8.3 b

[a]Numbers followed by a different letter are significantly different at $P < 0.01$ by Duncan's multiple-range test.
[b]Tested with three traps for each lure from October 23 to December 3, 1996.

major pheromone component secreted by the females of this species. Multicomponent lures tested in 1996 (Table 3), however, captured larger numbers of males than the single component lure, indicating the synergistic effect of two minor components identified in the virgin female extract, particularly the significant effect of *E*5,*Z*7-12:OH. A mixture of four components in the ratio that was observed in GC-MS analysis of the pheromone extract produced the strongest attraction among the tested combinations. The field attraction of four geometrical isomers of 5,7-dodecadienyl acetate was compared also in Chiba Prefecture (Table 4). *T. intermixta* males were selectively attracted by *E*5,*Z*7-12:OAc, and the activity of other isomers was much weaker than that. In Okinawa prefecture, *T. orichalcea* males were not attracted to a single component lure with *Z*7-12:OAc, the major component of *T. orichalcea* pheromone, and mixing *Z*7-12:OAc with *E*5,*Z*7-12:OAc was necessary for male attraction (Table 3). The possible synergistic effect of minor alcohol components on attraction is still unclear. In these field tests, no males of *T. intermixta* were caught in Okinawa prefecture, nor males of *T. orichalcea* in Chiba prefecture.

DISCUSSION

GC-MS analyses of female extracts have revealed that sex pheromones of *Thysanoplusia* species are composed of four common components (*Z*7-12:OAc, *Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:OH) in different ratios of 18:3:100:13 for *T. intermixta* and 100:6:11:1 for *T. orichalcea*. For attracting *T. intermixta* males, *E*5,*Z*7-12:OAc, the major pheromone component of this species, is essential, and the mixture of the four components in the natural occurrence ratio has most strongly captured male moths. For *T. orichalcea*, the major pheromone component is *Z*7-12:OAc, and *E*5,*Z*7-12:OAc as a minor component is indispensable for the male attraction. Therefore, it is concluded that these two species are reproductively isolated from each other,

not only geographically, but also by pheromonal communication. *T. intermixta* was found on Okinawa Island (Sugi, personal communication), but no males of this temperate-zone species were captured in our field trials on Ishigaki Island, which is located in a more southern part of Okinawa Prefecture than Okinawa Island. A possible explanation for the unsuccessful attraction may be that this species does not inhabit Ishigaki Island. Alternatively, lack of captures could be due to setting traps in an area with low population density, lures with components in an inappropriate ratio, and missed timing of the adult appearance. It is important to know whether or not *T. intermixta* is a sympatric species of *T. orichalcea* in Okinawa Prefecture, and whether or not minor alcohol components have some effects on the attraction of *T. orichalcea*. We are now planning to test several new lures in some islands in Okinawa Prefecture.

To date, sex pheromones have been chemically identified from 15 species in the subfamily Plusiinae. Nine species use Z7–12:OAc as a major pheromone component (Arn et al., 1992, 1997), and the present work with *T. orichalcea* makes the tenth time Z7–12:OAc has been identified as a major component for Plusiinae. Furthermore, field attraction of 22 species in this subfamily has been discovered by random screening tests with synthetic compounds, suggesting that the attractants of 17 species involve Z7–12:OAc as a major component (Arn et al., 1992, 1997). These findings indicate that this monoene acetate is a key substance of Plusiinae pheromones.

In this work we found *E*5,Z7–12:OAc and the parent alcohol in the pheromone gland extracts of two Plusiinae species, and it is the major pheromone component in *T. intermixta*. The 5,7-dodecadienyl compounds have been assumed to be a key substance of pheromones produced by females in the family Lasiocampidae, because the conjugated dienes were identified from 10 species of Lasiocampidae, (Arn et al., 1992, 1997). This is the first time 5,7-diene compounds have been identified as a pheromone component of species in the family Noctuidae. Some noctuid species in the subfamily Cuculliinae have been attracted to *E*5,Z7–12:OAc or a mixture of this diene and Z7–12:OAc in Canada (Reed et al., 1984).

Diversity of species-specific pheromones is due to the complexity of the biosynthetic systems of female moths and the receptors of male moths. During the long history of evolution, most lepidopteran females have developed the ability to produce multiple pheromone components in a strict ratio. Although there is no direct proof, the characteristics of Plusiinae pheromones identified suggest that the original species of Plusiinae utilized Z7–12:OAc for its sexual communication. These two *Thysanoplusia* species must have evolved an enzyme system to introduce one more double bond at position 5. In *Trichoplusia ni*, it has been confirmed that Z7–12:OAc (a main pheromone component of this Plusiinae species) is biosynthesized via the (Z)-11-hexadecenoate intermediate, which is chain-shortened to (Z)-7-dodecenoate (Bjostad and Roelofs, 1983), and

the Δ-11-desaturase of this species has been characterized (Wolf and Roelofs, 1986). Formation of the double bond at position 5 of the 5,7-dienes is possibly accomplished after the Δ-11-unsaturation, but the details remain to be determined. In addition to the 5,7-dienes, C_{12} monoene components unsaturated at position 5 also have been identified from some Plusiinae species (Arn et al., 1992, 1997). These desaturases, which form a double bond at position 5, possess different substrate specificities. It will be interesting in future work to examine the mode of action of the enzyme that makes a double bond at position 5 of the monounsaturated intermediate instead of at position 7 of a saturated intermediate. *T. intermixta*, which produces 5,7-diene pheromone components, is one of the desirable species for biosynthetic studies of a conjugated diene pheromone component and is suitable for the investigation of the differentiation of Plusiinae species at the enzymatic level.

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1-CYANO-2-HYDROXY-3-BUTENE, A PHYTOTOXIN FROM CRAMBE (*Crambe abyssinica*) SEEDMEAL[1]

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Abstract—Crambe (*Crambe abyssinica*) seedmeal was found to suppress seedling emergence and biomass accumulation when added to a sandy loam soil containing wheat (*Triticum aestivum* Cardinal) and hemp sesbania (*Sesbania exaltata*) seeds. Hexane, CH_2Cl_2, MeOH, and water extracts of the seedmeal were prepared and bioassayed against wheat and velvetleaf (*Abutilon theophrasti*) radicle elongation. The CH_2Cl_2 extract was the most inhibitory, while the other extracts inhibited the bioassay species only slightly (MeOH) or not at all (hexane, water). Fractionation of the CH_2Cl_2 extract identified the major phytotoxin as 1-cyano-2-hydroxy-3-butene (CHB), comprising 96.1% of the active CH_2Cl_2 fraction. Radicle elongation of wheat and velvetleaf were inhibited by CHB with I_{50} values of 2.1×10^{-4} M for wheat and 2.7×10^{-3} M for velvetleaf.

Key Words—Crambe, *Crambe abyssinica Triticum*, *Sesbania*, *Abutilon*, Brassicaceae, seedmeal, glucosinolate, epiprogoitrin, 1-cyano-2-hydroxy-3-butene, phytotoxicity, soil amendment.

INTRODUCTION

Alternatives to synthetic chemical pesticides need to be developed, especially for organic farming operations, landscape management systems, home gardens, and for situations where public policies mandate reduced pesticide use. Effective alternatives to synthetic herbicides are particularly important for organic producers, as their greatest costs are historically associated with weed control. One

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[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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approach is to utilize an allelopathic cover or green manure crop that can suppress weeds upon decay of its residues (Putnam et al., 1983). Members of the Brassicaceae, or crucifer family, have been examined as green manures or soil amendments for the control of weeds (Grossman, 1993; Boydston and Hang, 1995; Brown and Morra, 1995; Vaughn and Boydston, 1997; Al-Khatib et al., 1997), soil pathogens (Papavizas, 1966; Lewis and Papavizas, 1971; Papavizas and Lewis, 1971; Ramirez-Villapudua and Munnecke, 1988; Muelchen et al., 1990; Mayton et al., 1996; Williams-Woodward et al., 1997), nematodes (Mojtahedi et al., 1991, 1993), and insects (Brown et al., 1991). The pesticidal activity of crucifers has been found to be principally due to the presence of glucosinolates and/or their degradation products. Glucosinolates are a class of glucose and sulfur-containing organic anions whose biologically active degradation products are produced when the plant cells are ruptured and the glucosinolates present in vacuoles are hydrolyzed by the enzyme myrosinase (β-thioglucosidase glucohydrolase; EC 3.2.3.1) (VanEtten and Tookey, 1983). These products include substituted isothiocyanates, nitriles, thiocyanates, and oxazolidinethiones, which vary depending on the side-chain substitution, pH, and iron concentration (Cole, 1976; Fenwick et al., 1983; Uda et al., 1986; Chew, 1988). Seedmeals from glucosinolate-containing plants have also been identified as potential soil amendments for weed control (Brown and Morra, 1995; Vaughn et al., 1996).

Crambe (*Crambe abyssinica* Hochst. ex. R. E. Fries) is an industrial crop grown primarily in North Dakota for its seed oil, which is an excellent source of erucic acid (Carlson and Tookey, 1983; Carlson et al., 1985). Crambe seedmeal is the heat-toasted seed residue obtained after extraction of the oil and contains glucosinolates [primarily *epi*-progoitrin (2-hydroxy-3-butenyl glucosinolate)] and their degradation products (Carlson et al., 1985). Although crambe seedmeal is used as a protein source for animal feeds (Carlson and Tookey, 1983), its use for feedlot cattle is currently limited to levels not to exceed 4.2% (w/w) of the total diet because of toxicity concerns (Price et al., 1993). Following the completion of a new crambe processing plant in central North Dakota, the projected acreage of crambe grown in the United States was expected to increase to approximately 40,000 acres for 1997 (Gardner, 1996), resulting in a concurrent increase in the production of crambe seedmeal.

Recent research by Walker (1996) indicated that crambe meal was phytotoxic to tomato (*Lycopersicon esculentum* L.) and several other annual plants, while also suppressing plant parasitic nematodes. Tsao et al. (1996) found that crambe seedmeal extracts were toxic to several important agricultural and public health insect pests, including mosquito (*Aedes aegypti* L.), house fly (*Musca domestica* L.), and western corn rootworm [*Diabrotica virgifera virgifera* (LeConte)] larvae. Our interest in using crambe seedmeal as an alternative weed control agent prompted us to investigate further its use as a soil amendment for

weed suppression in high-value, low-acreage crops and to identify compounds responsible for phytotoxicity.

METHODS AND MATERIALS

Seedmeal Bioassays. Crambe seedmeal was obtained from Dr. Vernon Anderson, NDSU, Carrington, North Dakota, and was manufactured by National Sun Industries, Enderlin, North Dakota. The crambe seedmeal as supplied by the manufacturers was granular, with particle sizes ranging from approximately 2.0 mm to 125 μm, although the majority of the seedmeal particles were in the 500 to 850-μm range. Dry crambe seedmeal was thoroughly mixed with soil (Onarga sandy loam; Typic Argiudoll) at rates of 0.1, 0.5, 1.0, and 5.0% (w/w). The seedmeal–soil mixture was added to 200-ml cups, and 10 seeds of either wheat (*Triticum aestivum* L. Cardinal) or hemp sesbania [*Sesbania exaltata* (Ref.) Rydb. esc A. W. Hill] (species that we have found to be good indicators of suppressive activity in bioassays and that both exhibit nearly 100% germination) were added and covered with approximately 1 cm of the mixture. Nonamended soil was used as the control. Each cup received 10 ml of a solution containing 1 g/liter thiabendazole (excessive saprophytic fungal growth occurred at the higher seedmeal rates without this fungicide), and then additional water was added to bring the soil to field capacity. The cups were placed in the growth chamber at a 16-hr, 26°C day/8-hr, 21°C night regime. Emerged seedlings were counted after 14 days, and the above-ground tissue of the emerged seedlings was cut and weighed. Each treatment was replicated five times in a completely randomized design, and the experiment was repeated. Data were subjected to regression analysis (StatView, Abacus Concepts, Berkeley, California).

Chromatography and Spectroscopy. Routine gas chromatography (GC) was performed using a Hewlett-Packard (HP) 5890 Series II gas chromatograph with a HP 3396 integrator. Mass spectra were produced by a HP 6890 Series Mass Selective Detector. Columns used were fused silica HP-5MS capillaries (0.25-μm film thickness, 30 m × 0.25 mm ID). ^{1}H NMR spectra relative to tetramethylsilane were measured in $CDCl_3$ at 400 MHz with a Bruker ARX-400 spectrometer.

Extract Preparation. Seedmeal was first ground in a coffee grinder to a fine powder to facilitate extraction. Potential phytotoxins were extracted from 100-g samples of seedmeal using a Soxhlet apparatus, and the following solvents: hexane, CH_2Cl_2, and MeOH. Extracts were concentrated by rotoevaporation. A water extract was obtained by soaking the solvent-extracted seedmeal in 250 ml of distilled water overnight in a refrigerator at 2°C, after which the marc was washed with two additional 250-ml aliquots, and the extracts lyophilized. The crude CH_2Cl_2 extract subsequently found to be active in the bioassays

was separated on a lipophilic Sephadex LH-20 (Supelco, Inc., Bellefonte, Pennsylvania) column into three separate fractions using 100% $CHCl_3$; 50% $CHCl_3$/50% MeOH; and 100% MeOH as solvents.

Seedling Radicle Elongation Bioassay. Velvetleaf (*Abutilon theophrasti* Medicus; purchased from Valley Seed Service, Fresno, California) seeds were used in the radicle elongation bioassays instead of hemp sesbania, because of difficulties in measuring radicle lengths of the latter species. Wheat and velvetleaf seeds were surface sterilized with 0.5% commercial chlorine bleach for 15 min, after which they were rinsed with and then soaked in sterile distilled water for 2 hr. Seeds were wrapped in sterile paper towels saturated with water and incubated overnight in darkness at 25°C. All crude and column extracts were assayed by adding extracts to autoclaved water agar (1.0% w/v containing 500 mg/liter chloramphenicol) at the rate of 1 mg extract/ml agar (a concentration that is generally effective at identifying active fractions), after the agar had cooled to ~40°C. Fifteen milliliters of the agar–extract mixtures were placed in 9.0-cm plastic Petri dishes, and five germinated seedlings were placed on the agar in the Petri dishes. The dishes were incubated in darkness at 25°C on 45° slants for 24–48 hr, then evaluated for inhibition of radicle growth.

Solutions of purified 1-cyano-2-hydroxy-3-butene (CHB) dissolved in acetone were added to cooling water agar to give final concentrations of 0, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-6} M (the control contained acetone only). Radicle lengths (six plates of four seedlings each) were measured after 48 hr of incubation, and I_{50} values (the amount of each compound required to reduce radicle elongation by 50%) were determined from the intercept of 50% of the control with a best fit line of the data using nonlinear regression analysis (StatView, Abacus Concepts, Berkeley, California).

Glucosinolate Degradation Product Analysis. Because unhydrolyzed glucosinolates present in the seedmeal may break down further in the soil, residual glucosinolate concentrations were determined using a modified aglycone degradation product analysis procedure developed by Daxenbichler and VanEtten (1977). One hundred grams of seedmeal were blended in a Waring blender with 500 ml of 30% (v/v) aqueous methanol and allowed to stand for 1 hr. The eluate was filtered through folded Whatman 2V filter circles and the marc washed with additional aliquots (~200 ml each) of aqueous methanol. The eluate volume was reduced by rotoevaporation in which all of the methanol was removed. The residue was transferred to a centrifuge tube with water to which 2 ml of a 0.5 M barium acetate–lead acetate solution was added, and the mixture was centrifuged (2500 rpm, 10 min). The supernatant was decanted (the pellet was discarded), freeze-dried, and redissolved in 15 ml of 0.05 M potassium phosphate buffer, pH 7.0 to which 25 mg of thioglucosidase (Sigma) were added. Fifteen milliliters of CH_2Cl_2 containing 1 mg/ml butyl isothiocyanate as an internal standard were added to the buffered solution. All were shaken at 30°C

for 2 hr. After shaking, the organic layer was separated from the aqueous layer and analyzed by GC/GC-MS.

RESULTS AND DISCUSSION

Bioassays. Crambe seedmeal added to soil reduced both wheat and hemp sesbania emergence at all rates tested, although strong inhibition only occurred at the 1.0 and 5.0% levels for hemp sesbania, and only at the 5.0% level for wheat (Figure 1). Interestingly, the addition of the two lower percentages of seedmeal increased wheat biomass, while hemp sesbania fresh weight was suppressed by all the tested levels. It appeared that at the lower levels the seedmeal was suppressing germination, but once the seed germinated, then there was a stimulatory effect for wheat, but not for hemp sesbania. It has been shown that many allelochemicals stimulate growth at low concentrations, even though they may be highly toxic at higher concentrations (Rice, 1984).

Phytotoxin Identification. The crude CH_2Cl_2 extract strongly inhibited velvetleaf and wheat radicle elongation, the methanol extract slightly inhibited radicle growth, and the hexane and water extracts had no effect. Fractionation of the crude CH_2Cl_2 extract on the Sephadex LH-20 column yielded one fraction (100% $CHCl_3$) that was inhibitory to radicle elongation in bioassays when tested

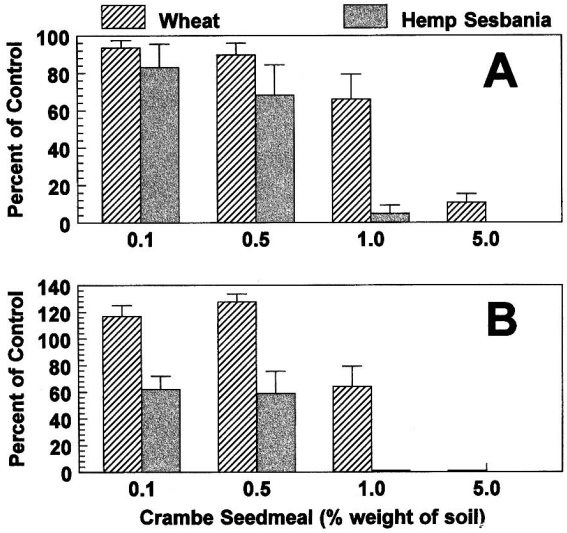

FIG. 1. Wheat and hemp sesbania emergence (A) and biomass (B) at 14 days after emergence after addition of crambe seedmeal to a sandy loam soil. Bars represent 1 SE.

at 1 mg extract/ml agar. This fraction contained one major peak as determined by GC. The compound was identified by GC-MS spectra, by comparison with published mass spectra (Spencer and Daxenbichler, 1980), and by NMR analysis as CHB (Figure 2). Prominent diagnostic mass spectral ions, their relative intensities, and ^{1}H NMR spectra for CHB are as follows: EI-MS [*m/z* (%)]: 97 (M^+, 1), 79(3), 69(4), 57(100), 55(15), 52(7). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.60 (d, 2H, J = 5.6 Hz); 3.90 (br.s, 1H); 4.52 (q, 1H, J = 5.8 Hz); 5.1-6.1 (m, 3H). The CHB peak accounted for 79.2% of the total peak area of the crude CH_2Cl_2 fraction and 96.1% of the active Sephadex column fraction. Two

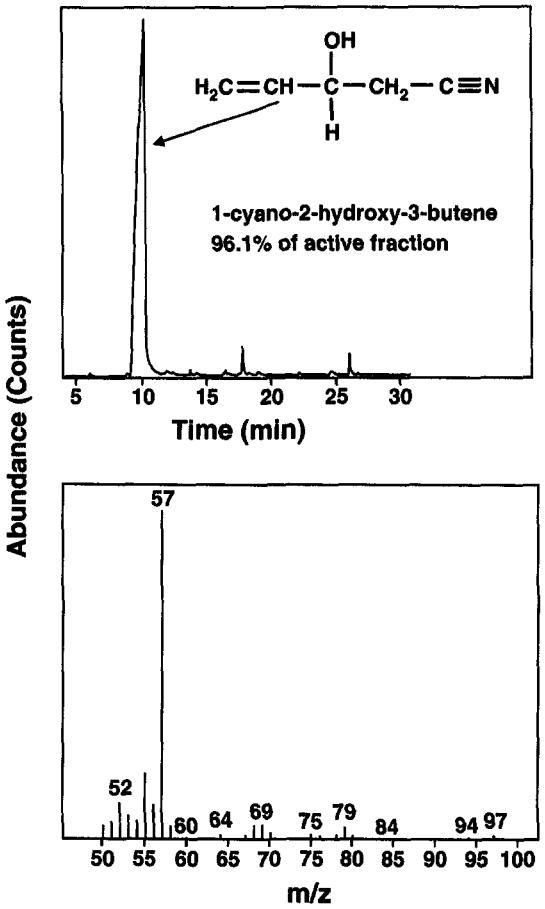

FIG. 2. Gas chromatograph of active CH_2Cl_2 fraction (top) and mass spectrum of CHB (bottom).

other compounds in this fraction were tentatively identified as benzenepropanenitrile (1.5% of the fraction) and 4-methyl-2,5-dimethoxybenzaldehyde (0.9%). Samples of the seedmeal we tested contained, on average, 189 mg of extractable CHB/100 g seedmeal.

Glucosinolate Analysis. Analysis of glucosinolate degradation products in the seedmeal indicated that the predominant glucosinolate remaining in the meal was *epi*-progoitrin, with minor amounts of sinigrin (allyl glucosinolate), gluconapin (3-butenyl glucosinolate), and gluconasturtiin (2-phenylethyl glucosinolate). 5-Vinyl-2-oxazolidinethione (5-vinyl OZT) was the major glucosinolate degradation product, accounting for 0.69 mg/g seedmeal (Figure 3). Other compounds detected were 1-cyano-2-hydroxy-3,4-epithiobutane (0.11 mg/g seedmeal), 3-butenyl isothiocyanate (0.09 mg/g seedmeal), 2-propenyl (allyl) isothiocyanate (0.08 mg/g seedmeal), and 2-phenylethyl isothiocyanate (0.06 mg/g seedmeal). However, no additional CHB was detected in the degradation products.

Toxicity of CHB. Purified CHB isolated from crambe seedmeal was bioassayed against velvetleaf and wheat seedling root elongation. The calculated I_{50}s for CHB were 2.1×10^{-4} M and 2.7×10^{-3} M for wheat and velvetleaf,

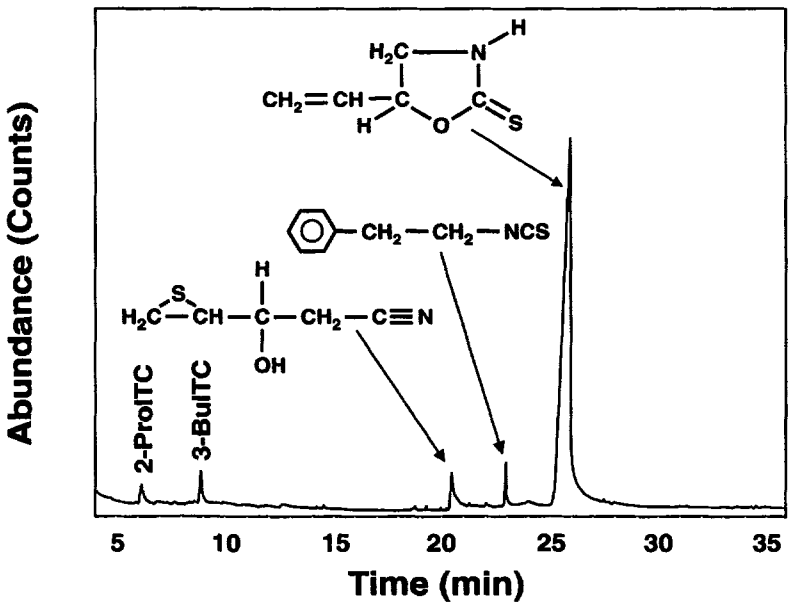

FIG. 3. Gas chromatograph of thioglucosidase-catalyzed degradation products of residual glucosinolates in seedmeal.

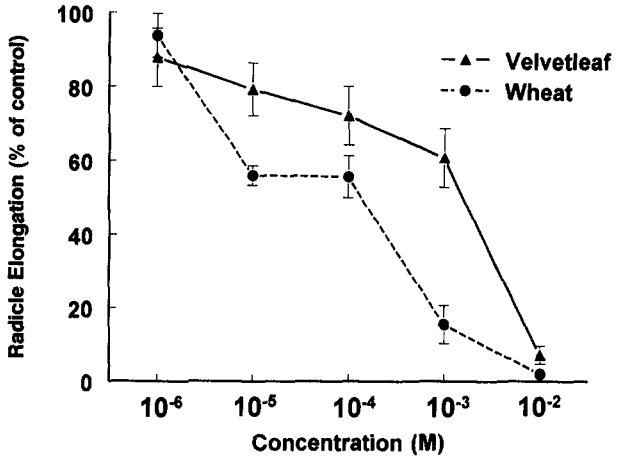

FIG. 4. Inhibition of velvetleaf and wheat radicle elongation by CHB. Bars represent ± 1 SE.

respectively (Figure 4). These figures are comparable to those found for (3-methoxyphenyl)acetonitrile (3-MPAN), a phytotoxin isolated from meadowfoam (*Limnanthes alba* Hartweg ex. Benth.) seedmeal (Vaughn et al., 1996) and for sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate), an enzymatic decomposition product of the glucosinolate glucoraphenin (Brinker and Spencer, 1993).

Donkin et al. (1995) reported that a water extract of crushed crambe seeds containing 26% *epi*-progoitrin was moderately toxic to the nematode *Caenorhabditis elegans*. They did not report, however, if this extract contained CHB, 5-vinyl OZT, or other decomposition products, which were likely present in the extract, as they made no attempt to inhibit myrosinase activity during extraction process. Tsao et al. (1996) found that 2-hydroxy-3-butenyl glucosinolate comprised over 90% of the total glucosinolates present in crambe seedmeal, regardless of whether water, MeOH, EtOH, or acetone was used as the extracting solvent. However, the MeOH and EtOH extracts were much more toxic to western corn rootworm larvae than the water extract. Although the authors did not identify specific compounds in these extracts, the water extract would be expected to contain *epi*-progoitrin but not CHB, while CHB would be extracted by both MeOH and EtOH, and this would help to explain why these extracts were more toxic. As in the case of 3-MPAN from meadowfoam seedmeal (Vaughn et al., 1996), CHB was not a potent phytotoxin, although as the research by Walker (1996) indicated, it may have greater activity against soilborne diseases and nematodes. Indeed, CHB has been shown to be both a hepatotoxin

and a pancreatotoxin at moderate doses in animals (Wallig and Jeffery, 1990). However, the relatively large amounts of CHB in the seedmeal suggest that the seedmeal might be useful as a soil amendment for weed control in situations where inhibitory levels of the seedmeal could be applied economically, such as for landscape ornamentals, high-value fruits and vegetables, or turf. Value-added utilization of the seedmeal, especially for higher returns than are obtained for its use as animal feed, would also help increase growers' profits.

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LIGANDS OF URINARY LIPOCALINS FROM THE MOUSE: UPTAKE OF ENVIRONMENTALLY DERIVED CHEMICALS

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Abstract—Mouse urine contains large quantities of proteins (major urinary proteins, MUPs) that are thought to function by binding lipophilic and volatile semiochemicals in a central calyx of the MUP. Two notable semiochemicals are 2-*sec*-butyl-4,5-dihydrothiazole and a brevicomin (3,4-dehydro-*exo*-brevicomin). MUPs derived from deposits of urine from wild caught mice contain neither of these ligands, but are replete with menadione. The menadione is probably incorporated in vitro from the environment, although some incorporation in vivo can also be demonstrated. These data show that the calyx of MUPs can bind other hydrophobic molecules derived from the environment, which may influence longevity of signal and deposition patterns of urinary scent marks. The ability to displace, rapidly and completely, the natural ligands by menadione also provides a new tool in the analysis of MUP function.

Key Words—Major urinary protein, menadione, olfactory communication, scent marking, mouse.

INTRODUCTION

Mouse urine contains large quantities of major urinary proteins (MUPs) that are members of the lipocalin family (Flower, 1996) and are products of a multigene

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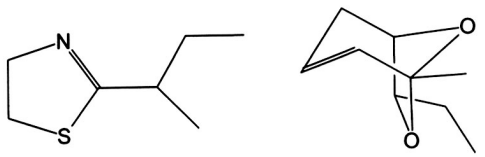
family highly expressed in liver (Finlayson et al., 1965; Knopf et al., 1983; Hastie and Held, 1978). The MUPs then enter the circulation, are efficiently filtered by the kidney, and are released in the urine. In urine from two different inbred mouse strains, there are at least 14 different MUPs. Full or partial cDNA sequence and mass spectral data from wild-caught individuals suggest the existence of more than 40 MUPs, but some of these have not been identified in urine. The total number of allellomorphs of these proteins must be large. Sequence variation is present in about 40% of residues, the majority of which are located at surface exposed residues.

MUPs derived from laboratory and wild house mice bind two ligands (Figure 1), a thiazole (2-*sec*-butyl-4,5-dihydrothiazole) and a brevicomin (3,4-dehydro-*exo*-brevicomin) (Bacchini et al., 1992; Robertson et al., 1993). X-ray electron density in the central calyx region is compatible with substantial occupation of the calyx with the thiazole (Bocskei et al., 1992). These ligands convey signals of male dominance (Novotny et al., 1985), and MUP binding may protect semiochemicals or facilitate a slow release of these otherwise volatile molecules. However, the precise role of the MUPs is untested, and current hypotheses are limited by the need for correlative biochemical and behavioral experiments.

In natural environments, mice deposit urine as scent marks in continuous streaks, small spots, or sometimes repeatedly in the same place, such that discrete posts are built (Hurst, 1987). These signals are used by males to advertise dominance over their territories (Gosling and McKay, 1990; Hurst, 1993). The purpose of the different deposition patterns is unclear but may be dictated by the need to replenish, in strategic sites, volatile molecules that are otherwise lost by evaporation. As part of a study of MUP heterogeneity and ligand binding in wild mice, we have examined the chemical composition of posts deposited by wild mice (*Mus domesticus*) in natural or seminatural environments. In this



I II

FIG. 1. Natural ligands bound to MUPs. The two ligands that have been observed to be bound to MUPs are I, 2-*sec*-butyl-4,5-dihydrothiazole, and II, 3,4-dehydro-*exo*-brevicomin.

manuscript, we report that MUPs derived from posts contain ligands derived from the environment that may modulate the signaling capabilities of these proteins or influence deposition patterns. Moreover, the ability to displace natural ligands opens up new opportunities for experiments to assess the structure–function relationships of this class of proteins.

METHODS AND MATERIALS

Recovery of Urine Post Material. Urine posts were collected from single wild male mice maintained in large enclosures. Portions (1 g) of the post material were homogenized in 5 ml of deionized water. The homogenate was clarified by centrifugation at 12,500*g* for 5 min and the supernatant was removed. MUPs (typically 100–200 μl) were recovered from the supernatant by size exclusion chromatography (SEC) on spun columns of Sephadex G25. MUPs were recovered from the fresh urine of wild mice and BALB/C inbred mice in the same way.

SDS-PAGE. SDS-PAGE was performed as described in Laemmli (1970). All samples were run under reducing conditions in 17.5 (w/v) acrylamide gels. Gels were run for 1 hr at 100 V, and the proteins were stained with Coomassie blue. Separated proteins were electroblotted to a nitrocellulose membrane. In this case, the gel was equilibrated in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3] for 30 min and subsequently overlaid with the nitrocellulose membrane in a blotting cassette. Electroblotting was subsequently conducted at 30 V overnight. The membrane was then probed with a 1 : 1000 dilution of polyclonal antibody to MUPs, purified from inbred BALB/C mice, in PBS–Tween (10 mM sodium phosphate, 0.15 M sodium chloride, 0.05% Tween 20, pH 7.4).

Electrospray Mass Spectrometry. The MUPs isolated from urine posts by SEC were further analyzed by electrospray ionization mass spectrometry (ESI-MS). The desalted urine post extract was diluted 1 : 10 with an aqueous solution of 50% (v/v) acetonitrile, 0.1% (v/v) formic acid. This solution was introduced into the mass spectrometer as a continuous infusion at 5 μl/min. The instrument used for this analysis was a VG-Quattro I, triple quadrupole mass spectrometer, upgraded to Quattro-II specifications, and fitted with an electrospray ionization source. Raw data were acquired between m/z 900 and 1600. These data were transformed to a true mass scale and refined with Maximum Entropy software. The Maximum Entropy refinement was conducted between masses 18,500 and 19,000 Da at 1 Da/channel with a peak width parameter of 0.75 Da. The instrument was calibrated with a 2 pmol/μl solution of horse heart myoglobin (Sigma Chemicals), the spectrum for which was acquired immediately after the urine post sample under identical conditions. The calibration spectrum also was

used to determine the peak width parameter used during Maximum Entropy processing (Micromass, Altringham, UK).

Volatile Ligand Analysis. The ligands associated with MUP fractions from urine posts and from freshly collected urine samples were analyzed by chloroform extraction and gas chromatography–mass spectrometry (GC-MS). Three volumes (typically 300 μl) of desalted MUPs were added to two volumes of chloroform and vortexed for 10 sec. The vortexed solution was allowed to stand at room temperature for 60 min prior to removal of the chloroform. A 2-μl aliquot of this extract was injected onto a Carbowax 20 M column in an HP 5890 gas chromatograph equipped with an HP 5971A mass selective detector and an on-column injector. The column head was attached to 45 cm of deactivated silica retention gap precolumn, and the column was run in a stream of pure helium at a head pressure of 10 psi. The oven temperature at the time of injection was 60°C, which was maintained for 7 min after injection. The temperature was then increased to 200°C at 10°/min and maintained at 200°C for 5 min. The mass selective detector was run in scan mode, between *m/z* 50 and 550. Menadione was identified by comparison of its sample mass spectrum to the Wiley library of mass spectra and to that of authentic menadione (Sigma Chemicals).

Menadione Incorporation Studies. Stock solution of 20, 10, 4, 2, 1, and 0.4 mg/ml menadione were made in absolute ethanol. A 10-μl aliquot of each solution was added to 190 μl of desalted MUPs isolated from BALB/C mice maintained on standard laboratory diet (dietary content of menadione = 30 mg/kg). This resulted in the MUPs being exposed to 1, 0.5, 0.2, 0.1, 0.05, and 0.02 mg/ml concentrations of menadione in 5% (v/v) ethanol. The MUPs were then recovered by SEC and the bound menadione and thiazole were extracted into chloroform as described above. The chloroform extract was then analyzed by GC-MS in selected ion monitoring (SIM) mode, where the selected ions were *m/z* 60 (thiazole) and *m/z* 172 (menadione).

Male BALB/C mice (N = 8) were injected subcutaneously with 100 μl of 50% (v/v) ethanol containing 0.2 mg of menadione or with solvent alone. At time intervals, urine samples were obtained from the animals by gentle bladder massage, and the MUPs were isolated by SEC. Bound menadione, thiazole, and brevicomin were analyzed by chloroform extraction and GC-MS with selected ion monitoring as described above.

Molecular Modeling. All structural analyses and simulations were performed with the X-ray crystal structure of MUP (PDB code: 1MUP) determined by Bocskei et al. (1992). The cavity of the MUPs was analyzed with the MSP program suite of Connolly (1993) to determine cavity size and volume. Docking simulations involving the menadione ligand and water molecules in the MUP cavity were performed with the Biased Probability Monte Carlo method of the

ICM program (Abagyan and Totrov, 1994; Abagyan et al., 1994). Simulations were propagated for 100,000 steps by using the standard ICM force field with the menadione ligand placed in the central cavity along with either 0, 1, 2, or 3 water molecules (all assigned initial random positions) at a temperature of 300°K. The ICM force field contains specific terms for van der Waals forces, electrostatic interactions, torsion angles, hydrogen bonds and atomic solvation. For each simulation, the menadione ligand and water molecules were assigned a random conformation within the cavity boundary and were unconstrained (except for a weak restraining term to keep them within the vicinity of the cavity). All atoms in the protein were fixed except for side chain moieties of amino acids forming the cavity surface. The lowest energy conformation from each simulation was further considered.

RESULTS AND DISCUSSION

Urine posts contained solid fecal matter and food residues, in addition to substantial quantities of protein (38 ± 6 mg/g wet weight post material, mean ± SEM, $N = 4$). The protein was predominantly an 18-kDa species that is strongly immunoreactive to a MUP antiserum and therefore highly likely to be MUP (Figure 2). Electrospray mass spectrometry confirmed that the MUPs were of the masses predicted from cDNA sequences (Robertson et al., 1996) and were thus largely intact in the posts. This confirms the belief that the posts are formed by urine marking (Hurst, 1987). The samples from wild-caught animals contained MUPs of masses 18,647 ± 2 Da, 18,691 ± 2 Da, and 18,707 ± 2 Da that had been observed previously (Robertson et al., 1996, 1997), and additional proteins, notably those of 18,808 Da, 18,971 Da, and 19,032 Da that might represent yet further alleles of this multigene family (Pes et al., 1998). The total number of MUPs is unknown, but from our observations on inbred and wild strains there must be in excess of 20 allelic variants that are expressed. Whether the different alleles exert subtly different functional characteristics is unknown.

The proteins recovered from urine posts were also analyzed by GC-MS for their associated volatiles. In contrast to MUPs recovered from fresh urine samples derived from either inbred mice or wild mice, we were unable to observe bound components with GC retention times or mass spectra characteristic of the thiazole and the brevicomin. Instead, less volatile compounds were evident. In particular, one component ($R_t = 22.99$ min) was abundant and gave a discrete mass spectrum that was consistent with that of a quinone. Library searches revealed that the best match was to menadione (vitamin K_3); the identification was substantiated by GC-MS analysis of the authentic compound (Figure 3).

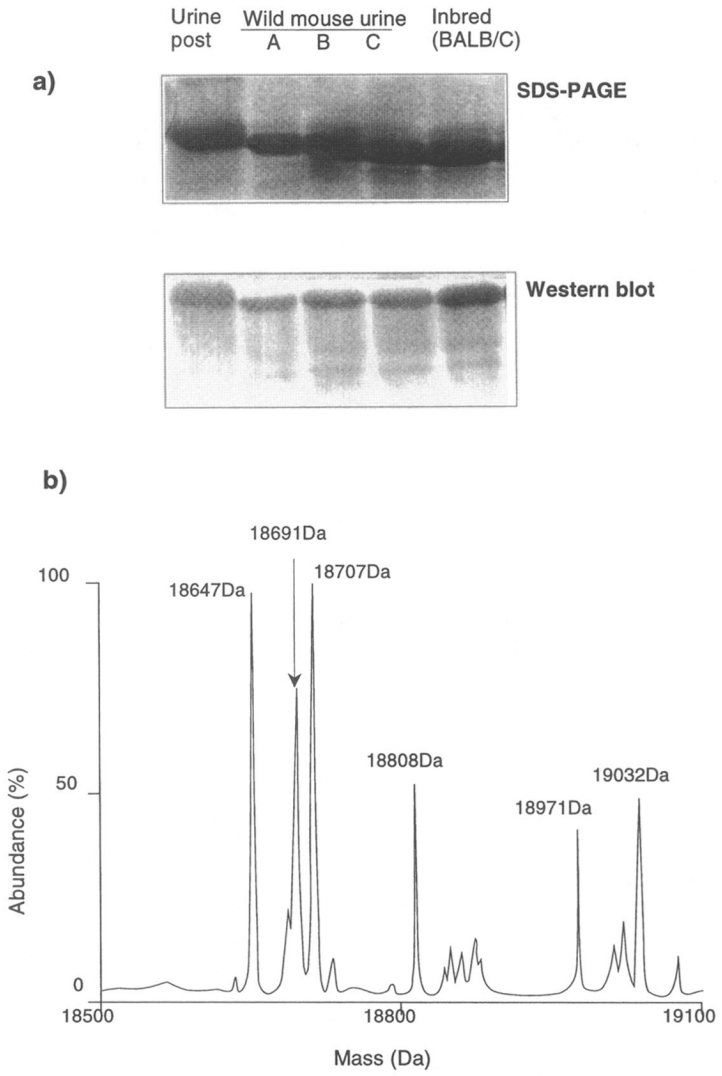


FIG. 2. Protein components in urine posts. (a) The SDS-PAGE analysis and western blot of proteins derived from the urine post and fresh urine samples from wild and inbred mice; (b) electrospray ionization mass spectrometry analysis of the proteins recovered in urine posts.

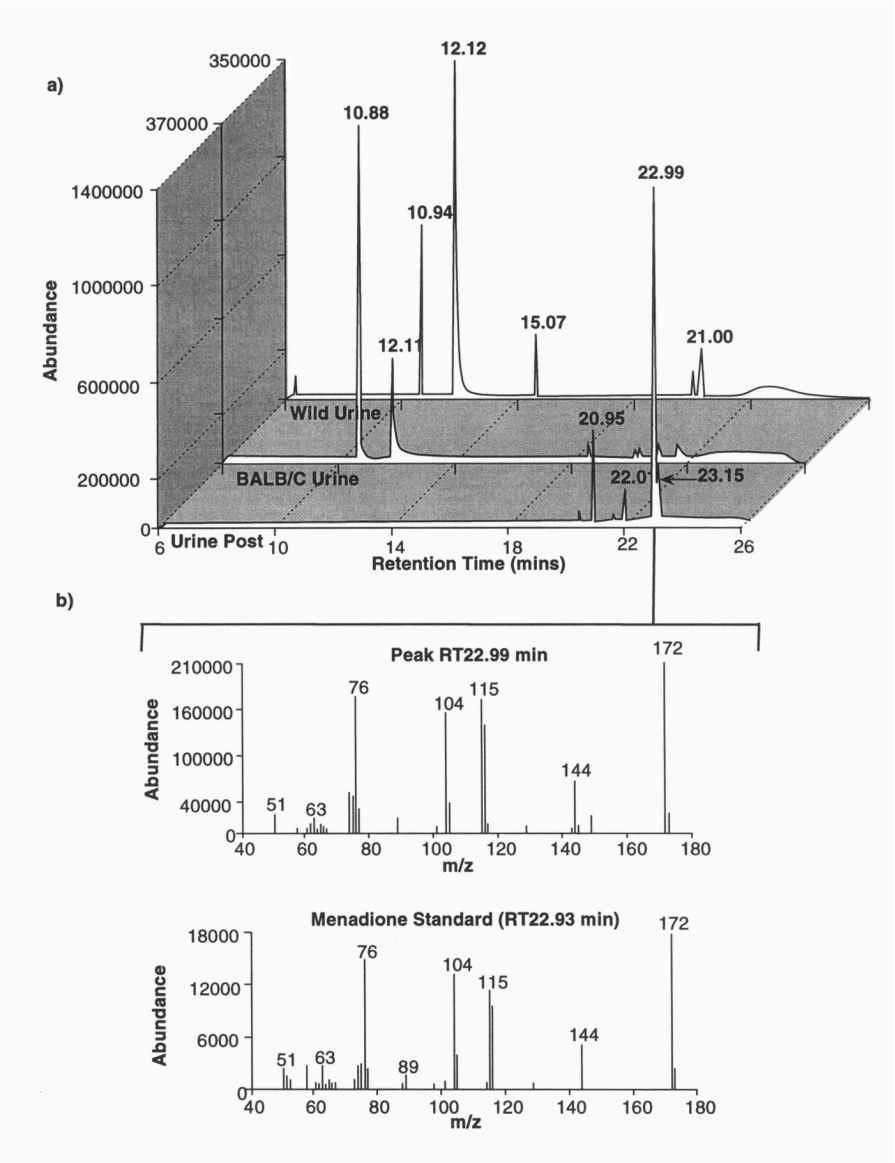


FIG. 3. Identification of ligands in urine samples and posts from wild-caught mice. (a) Gas chromatograms (GC) of bound ligands from urine posts, fresh urine from an inbred male mouse (BALB/C), and fresh urine from a wild male mouse maintained in captive conditions, (b) electron impact mass spectrum of the component with a GC retention time of 22.99 min from urine posts is compared with the mass spectrum of authentic menadione, which showed the same retention time.

The data are consistent with occupancy of the calyx by menadione in place of the thiazole and brevicomin. Upon inspection, trace amounts of menadione were also associated with MUPs in urine samples freshly collected from mice that had been fed laboratory diet (results not shown).

For menadione to displace MUPs, the central calyx of the protein must be able to accommodate the larger quinoid molecule. The overall orientation of MUP and the central cavity are illustrated in Figure 4A. Structural analysis of the ligand binding calyx of the crystal structure of MUP (Bocskei et al., 1992) revealed a large hydrophobic void (475 $Å^3$) into which the small thiazole molecule (volume 136 $Å^3$) was a loose fit, with a further two putative water molecules assigned within the cavity (Figure 4B). The slightly bulkier menadione (volume 151 $Å^3$) was also readily accommodated in the cavity (Figure 4C), and the optimal binding and orientation were analyzed by molecular dynamics with the ICM program. The lowest energy conformer from computer docking analyses repeatedly oriented the menadione in the same position in the cavity, regardless of the starting orientation and the solvent occupancy in the simulation. No unfavorable geometric or spatial constraints between the ligand and the MUP calyx were observed. Furthermore, menadione makes favorable interactions with surrounding hydrophobic protein side chains including a hydrogen bond, via a water molecule, to one of the quinoid oxygens in the otherwise unsatisfied hydroxyl group of Tyr_{124} (Figure 5). The molecular interactions of menadione and the binding pocket are consistent with a putative ligand and are at least as favorable as those made by thiazole in the crystal structure. The calyx is able to accommodate this ligand with ease. It is unclear whether the displacement is a consequence of tighter binding of menadione, or whether this reflects a much higher concentration of the displacer molecule (i.e., menadione) than the natural ligands. The insolubility of menadione and the lack of ligand-free protein preclude direct measurement of the dissociation constants for the individual ligands.

Menadione was identified at high levels only in urine post samples and not in freshly collected urine samples, whether from wild or inbred animals. This raises the issue of the mechanism of incorporation of menadione into the calyx. It was likely that the menadione was bound from a source in the environment, and the most likely source was the diet upon which these animals were maintained, since the artificial laboratory diet contained 150 mg/kg vitamin K_3. Because the posts contained solid food residues, the menadione could have become incorporated into the protein calyx after the urine was deposited. To test this idea, we isolated MUPs from inbred animals and incubated them with menadione (Figure 6). Menadione was able to bind rapidly (within 60 sec, data not shown) to MUPs in a saturable fashion, and the binding was coincident with virtually complete displacement of the natural ligands from the protein calyx in vitro. Because menadione is largely insoluble in water, it is not possible to estimate the binding constant for this ligand. However, the proteins are approx-

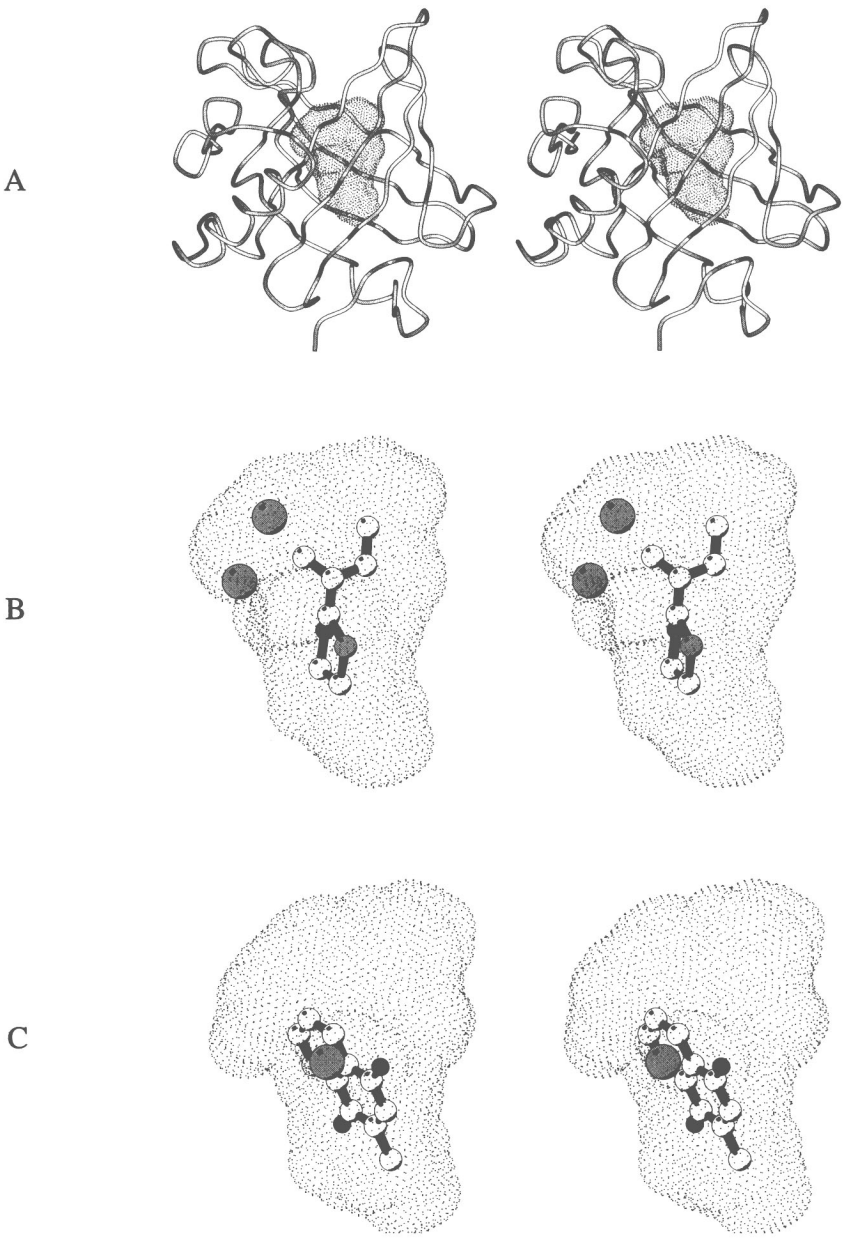


FIG. 4. Thiazole and menadione binding to MUPs. (A) The MUP ribbon structure encloses the binding cavity (dotted area); (B) a close-up of the cavity shows the relatively loose fit of the thiazole ligand into the cavity in a ball-and-stick representation, with the two solvent molecules depicted as grey spheres, (C) the lowest energy conformation of a number of docking simulations involving the menadione ligand and a single water molecule in the MUP cavity.

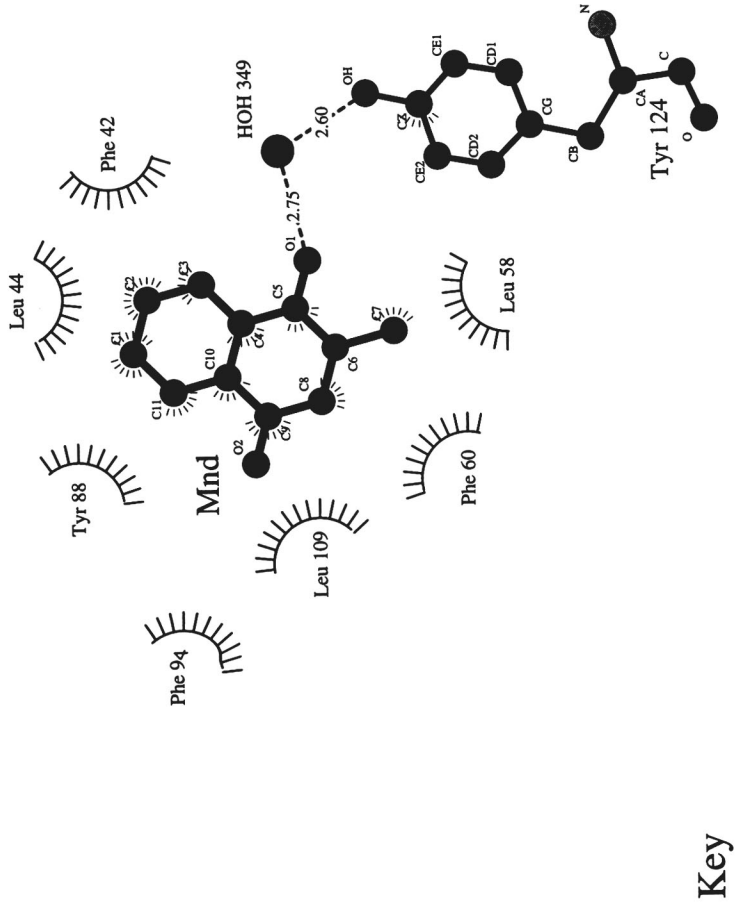


FIG. 5. Putative interactions between MUP and menadione. A LIGPLOT diagram (Wallace et al., 1995) of the modeled MUP–menadione complex indicates that the interactions are predominantly hydrophobic but that a stabilizing hydrogen bond can be formed between one of the quinoid oxygen atoms and the side chain of Tyr 124 via a water molecule.

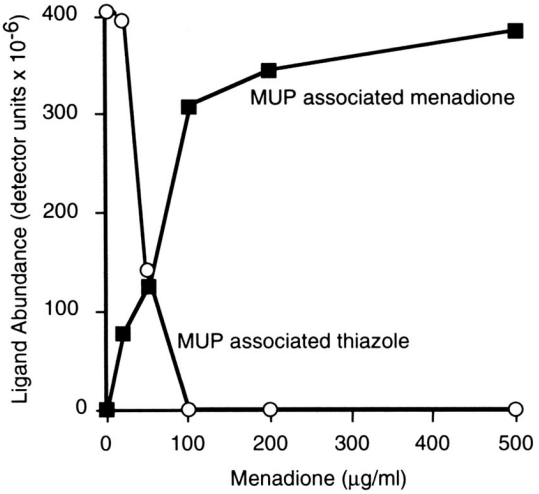


FIG. 6. Binding of menadione to MUPs in vitro. MUPs were isolated by Sephadex G25 size exclusion chromatography from BALB/C mice maintained on standard laboratory diet (menadione = 30 mg/kg). The MUPs were exposed to increasing quantities of menadione before being recovered for analysis of bound menadione and thiazole by GC-MS with selected ion monitoring.

imately 50% saturated at nominal ligand concentrations of 100 μg/ml. At 100 μg/ml menadione, thiazole was totally displaced from the ligand–protein complex.

Some menadione, absorbed across the gut, might have been incorporated into the MUPs during biosynthesis and secretion. Such a mechanism would allow for the variation of ligand content in MUPs, perhaps associated with changed metabolic or physiological status (for example, changes attendant upon diet, infection, or after puberty). To assess the possibility of incorporation in vivo, inbred mice were injected with menadione, and MUPs were monitored for protein-bound menadione over the next four days (Figure 7). Within 4 hr, the released MUPs contained menadione, although the thiazole and brevicomin signals were not appreciably reduced. After 24 hr, the menadione signal had returned to zero, attesting to the rapidity of association of menadione with MUPs and the apparently high turnover of these proteins. Although incorporation in vivo is demonstrable, it seems unlikely that it is responsible for the high degree of occupancy seen in the urine posts.

MUPs are probably capable of binding a broad range of lipophilic molecules and may modulate complex olfactory signals through a wide repertoire of volatiles. The rat equivalent, α-2u proteins, are known to bind a range of xeno-

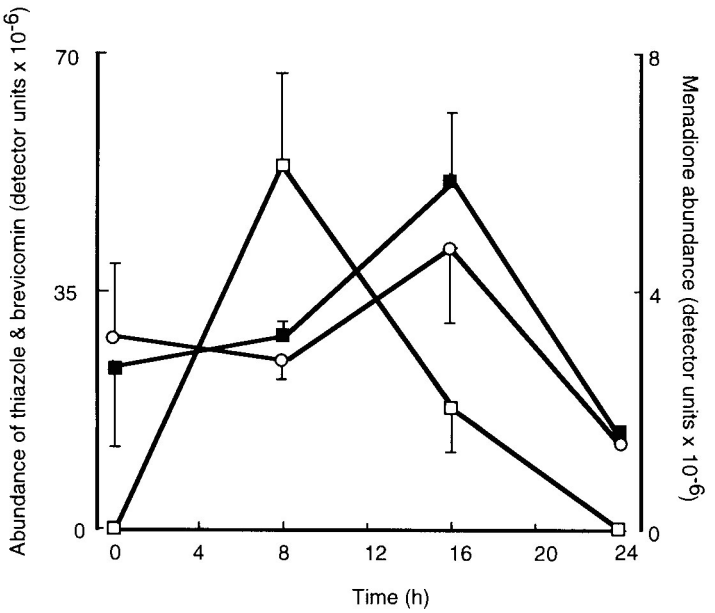


FIG. 7. Binding of menadione to MUPs in vivo. Male BALB/C mice were injected subcutaneously with 100 μl of 50% (v/v) ethanol containing 0.2 mg of menadione or with solvent alone. At time intervals, urine samples were recovered, the urinary MUPs were isolated by SEC, and bound menadione, thiazole, and brevicomin were analyzed by chloroform extraction and GC-MS with selected ion monitoring. The data are presented as mean $\pm$ SEM (N = 3–8, depending upon availability of urine samples from individual mice at each time point) (open squares, menadione; filled squares, brevicomin; open circles, thiazole).

biotics (Borghoff et al., 1991; Lehman-Keeman and Caudhill, 1992). Since association may occur in vivo or in vitro, these volatiles may derive from the diet, or from other lipophilic molecules present in the environment and accumulated after the MUPs have been released. It is likely that most of the menadione in urine post MUPs was incorporated in vitro, as the posts contained material (including food particles) derived from the environment and held in place by the sticky, semi-dry, protein-rich mass.

The consequence of menadione binding to deposited MUPs is the attendant loss of natural ligands. If the rate of loss of these ligands is greater than would occur by natural dissociation and evaporation, then repeated urine marking, leading to the formation of urine posts, may reflect the need to replenish natural signals because of accelerated displacement of natural semiochemicals by lipophilic molecules from the environment. At present, we have no evidence for a

specific or natural signaling or displacer role of menadione; its incorporation into MUPs in vitro might simply reflect its abundance in the single food source to which these animals have access. This is consistent with our inability to observe the thiazole and brevicomin ligands in urine posts, whether derived from wild or seminatural environments.

MUPs are expressed as a multigene family (Hastie and Held, 1978), and there are at least 10 allelic variants of MUPs in inbred mice (Robertson et al., 1996) and many more in wild-caught mice (Pes et al., 1998). Differences in the structure of the various allelomorphs may modulate specificity of ligand binding or kinetics of release of volatile molecules and thus add a new dimension to the subtleties of chemical signaling. The demonstrable ability of MUPs to bind other nonpolar molecules also suggests that they may find utility in the slow release or protection of a broad range of lipophilic semiochemicals, which in turn may be of value in biological control of mice and other species. Finally, displacement of natural ligands by lipophilic competitors such as menadione offers new possibilities for exploration of the role of the urinary proteins in semiochemical expression. Indeed, the utility of this tool is apparent in our recent work and has provided direct evidence, for the first time, in support of the hypothesis that MUPs act as a slow release mechanism for their associated volatiles (Hurst et al., 1998).

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SEX PHEROMONE OF FEMALE VINE BUD MOTH, *Theresimima ampellophaga* COMPRISES (2*S*)-BUTYL (7*Z*)-TETRADECENOATE

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Abstract—The sex pheromone of the vine bud moth, *Theresimima ampellophaga*, released at the 3rd–5th abdominal tergites, was identified by coupled GC-EAG, GC-MS, and synthesis as (2*S*)-butyl (7*Z*)-tetradecenoate. For the first time, full stereochemistry is unambiguously defined for the sex pheromone of a member of the Zygaenidae. The synthetic compound caught significant numbers of males in field-trapping experiments.

Key Words—*Theresimima ampellophaga*, Zygaenidae, Lepidoptera, sex pheromone, (2*S*)-butyl (7*Z*)-tetradecenoate, electroantennogram.

INTRODUCTION

The vine bud moth, *Theresimima ampellophaga* Bayle-Barelle (Lepidoptera: Zygaenidae), is a pest of the grape vine, *Vitis vinifera* (Vitaceae), and in some years causes serious damage in vineyards, particularly in Bulgaria, Caucasus,

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Crimea, Georgia, Hungary, and Italy (Pucci and Dominici, 1986). Dolidze et al. (1980) first reported the attraction of *T. ampellophaga* males by conspecific females in the field, and further studies confirmed this activity (Subchev and Harizanov, 1990; Harizanov and Harizanova, 1991). In addition, an unusual calling behavior was observed (Subchev and Harizanov, 1990); the site of the pheromone gland in the females was subsequently shown to be situated on the anterior part of the 3rd–5th abdominal tergites (Hallberg and Subchev, 1997), rather than on the ventral intersegmental membrane between the 8th and 9th abdominal segments as seen in most Lepidoptera. In this paper, the chemical identity of the sex pheromone of *T. ampellophaga* is investigated.

METHODS AND MATERIALS

Insects

For each year of the study, second or third instars of *T. ampellophaga* were collected in spring from vineyards in Bulgaria, and two generations were reared under ambient laboratory conditions. Larvae were mass-reared on loose vine leaves, which were regularly replaced to ensure that sufficient amounts were always present. Under these conditions, the time from egg to adult was approximately seven weeks. The sexes were identified at the pupal stage and maintained separately in 0.4-liter glass jars until eclosion. Pupae were sent to the United Kingdom and to Germany for electrophysiological studies and chemical investigations. Adult moths, which do not feed, were supplied with water in glass vials containing cotton wool to maintain humidity.

Preparation of Female Extracts

As maximum calling (Subchev, unpublished) and male attraction (Harizanov and Harizanova, 1991) occurred between 09:00 and 12:00 hr, females were collected during this period and kept in the freezer for about 2 hr. The dorsal part of the abdominal cuticle up to the 5th tergite, containing the pheromone gland, was then excised, cleaned on a filter paper, and placed in 0.5 ml hexane for 15–20 min. The solvent was removed, placed in a glass vial, and stored in a freezer until required. The extract for coupled GC-EAG studies contained 20 female glands and was concentrated to 100 μl before use.

Electrophysiology

Electroantennograms (EAG) were recorded from antennae of 1- to 2-day-old unmated males using Ag–AgCl glass electrodes filled with saline (as in Maddrell, 1969, but without glucose). The antenna was excised and suspended between the two electrodes. The tip of the recording electrode had an inside

diameter just wide enough to accept the uncut terminal process. Signals were passed through a high-impedance amplifier (Syntech UN-06, Hilversum, Netherlands), and data storage and processing were carried out with a PC-based interface and software package (Syntech). The effluent from the gas chromatograph was delivered into an airstream (1 liter/min), purified but not humidified, flowing continuously over the preparation.

Coupled Gas Chromatography–Electroantennography (GC-EAG)

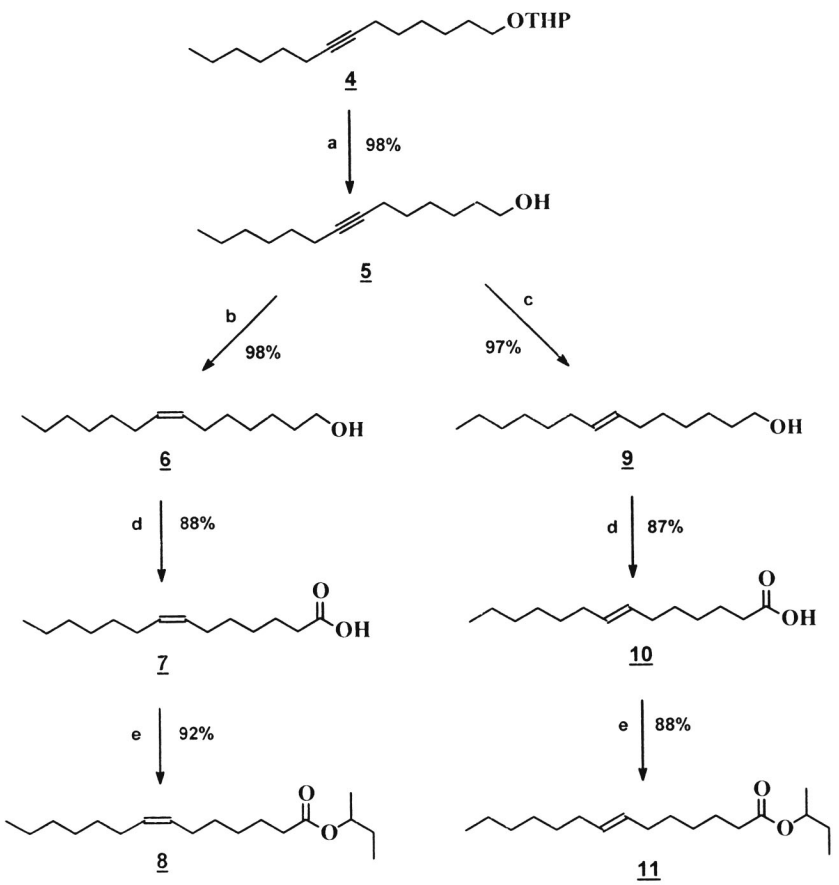
The coupled GC-EAG system, in which the effluent from the capillary column GC is delivered simultaneously to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Gas chromatography of female gland extracts (1 μl) and synthetic samples (100 ng) was carried out on an AI 93 instrument (AI Scientific, Cambridge, UK) equipped with a cold on-column injector and a flame ionization detector (FID). The column (50 m $\times$ 0.32 mm ID HP-1) was maintained at 40°C for 1 min and then programmed at 5°/min to 100°C, then at 10°/min to 250°C. The carrier gas was hydrogen.

Chemical Analysis

Coupled gas chromatography–mass spectrometry (GC-MS) was performed with a VG 70-250 double focusing mass spectrometer (Vacuum Generators, Manchester, UK) linked to a HP 5890 gas chromatograph (Hewlett Packard), equipped with a 30 m $\times$ 0.25 mm ID DB-5 fused silica column, with temperature programming at 4°/min from 60 to 300°C. The position of the double bond was determined by the dimethyl disulfide (DMDS) method (Buser et al., 1983) on crude extracts. Chirality was determined by GC with a chiral custom-made 25 $\times$ 0.25 mm ID fused silica column coated with heptakis (6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-γ-cyclodextrin at 130°C (König et al., 1994).

Chemical Synthesis

Syntheses of the reference samples followed conventional methods (see Scheme 1). 6-Bromohexanol (**1**) (Aldrich) was protected with dihydropyran and the reaction product **2** was coupled to 1-octyne (**3**) (Fluka) according to the standard procedure (Brandsma, 1988). The resulting compound **4** was deprotected with acidic methanol to yield 7-tetradecyn-1-ol (**5**). Lindlar hydrogenation of **5** to (7*Z*)-tetradecenol (**6**), oxidation to the acid (Millar et al., 1983) (**7**), followed by esterification with the appropriate alcohol, i.e., (2*S*)-butanol, (2*R*)-butanol, or 2-methylpropanol (all purchased from Aldrich), yielded a series of esters, e.g., **8** for 2-butanol, showing *Z* stereochemistry at the double bond. Reduction of **5** with lithium tetrahydridoaluminate in diglyme at 200°C (Rossi



SCHEME 1. Synthesis of 2-butyl (7*Z*)-tetradecenoate (**8**) and 2-butyl (7*E*)-tetradecenoate (**11**). (a) MeOH, H^+; (b) H_2/Lindlar, 1013 mbar, hexane; (c) $LiAlH_4$, diglyme, 200°C; (d) Jones-reagent, acetone; (e) $1.C_2Cl_2O_2/CH_2Cl_2$(cat.DMF) 2.2-butanol/Py, 0°C.

and Carpita, 1977) produced (7*E*)-tetradecenol (**9**), which served as the starting material for a series of *E* configured esters, e.g., **11** for 2-butanol. Structures of the compounds were confirmed by NMR.

NMR Data for Synthetic Compounds

2-(7-Tetradecynyl-1-oxy)tetrahydropyran (4). ^1H (400 MHz, $CDCl_3$): δ = 0.89 (t, 3H, J = 6.9 Hz, 14-H); 1.20–1.90 (m, 22H, 2-H, 3-H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H, 3′-H, 4′-H, 5′-H); 2.10–2.22 (m,4H, 6-H + 9-H); 3.38 (dt, 1H, J = 9.7/6.6 Hz), 3.46–3.54 (m, 1H), 3.73 (dt, 1H, J = 9.7/6.6

Hz); 3.83–3.5O (m,1H), 2-H + 6′-H; 3.83–3.50 (m, 1-H); 4.55–4.59 (m, 1H, 2′-H).

^{13}C (101 MHz, $CDCl_3$): δ = 14.06 (q, 14-C) 18.74, 18.79, 19.71, 22.59, 25.54, 25.85, 28.57, 28.72, 29.14, 29.16, 29.71, 30.81, 31.40, 62.33, 67.6 (15t, 15CH_2); 80.12, 80.33 (2s, 7-C + 8-C); 98.86 (d, 2′-C).

7-Tetradecyn-1-ol (5). ^{13}H (400 MHz, $CDCl_3$): δ = 0.89 (t, 3H, J = 6.9 Hz, 14-H); 1.20–1.53 (m, 14H, 3-H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H); 1.57 (quin, 2H, J = 6.6 Hz, 2-H); 1.76 (br s, 1H, OH); 2.11–2.18 (m, 4H, 6-H + 9-H); 3.63 (t, 2H, J = 6.6 Hz, 1-H).

^{13}C (101 MHz, $CDCl_3$): δ = 14.06 (q, 14-C); 18.72, 18.79, 22.61, 25.34, 28.57, 28.64, 29.17, 31.41, 32.70 (10t, 2-C, 3-C, 4-C, 5-C, 6-C, 9-C, 10-C, 11-C, 12-C, 13-C); 62.88 (t, 1-C); 80.06, 80.41 (2s, 7-C + 8-C).

(7Z)-Tetradec-7-en-1-ol (6). ^{1}H (400 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J = 6.9 Hz, 14-H); 1.20–1.42 (m, 15H, 3-H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H, OH); 1.57 (quin, 2H, J = 6.6 Hz, 2-H); 1.93–2.08 (m, 4H, 6-H + 9-H); 3.64 (t, 2H, J = 6.6 Hz, 1-H); 5.30–5.40 (m, 2H, 7-H + 8-H).

^{13}C (101 MHz, $CDCl_3$): δ = 14.10 (q, 14-C); 22.68, 25.68, 27.16, 27.26, 29.01, 29.10, 29.74, 29.76, 31.81, 32.81 (10t, 2-C, 3-C, 4-C, 5-C, 6-C, 9-C, 10-C, 11-C, 12-C, 13-C); 63.02 (t, 1-C); 129.71, 130.08 (2d, 7-C + 8-C).

(7Z)-Tetradec-7-enoic Acid (7). ^{1}H (400 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J = 6.9 Hz, 14-H); 1.24–1.40 (m, 12H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H); 1.59–1.70 (m, 2H, 3-H); 1.95–2.08 (m, 4H, 6-H + 9-H); 2.35 (t, 2H, J = 6.9 Hz, 2-H); 5.30–5.40 (m, 2H, 7-H + 8-H); >10 (br s, 1H, OH).

^{13}C (101 MHz, $CDCl_3$): δ = 14.10 (q, 14-C); 22.67, 24.61, 26.99, 27.27, 28.73, 29.01, 29.37, 29.74, 31.80, 34.09 (10t, 2-C, 3-C, 4-C, 5-C, 6-C, 9-C, 10-C, 11-C, 12-C, 13-C); 63.02 (t, 1-C); 129.41, 130.29 (2d, 7-C + 8-C); 180.33 (s, C=O, 1-C).

2-Butyl (7Z)-tetradecenoate (8). ^{1}H (400 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J = 7.2 Hz), 0.89 (t, 3H, J = 7.2 Hz, 1.20 (d, 3H, J = 6.3 Hz, OCHC$\underline{H}_3$); 1.24–1.40 (m, 12H) and 1.48–1.66 (m, 4H, 3-H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H, OCHC$\underline{H}_2$); 1.95–2.07 (m, 4H, 6-H + 9-H); 2.28 (t, 2H, J = 7.6 Hz, O=CC$\underline{H}_2$); 4.84 (sext, 1H, J = 6.3 Hz, O—C$\underline{H}$); 5.33–5.40 (m, 2H, 7-H + 8-H).

^{13}C(101MHz,$CDCl_3$):δ=8.68,13.08,18.49[3q,14-C, OCH($\underline{C}H_3$)$CH_2\underline{C}H_3$]; 21.63, 24.00, 26.00, 26.21, 27.77, 27.81, 27.97, 28.37, 28.70, 30.76, 33.69 [11t, 2-C, 3-C, 4-C, 5-C, 6-C, 9-C, 10-C, 11-C, 12-C, 13-C, OCH(CH_3)-$\underline{C}H_2CH_3$], 70.88 [d, O$\underline{C}$H(CH_3)CH_2CH_3]; 128.41, d, 129.18 (d, 7-C + 8-C); 172.51 (s, C=O, 1-C).

(7E)-Tetradec-7-en-1-ol (9). ^{1}H (400 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J = 6.9 Hz, 14-H); 1.20–1.43 (m, 15H, 3-H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H, OH); 1.57 (quin, 2H, J = 6.6 Hz, 2-H); 1.90–2.20 (m, 4H, 6-H + 9-H); 3.63 (t, 2H, J = 6.6 Hz, 1-H); 5.35–5.44 (m, 2H, 7-H + 8-H).

^{13}C (101 MHz, $CDCl_3$): δ = 14.10 (q, 14-C); 22.68, 25.63, 28.86, 28.93, 29.59, 29.63, 31.78, 32.52, 32.63, 32.79 (10t, 2-C, 3-C, 4-C, 5-C, 6-C, 9-C, 10-C, 11-C, 12-C, 13-C); 63.06 (t, 1-C); 130.17, 130.56 (2d, 7-C + 8-C).

(7E)-Tetradec-7-enoic Acid (10). ^1H (400 MHz, $CDCl_3$): δ = 0.88 (t, 3H, J = 6.9 Hz, 14-H); 1.24–1.40 (m, 12H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H); 1.59–1.70 (m, 2H, 3-H); 1.92–2.02 (m, 4H, 6-H + 9-H); 2.35 (t, 2H, J = 6.9 Hz, 2-H); 5.33–5.45 (m, 2H, 7-H + 8-H); >10 (br s, 1H, OH).

^{13}C (101 MHz, $CDCl_3$): δ = 14.10 (q, 14-C); 22.65, 24.56, 28.53, 28.85, 29.21, 29.60, 31.75, 32.33, 32.59, 33.86 (10t, 2-C, 3-C, 4-C, 5-C, 6-C, 9-C, 10-C, 11-C, 12-C, 13-C); 63.02 (t, 1-C); 129.88, 130.77 (2d, 7-C + 8-C); 179.22 (s, C=O, 1-C).

2-Butyl (7E)-tetradecenoate (11). ^1H (400 MHz, $CDCl_3$): δ = 0.875 (t, 3H, J = 7.2 Hz), 0.89 (t, 3H, J = 7.2 Hz), 1.19 (d, 3H, J = 6.3 Hz, $OCHC\underline{H}_3$); 1.22–1.41 (m, 12H) and 1.48–1.66 (m, 4H, 3-H, 4-H, 5-H, 10-H, 11-H, 12-H, 13-H, $OCHC\underline{H}_2$); 1.95–2.07 (m, 4H, 6-H + 9-H); 2.30 (t, 2H, J = 7.6 Hz, $O{=}CC\underline{H}_2$); 4.85 (sext, 1H, J = 6.3 Hz, $O{=}C\underline{H}$); 5.33–5.40 (m, 2H, 7-H + 8-H).

^{13}C (101 MHz, $CDCl_3$): δ = 9.70, 14.10, 19.51 [3q, 14-C, $OCH(\underline{C}H_3)CH_2\underline{C}H_3$]; 22.65, 25.00, 28.64, 28.86, 29.16, 29.27, 29.62, 31.77, 32.39, 32.61, 34.73 [11t, 2-C, 3-C, 4-C, 5-C, 6-C, 9-C, 10-C, 11-C, 12-C, 13-C, $OCH(CH_3)\underline{C}H_2CH_3$]; 71.90 [d, $O\underline{C}H(CH_3)CH_2CH_3$]; 129.98, 130.68 (2d, 7-C + 8-C); 173.56 (s, C=O, 1-C).

The optically active esters prepared from (2*S*)- or (2*R*)-butanol showed the following rotation values in chloroform: (*R*)-8: $[\alpha]_D^{21}$ = −9.60 (c = 3.54); (*S*)-8: $[\alpha]_D^{22}$ = +9.30 (c = 3.87); (*R*)-11: $[\alpha]_D^{21}$ = −9.75 (c = 5.4); and (*S*): $[\alpha]_D^{22}$ = +9.50 (c = 7.3).

Field Trapping

The attractant activity of the synthetic (2*S*)-butyl (7*Z*)-tetradecenoate to *T. ampellophaga* males was tested by trapping in vineyards in three areas of South Bulgaria (Korten, Shivachevo, and Rupki). Two doses, 10 μg and 100 μg in hexane, were applied to rubber septa from penicillin vial caps. These lures were placed in home-made delta traps consisting of transparent PVC foil with sticky layers measuring 10 × 15 cm. Three replicates, each comprising traps with the doses and a control trap, were placed in a line, with 10 m between the traps and more than 100 m between replicates in the three areas. Traps were checked twice weekly.

RESULTS

Coupled GC-EAG analysis of the female *T. ampellophaga* extract showed one peak associated with activity on the male antenna (Figure 1A). On GC-MS

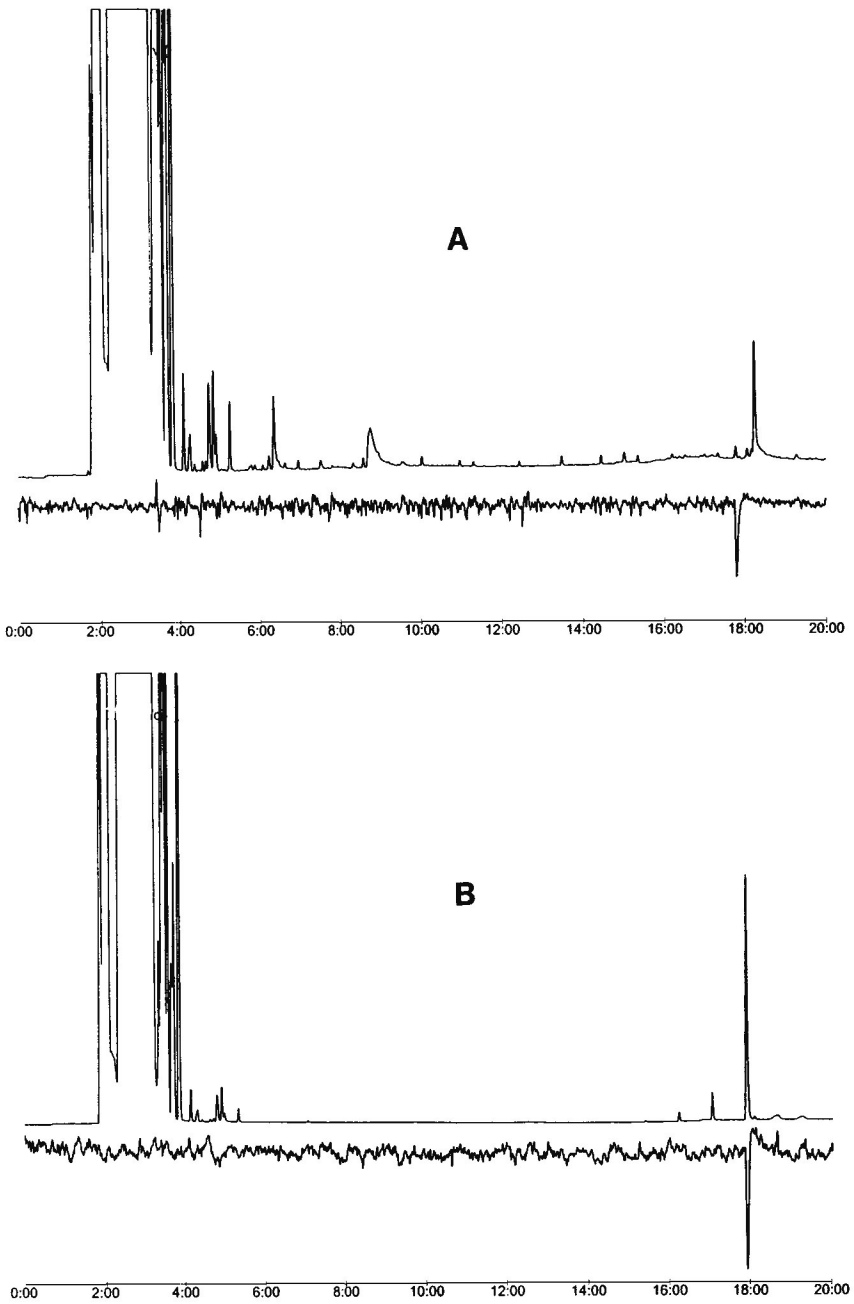


FIG. 1. Coupled GC-EAG with a *T. ampellophaga* male: consecutive runs on the same antenna with: (A) 1 μl female gland extract, and (B) 100 ng of (2*S*)-butyl (7*Z*)-tetradecenoate. In both cases, the upper trace is the FID of the sample and the lower trace is the EAG response.

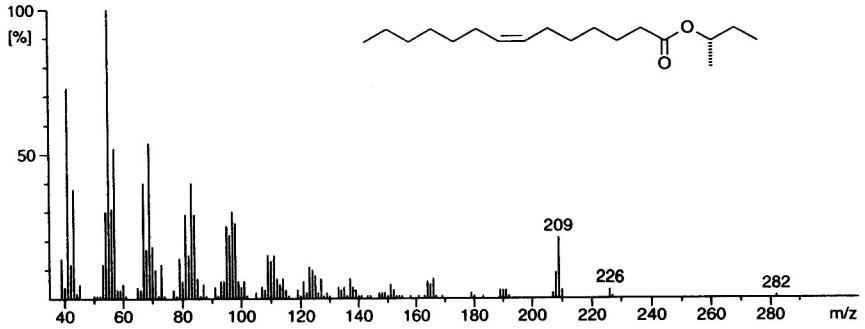


FIG. 2. Mass spectrum for female-derived sex pheromone of *T. ampellophaga*, (2*S*)-butyl (7*Z*)-tetradecenoate.

(Figure 2), this peak yielded a molecular ion at *m/z* 282, confirmed as $C_{18}H_{34}O_2$ by high resolution MS (found: *m/z* 282.2494; required 282.2559). Diagnostic fragments at *m/z* 226 ($C_{13}H_{25}CO_2H^+$) and *m/z* 209 ($C_{13}H_{25}CO^+$) suggested tetradecenoic acid as a substructure, indicating the target compound to be a butyl tetradecenoate. After DMDS treatment of the crude extract, the derivative of the ester was found to show a molecular ion at *m/z* 376 (282 + 94). The diagnostic fragment at *m/z* 145 [$CH_3(CH_2)_5CH:SMe^+$] clearly revealed a double bond in the 7 position (Dunkelblum et al., 1985).

The GC retention time for *n*-butyl tetradecenoate was longer than that for the natural product, and so 2-butyl and 2-methylpropyl esters of synthetic *E* and *Z* isomers of 7-tetradecenoic acid were prepared. Comparison of retention times for these compounds and the DMDS derivatives showed the natural compound to be the *Z* configured ester (**8**) of the chiral alcohol, 2-butanol. The mass spectrum of the synthetic compound was identical with the natural product. On a chiral GC column the ester of (2*R*)-butanol had a retention time of 88.2 min, while its enantiomer eluted 2.5 min later. Each enantiomer was separately coinjected with the natural product, which showed the latter to be the (2*S*)-butyl ester of about 96–98% ee. Coupled GC-EAG of the synthetic (2*S*)-butyl (7*Z*)-tetradecenoate confirmed the neurophysiological activity of this compound (Figure 1B).

High numbers of *T. ampellophaga* males were caught in pheromone traps at all three localities (Table 1). Catches in traps baited with 100 μg of the synthetic (2*S*)-butyl (7*Z*)-tetradecenoate were significantly higher than in traps with the 10-μg dose, and both were significantly higher than in unbaited control traps.

TABLE 1. CATCHES OF *T. ampellophaga* MALES IN TRAPS WITH TWO DOSES OF (2*S*)-BUTYL (7*Z*)-TETRADECENOATE

Dose (μg)	Total number of moths caught[a]		
	I	II	III
0	1a	4a	2a
10	497b	567b	363b
100	1208c	972c	1261c

[a]Numbers followed by the same letter are not significantly different at $P = 5\%$ by Duncan's NMRT. Three replicates at each site. I: Korten, June 14–July 14, 1996. II: Shivachevo, June 21–July 14, 1996. III: Rupki, June 14–July 14, 1996.

DISCUSSION

The family Zygaenidae is now considered to comprise three subfamilies, Zygaeninae, Chalcosinae, and Procridinae (Leraut, 1980), with the latter, incorporating *T. ampellophaga*, being the most ancient (Efetov, personal communication). For the species of Zygaeninae investigated to date, e.g., *Zygaena filipendulae* L. (Zagatti and Renou, 1984), the pheromone gland is situated, as for most Lepidoptera, on the ventral intersegmental membrane between the 8th and 9th abdominal segments. Furthermore, the chemical structures of their pheromones are commonly found in other species and, when present as esters, usually comprise acetates of long-chain unsaturated alcohols (Arn et al., 1992).

The first sex pheromone to be identified for a species in the subfamily Procridinae, *Harrisina metallica* Stretch (= *H. brillians* Barnes & McDunnough), comprises four components. The major one is 2-butyl (7*Z*)-tetradecenoate, a highly volatile alcohol with as yet unknown absolute configuration, esterified with a long-chain unsaturated carboxylic acid (Myerson et al., 1982). In field tests, the enantiomers of this ester were shown to attract six zygaenid species, all belonging to the same subfamily. The ester of (2*R*)-butanol attracted *H. americana* (Guerin-Meneviles) and *Acoloithus falsarius* Clemens, while the (2*S*)-enantiomer attracted *H. metallica*, *H. coracina* (Clemens), *H. guatemalena* (Druce), and *A. novaricus* (Barnes & McDunnough) (Soderstrom et al., 1985; Landolt et al., 1986; Landolt and Heath, 1991). The identification of the sex pheromone of *T. ampellophaga* as (2*S*)-butyl (7*Z*)-tetradecenoate is the first identification, based on unequivocal analytical methods, of the enantiomeric composition of the natural pheromone. Behavioral effects of unnatural stereoisomers have not been tested for this species. The pheromones for *T.*

ampellophaga and *H. metallica* are chemically related to (2*R*)-2-pentyl decanoate (Leonhardt et al., 1983), produced by the psychid *Thyridopteryx ephemeraeformis* (Haworth); in the latter species, the pheromone glands also were found to be atypically sited for Lepidoptera, in this case on the female thorax (Leonhardt et al., 1983). The semiochemistry described in the present study, combined with the finding of an unusual pheromone gland in *T. ampellophaga* females (Hallberg and Subchev, 1997), demonstrates how radically the pheromone system of the subfamily Procridinae differs from that of most other Lepidoptera and even from other members of the zygaenid family.

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SPRUCE BUDWORM LARVAL PROCESSING OF PIPERIDINE ALKALOIDS FROM SPRUCE NEEDLES[1]

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Abstract—New needle bundles of *Picea englemannii* (Engelmann spruce) and *Picea pungens* (blue spruce) contain disubstituted piperidine alkaloids. No alkaloids were found in western spruce budworm larvae (*Choristoneura occidentalis*), which were feeding on the needles, but needle alkaloids were present in the budworm frass. Excretion of the alkaloids by the budworm larvae, either intact or after a metabolic change, was the common processing pathway. No sequestration could be demonstrated.

Key Words—*Picea*, *Choristoneura*, Pinaceae, spruce, budworms, alkaloids, piperidines, larvae, metabolism.

INTRODUCTION

Pinaceae species in the genera *Pinus* (pines) and *Picea* (spruce) relatively recently have been found to contain an array of 2,6-disubstituted piperidine alkaloids (Schneider and Stermitz, 1990; Stermitz et al., 1990, 1994; Schneider et al., 1991, 1995; Tawara et al., 1993, 1995; Todd et al., 1995). Conifers are susceptible to many diseases and are attacked by a wide variety of herbivores (Sinclair et al,, 1987; Johnson and Lyon, 1988; Cranshaw et al., 1993). Some plant alkaloids profoundly affect herbivores and are involved in a variety of natural plant–insect interactions (Hartmann, 1991; Saunders et al., 1991; Brown and Trigo, 1995). Piperidine alkaloids have such bioactivity (Fodor and Colasanti, 1985; Schneider, 1996).

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Several of the alkaloids found in conifers have also been isolated from beetles, in which they are synthesized de novo (Brown and Moore, 1982; Eisner et al., 1986; Proksch et al., 1993; Attygalle et al., 1993). Figure 1 gives structures of the disubstituted piperidines so far described from conifers and indicates which are also known from insects.

In the present study, perhaps the most important bioactivities are those of 6-methyl-9-norgranatanone, **2**, and epidihydropinidine, **8**. Alkaloid **2**, a known constituent of the plant *Euphorbia atoto* (Hart et al., 1967), was identified in the defensive secretion of two beetles: *Cryptolaemus montrouzieri* (Brown and Moore, 1982) and *Epilachna varivestis* (Eisner et al., 1986). In the latter paper, **2** was dubbed euphococcinine and shown to be deterrent against spiders and ants. In a preliminary report, a spruce alkaloid mixture dominated by epidihydropinidine, **8**, was reported to have moderate to high antifeedant activity against eastern spruce budworm (Schneider et al., 1991).

The research reported here was initiated to determine the pathway(s) of alkaloid processing by spruce budworm larvae through analysis of the conifer needles consumed, the larvae, and larval frass. Sequestration, metabolism, and simple excretion (or a combination of these) have been observed in studies on other insect herbivores (Hartmann, 1991; Saunders et al., 1991; Brown and Trigo, 1995). We studied Western spruce budworm, *Choristoneura occidentalis* Freeman, feeding on *Picea pungens* Engelm. (the Colorado blue spruce), and on *Picea engelmannii* Parry ex Engelmann (Engelmann spruce). Previous analyses showed the two spruce species to have similar needle alkaloid patterns,

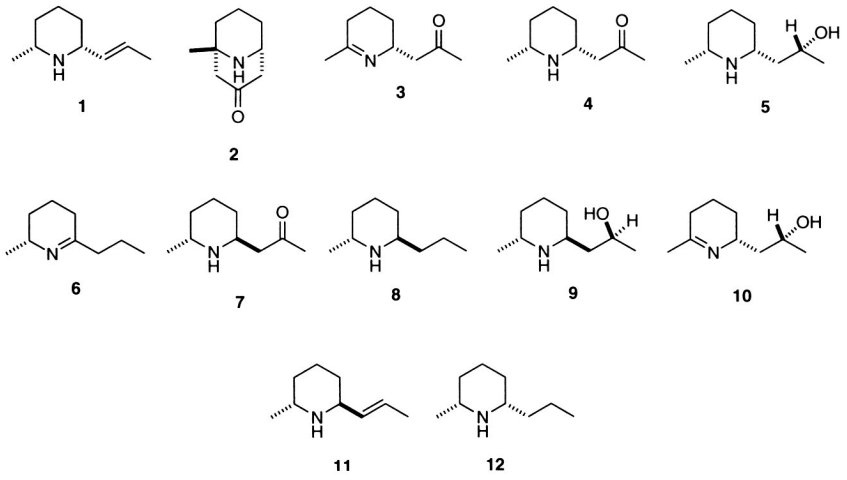


FIG. 1. Disubstituted piperidine alkaloids from conifers. Alkaloids **2**, **3**, **4**, **5**, and **12** are also known from beetles (stereochemistry not always established).

with the major exception that euphococcinine, **2**, was a prominent alkaloid of the blue spruce needles (Todd et al., 1995), while it was absent from Engelmann spruce (Todd, 1994).

METHODS AND MATERIALS

Collections. Choristoneura occidentalis late instars were collected on June 23, 1990, from several trees in Chaffee County, Colorado, near county road 217–218 intersection (Castle Rock gulch, SE of Buena Vista) where the budworms were feeding on *Picea pungens*. Budworms were held 12–24 hr without food prior to preparing them for analysis so that any gut contents could be voided. New needles from buds being consumed and budworm frass from within the webs were also collected. Budworms, needles, and frass were collected from *Picea engelmannii* on June 16 and 17, 1996, 41 miles west of Montrose, Montrose County. Colorado off Forest Road (FR) 402, and from Trout Creek Pass, Chaffee County, Colorado, off FR 311 east of Buena Vista. Similar samples were taken on June 15, 1996, from a single Engelmann spruce (labeled tree 9) near the junction of FR 622 and FR 630 north of US 160 west of Walsenburg, Huerfano County, Colorado. Tree species were identified by R. Moench, Colorado State Forest Service, Fort Collins.

Alkaloid Isolation and Analyses. Isolations from the blue spruce system were performed early in our studies (1991) with a relatively simple procedure, while the Engelmann spruce samples were treated later by a more detailed procedure.

Fresh blue spruce needles, budworm larvae, or frass were weighed samples that had been refrigerated in vials of MeOH prior to analysis. Samples were filtered, the MeOH was evaporated, and the residues were taken up in 0.5 M aq. HCl. The solutions were extracted with $CHCl_3$ and then made basic with NaOH pellets and extracted four times with $CHCl_3$. The $CHCl_3$ solutions were combined, dried over Na_2SO_4, evaporated to a small volume in vacuo, and then allowed to evaporate in air at room temperature. Samples of the residue (purified total alkaloid mixture) were weighed and analyzed by GC-MS. This provided an approximate total alkaloid content (by weight) and a relative individual alkaloid content (by relative ratios of GC-MS peaks).

Engelmann spruce samples were composite collections except for those from the single Engelmann spruce at the FR 622/630 junction (see above). These were analyzed as follows. Approximately 2-g weighed samples were ground in liquid nitrogen and allowed to stand with occasional shaking in 10 ml MeOH for three days. For the insect or insect frass samples, the methanol contained three drops of conc. aq. HCl to convert any alkaloids present as free bases (because of the usual larval gut pH 9) to acid salts. Samples were filtered and

evaporated to near dryness in a round bottom flask. Ten milliliters of 0.5 M aq. HCl were added to each, and each was extracted three times with 10 ml $CHCl_3$. The retained aqueous layer was made basic to pH 11 with 1 M K_2CO_3 and extracted (4 × 10 ml) with $CHCl_3$. The $CHCl_3$ layers were combined, dried over Na_2SO_4, filtered, and evaporated to about 2–3 ml that was transferred to a small vial and carefully evaporated with a jet of N_2 to a premarked 1.0 ml level. This solution was analyzed quantitatively by GC with the use of response factors based on an average determined for **1**, **2**, and **5**. GC and GC-MS methods and parameters were similar to those previously described (Tawara et al., 1993; Todd et al., 1995).

RESULTS

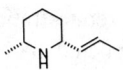
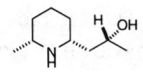
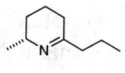
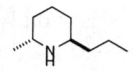
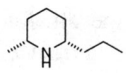
Colorado Blue Spruce. A composite sample of new growth needles being consumed by budworms (56.5 g fresh weight) yielded 119 mg of purified alkaloid mixture composed of approximately 60% **2**, 16% **3**, 9% **1**, 7% **5**, 4% **10**, and about 4% unknown alkaloids. A group of budworms (3.20 g live weight total; individuals not weighed) collected from several spruce trees was analyzed and no alkaloids were detected. Larval frass (fresh weight 6.45 g) from the same trees yielded 6.6 mg of a purified alkaloid mixture, which was found to be composed of approximately 74% **2**, 25% **3**, and 1–2% combined of **4** (or **7**), **5**, **9**, and **10**.

The needle analysis of these trees was comparable to that reported (Todd et al., 1995) for first year needles of two Fort Collins blue spruce trees, with the exceptions that the Fort Collins trees contained appreciable **8**, which was not detected in the present collection, and that less **1** was found in the Fort Collins samples. Larval frass did not contain **1**, but otherwise was relatively similar in alkaloid content and pattern to the needles, considering the nature of the composite samples and that the analyses were performed by a 1991 procedure.

Engelmann Spruce. Budworm larvae from the composite collection and larvae and pupae from tree 9 yielded no detectable alkaloids. Alkaloids were found, however, in frass and infested needles of both systems (Table 1). For comparison, alkaloids in uninfested needles from tree 9 were also determined (Table 1). Of particular interest was the finding that *cis*-pinidine, **1**, and *cis*-dihydropinidine, **2**, were major components of the budworm frass even though neither was detected in tree needles. This was true of frass from budworms feeding on both tree 9 and the composite collection.

A previous study established that there was a quantitative inter- and intra tree variability in piperidine alkaloid patterns and content (Todd et al., 1995). Replicate samples were not analyzed in the present work, however, and detailed quantitative comparisons of alkaloid content would not be justified without fur-

TABLE 1. MAJOR ALKALOIDS IN SPRUCE NEEDLES AND FRASS FROM SPRUCE BUDWORMS CONSUMING SPRUCE NEEDLES

	Alkaloid (μg/g, fresh weight basis)				
					
Sample	**1**	**5**	**6**	**8**	**12**
Composite infested needles	ND*	490	45	41	ND
Composite frass	550	510	33	30	72
Tree 9 infested needles	ND	260	220	150	ND
Tree 9 uninfested needles	ND	400	340	170	ND
Tree 9 frass	2500	1300	ND	440	1700

*ND = not detected.

ther study. Although expected intertree variability for the composite collection would make quantitative comparisons for that sample particularly hazardous, some of the data for tree 9 may be useful to emphasize. Of particular note was the relatively high content of the 1,6-imine, **6**, in the needles (both infested and uninfested) and its absence from the frass of budworms consuming those needles.

A number of the minor alkaloids (Figure 1) were also detected in small amounts in some of the samples (Kamm, 1997). A piperidine of unknown structure (MW 153 by MS) was found in needles of both collections (19–210 μg/g) but not in the frass from budworms on tree 9. It was barely detectable (1 μg/g) in the composite frass sample. Alkaloid **3** has the same molecular weight as this unknown and a similar, but not identical, GC retention time and has a different MS fragmentation pattern.

DISCUSSION

The first important result of this work was negative, in the sense that we found no evidence that budworm larvae sequester any of the ingested alkaloids. Beetles contain some of these alkaloids in hemolymph or defensive glands (Brown

and Moore, 1982; Eisner et al., 1986; Proksch et al., 1993; Attygalle et al., 1993). At least one of them, **2**, (Eisner et al., 1986) has been shown to be directly deterrent to ants and spiders, and we thought that budworms might take advantage of such activity by sequestering **2**. This proved not to be the case. When **2** was encountered by larvae feeding on Colorado blue spruce needles, the alkaloid was simply excreted. The lack of sequestration of any possibly deterrent or toxic alkaloids might have been expected based upon the cryptic nature of spruce budworms as opposed to the aposematic coloring of beetles that synthesize some of the same alkaloids de novo. Pine butterflies, *Neophasia menapia* (Felder and Felder), whose larvae consume ponderosa pine needles (which also contain piperidine alkaloids), have wings that are patterned boldly in black and white, and the females also have red striping on the wings. Males and females of this aposematic conifer feeder also were found in preliminary work to be devoid of any sequestered piperidines (Kamm, 1997).

A number of other piperidine alkaloids, structurally related to those in this study, are known to have toxic or deterrent activity (Figure 2). Thus, solenopsin A, **13**, is one of the active components of fire ant venom (MacConnell et al., 1971), while **14** was reported to have strong insecticidal activity against termites and ants (Jones et al., 1990). Coniine, **15**, from the plant *Conium maculatum* has a mammalian LD_{50} of less than 1 mg/kg, and it is also the insect-paralyzing component of a pitcher plant, active at the nanogram level (Mody et al., 1976). Both **15** and coniceine, **16**, are potent teratogens in mammals, *cis*-Pinidine, **1**, was active as a teratogen in a frog embryo test (Tawara et al., 1993), but activity against insects has not been explored.

Isolation of large quantities of *cis*-pinidine, **1**, and *cis*-dihydropinidine, **12**, from frass of budworms feeding on Englemann spruce needles was remarkable since neither alkaloid was found in needle samples (Table 1). A small amount

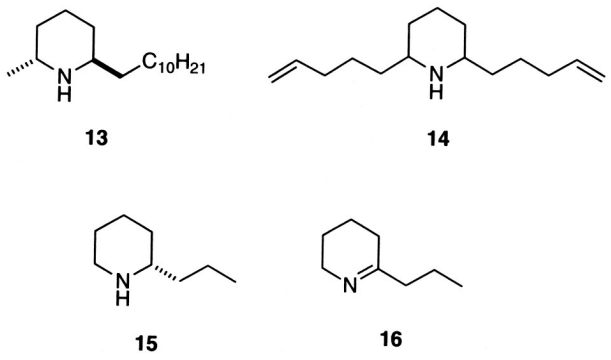


FIG. 2. Some bioactive piperidine alkaloids.

of **1** was found in needles of blue spruce, but no **12**, although a trace of **12** was found by Todd et al. (1995) in blue spruce samples. Genesis of **12** might be from budworm reduction of the imine ***6***, which was present in the needles but absent from the frass. In the plant biosynthesis, we have presumed a reduction of **6** by $NADPH^+$ to be the step that gives *trans* compound **8** (Stermitz et al., 1994), but a budworm enzyme might produce the opposite stereochemistry (**6** to **12**). A reductase catalyzes a coniceine–coniine reduction–oxidation equilibrium (**15–16**) in *Conium maculatum* (Roberts, 1975). Alkaloid **1** might arise from dehydration of alcohol **5.** In general, herbivores utilize oxidations to convert ingested secondary metabolites to more polar, more easily excreted compounds, but metabolic reductases also are known (Brattsen, 1992).

Despite the large literature on spruce budworms, we could find none, other than pheromone studies, that reported chemical analysis of the insect or its secretion products. It seems likely that more detailed studies of budworm metabolism and the possible ecological effects of the conifer piperidine alkaloids are warranted.

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HEADSPACE ANALYSIS IN CHEMICAL ECOLOGY: EFFECTS OF DIFFERENT SAMPLING METHODS ON RATIOS OF VOLATILE COMPOUNDS PRESENT IN HEADSPACE SAMPLES

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Abstract—The effects that different headspace sampling methods have on the analysis of the ratios of compounds present in the headspace of a synthetic mixture and a biological sample were evaluated using the following methods: solid-phase microextraction (SPME), a syringe, and the porous polymers Porapak Q, Tenax TA, and Hayesep Q (thermal and solvent desorption). The performance of each method was only evaluated for a certain sampling period and under given experimental conditions. The test mixture comprised pentan-3-ol, 4-penten-1-ol, (*E*)-2-hexenal, hexan-1-ol, heptanal, (1*S*,5*S*)-(−)-α-pinene, 6-methyl-5-hepten-2-one, hexyl acetate, (*R*)-(+)-limonene, and undecane. SPME gave significantly different ratios of pentan-3-ol, 4-penten-1-ol, hexan-1-ol, heptanal, (1*S*,5*S*)-(−)-α-pinene, 6-methyl-5-hepten-2-one, hexyl acetate, limonene, and undecane compared with the other methods. No significant differences were observed between the syringe and the porous polymers or between the three different porous polymers used. The method of release of the trapped analytes from the polymers, thermal or solvent desorption, did not affect the ratios of compounds. The effects that different sampling methods have on the ratios of compounds present in the headspace of a biological sample, ripe banana, were evaluated. The headspace was sampled using SPME and the porous polymers Porapak Q and Tenax TA (thermal desorption). The following compounds were identified in the headspace of ripe bananas: 2-methylbutyl acetate, pentyl acetate, 2-methylbutyl propanoate, butyl butanoate, and pentyl butanoate. SPME gave significantly different ratios of 2-methylbutyl acetate, pentyl acetate, butyl butanoate, and pentyl butanoate when compared to Porapak Q and Tenax TA. No significant differences on the ratios of compounds present in the headspace of ripe banana

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were observed when the two polymers were compared. The use of different sampling methods in headspace analysis of biological samples is discussed.

Key Words—Headspace analysis, volatile chemicals, bananas, solid-phase microextraction, porous polymers, gas syringe, Porapak Q, Tenax TA, Hayesep Q, thermal desorption, solvent desorption.

INTRODUCTION

Volatile compounds produced by an organism and released into the atmosphere can be perceived at a distance from their release point by a number of other organisms of the same or different species, thus mediating a wealth of interactions. Identity, quantity, and relative amounts (ratios) of such chemicals convey specific information to the receivers about the identity and physiological state of the emitter (Cardé and Bell, 1995; Visser, 1986).

The techniques employed in chemical ecology for identification and quantification of volatile semiochemicals are the common preconcentration techniques used for headspace analysis in gas chromatography, modified specifically to serve the needs of the living organisms under study (for an overview see: Núñez et al., 1984; Thomas, 1991; Charmas et al., 1994). Routinely, dynamic headspace analysis is employed, although static headspace analysis is sometimes used (Jakobsen, 1997). In dynamic headspace analysis, the sample is confined in an entrainment chamber and a carrier gas (usually purified air) is passed over the sample. The volatile chemicals released by the sample are carried by the gas to a solid trap, usually a porous organic polymer such as Porapak Q, Tenax TA, or activated charcoal, where the analytes are adsorbed and preconcentrated. The desorption of the analytes from the solid trap for gas chromatography can be achieved by either elution with a solvent (solvent desorption) or rapid heat treatment (thermal desorption). In static headspace analysis, the sample is tightly closed into a vessel, where it comes into equilibrium with its vapors at a predetermined temperature. The headspace can be sampled using a syringe or a similar device and injected directly into the gas chromatograph.

Solid-phase microextraction (SPME) is a new isolation method that can be used to extract and concentrate a wide range of volatile and semivolatile organic compounds from various matrices such as air, water, and soil (Zhang and Pawliszyn, 1993). The technique was developed for sampling organic contaminants in water by directly immersing the fiber into the sample, but it has also been applied with success to sampling headspace of liquid and solid samples. SPME has also found applications in environmental and flavor analysis (Steffen and Pawliszyn, 1996) and has been used for recording the release of insect pheromones (Malosse et al., 1995). The technique uses a polymer-coated fused silica fiber that has the ability to adsorb chemicals relying on a three-phase equilibrium

between the sample, its vapors, and the fiber. The analytes adsorbed onto the SPME fiber are released by thermal desorption.

Volatile chemicals released by biological samples are not released continuously, but most of them show a time course of release related to the physiological state of the organism. Some compounds are only released in very short windows of time. The aim of this research is to evaluate, for a given sampling period and under given experimental conditions, the effects that different headspace sampling methods have on the ratios of volatile compounds present in the headspace of biological samples. We concentrate on ratios of compounds because of their importance in insect behavior. Using a standard mixture composed of 10 compounds frequently encountered in studies of chemical ecology, we evaluated the impact of sampling by SPME, a syringe, and three different organic porous polymers on the ratios of compounds present in the headspace. In the case of the organic polymers, solvent and thermal desorption were compared as different desorption methods to release the trapped analytes. SPME was also compared with the porous polymers by using a biological sample (a ripe banana).

METHODS AND MATERIALS

Test Mixture. The test mixture comprised the following compounds: pentan-3-ol, 4-penten-1-ol, (*E*)-2-hexenal, hexan-1-ol, heptanal, (1*S*,5*S*)-(−)-α-pinene, 6-methyl-5-hepten-2-one, hexyl acetate, (*R*)-(+)-limonene, and undecane. The test compounds were purchased from commercial sources (Aldrich Co. Ltd., Fluka Chemie AG) and were ≥97% pure. Five microliters of each compound were placed in a 2-liter glass headspace jar and the jar was sealed with a Teflon-coated silicone septum. The headspace was sampled using the following methods: (1) solid-phase microextraction (SPME) using the 100-μm poly(dimethylsiloxane) fiber (Supelco, Inc.); (2) a glass syringe (5 ml); (3) the porous polymers Porapak Q (50 mg, 60–80 mesh), Tenax TA (50 mg, 60–80 mesh), and Hayesep Q (50 mg, 60–80 mesh), where the trapped analytes were released using thermal desorption; and (4) the porous polymers Porapak Q (50 mg, 60–80 mesh), Tenax TA (50 mg, 60–80 mesh), and Hayesep Q (50 mg, 60–80 mesh), where the trapped analytes were released by solvent desorption. After production, the mixture was left to equilibrate for 1 hr before sampling. The sampling procedure had the following order: SPME, syringe, polymers for thermal desorption, polymers for solvent desorption. Half an hour was left between sampling by the different methods to allow equilibration of the headspace. The experiments were conducted in a temperature-controlled room at 15°C ± 3°C and the headspace of the original mixture was sampled three times.

Biological Sample. Two ripe bananas (yellow) (*Musa* sp.) were confined in a 2-liter glass entrainment jar. The jar was sealed as before and left to

equilibrate for 2 hr. The headspace was sampled using: (1) SPME [100 μm poly(dimethylsiloxane) fiber], (2) Porapak Q (50 mg, mesh 60–80), and (3) Tenax TA (50 mg, mesh 60–80). The volatiles trapped on the porous polymers were released by thermal desorption. The experiment was conducted in a temperature controlled room (15°C) and was repeated three times on the same day using the same sample banana. To avoid depletion of the analytes by previous sampling, the time between sampling by the different methods was 1 hr.

Sampling. The SPME device (manual holder) pierced the top septa of the jar, and the fiber was extended into the headspace of the sample. For the test mixture, the fiber was left to adsorb for 30 sec and for the banana sample, the fiber was left to absorb for 1 min. After adsorption, the fiber was immediately introduced into the injector where it was left to desorb for 5 min (temperature of injector 250°C).

For thermal desorption, the porous polymers were packed individually into glass tubes (8 cm length, 3 mm ID) and were kept in place by plugs of silanized glass wool. The tubes used were the glass injector port liners of the temperature vaporization injector. Volatile chemicals from the headspace of the test mixture were drawn onto the polymer for 30 sec by using a mini pump (NMP 08 S, 6V, ATAS, Inc.) equipped with a flowmeter (flow: 20 ml/min). Volatile chemicals from the headspace of the bananas were drawn onto the polymer for 1 min by using a mini pump under the same conditions. After the end of the entrainment, the tube was immediately placed into the programmed temperature vaporization (PTV) unit for analysis.

For solvent desorption, the polymers were packed individually into glass tubes (8 cm length, 2.5 mm ID) and kept in place by using plugs of silanized glass wool. Volatile chemicals from the headspace of the test mixture were drawn onto the polymer for 30 sec by using the mini pump and flowmeter (flow: 20 ml/min). Similarly, the volatile chemicals from the headspace of the banana were drawn onto the polymer for 30 sec, again by using a mini pump and flowmeter (flow: 20 ml/min). After the end of entrainment, the trapped analytes were eluted from the polymer with 500 μl of redistilled diethyl ether. Using the gas syringe, 5 ml of the headspace of the test mixture were drawn and immediately injected into the PTV unit equipped with an empty glass liner.

Gas Chromatography and Mass Spectrometry. For the SPME, gas chromatography was performed on an AI 92 model gas chromatograph fitted with a nonpolar HP-1 methylsilicone column (30 m, 0.53 mm ID, 2.65-μm film thickness) and a flame ionization detector (FID). The carrier gas was nitrogen and the oven temperature was maintained at 40°C for 5 min (the period in which the fiber was desorbing) and was then programmed at 10°C/min to 250°C.

The samples collected for thermal and solvent desorption of the porous polymers, and the gas syringe were analyzed on an AI 94 model gas chro-

matograph fitted with an Optic 400 PTV injector (ATAS, Inc.) (Lewis et al., 1995; Bartle and Lewis, 1996), the nonpolar HP-1 methyl silcone column (30 m, 0.53 mm ID, 2.65-μm film thickness), and a FID. Desorption inside the PTV unit was performed using a rapid temperature ramp starting at 30°C (temperature of the PTV injector during introduction of the sample) and reaching 200°C in 1 min. The carrier gas was nitrogen. The oven temperature was held at 30°C for 1 min and then programmed at 10°C/min to 250°C where it stayed for 30 min. The compounds present in the volatile profile of bananas were tentatively identified using coupled gas chromatography-mass spectrometry (GC-MS) (VG Autospec, Fisons). Ionization was by electron impact at 70 eV, 250°C, and the GC column (HP-1, cross-linked methyl silicone, 50 m × 0.32 mm ID) was maintained at 30°C and then programmed at 5°C/min to 250°C.

The ratios of compounds were represented by GC peak areas, normalized so that the total peaks of interest equaled 100. The effects of the different sampling methods on the ratios of test compounds were compared using the t test for means of paired samples ($P \leq 0.01$).

RESULTS

Test Mixture. SPME significantly affected ($P \leq 0.01$) the ratios of the following compounds present in the headspace of the test mixture when compared to the porous polymers (thermal and solvent desorption): pentan-3-ol, 4-penten-1-ol, hexan-1-ol, 6-methyl-5-hepten-2-one, hexyl acetate and limonene (Table 1; Figures 1 and 2). The ratios of pentan-3-ol, hexan-1-ol, (1*S*,5*S*)-(−)-α-pinene, 6-methyl-5-hepten-2-one, hexyl acetate, limonene, and undecane were affected when SPME was compared to the syringe. No significant differences ($P \leq 0.01$) were observed between the syringe and the polymers or between the three different polymers. The method of release of the trapped analytes, thermal or solvent desorption, did not significantly affect the ratios of compounds ($P \leq 0.01$).

Biological Sample. Five compounds present in the headspace of the ripe banana were tentatively identified by GC-MS: 2-methylbutyl acetate, pentyl acetate, 2-methylbutyl propanoate, butyl butanoate, pentyl butanoate. SPME significantly ($P \leq 0.01$) affected the ratio of 2-methylbutyl acetate, pentyl acetate, butyl butanoate, and pentyl butanoate when compared to Porapak Q and Tenax TA. No significant differences ($P \leq 0.01$) in the ratios of the identified compounds were observed between the two polymers (Table 2). The ratio of the compound 2-methylbutyl propanoate was not significantly affected by the sampling methods used.

TABLE 1. EFFECTS OF DIFFERENT SAMPLING METHODS ON RATIOS (MEAN ± SE) OF COMPOUNDS PRESENT IN HEADSPACE OF TEST MIXTURE ($N = 3$)[a]

Compound	Syringe	SPME	Thermal desorption			Solvent desorption		
			Porapak	Hayesep	Tenax	Porapak	Hayesep	Tenax
Pentan-3-ol[ab]	23.87 ± 0.26	8.17 ± 0.19	23.97 ± 0.39	24.07 ± 0.39	24.12 ± 0.17	24.9 ± 0.60	25.38 ± 0.53	24.02 ± 0.46
4-Penten-1-ol[b]	9.87 ± 0.49	8.77 ± 0.11	12.41 ± 0.14	12.38 ± 0.27	12.52 ± 0.03	10.67 ± 0.26	10.23 ± 0.46	11.07 ± 0.28
(*E*)-2-Hexenal	16.1 ± 0.21	15.89 ± 0.15	15.21 ± 0.12	15.27 ± 0.06	15.28 ± 0.15	15.47 ± 0.09	15.67 ± 0.12	15.28 ± 0.05
Hexan-1-ol[ab]	2.46 ± 0.27	5.79 ± 0.01	3.66 ± 0.14	3.98 ± 0.23	3.70 ± 0.22	3.54 ± 0.12	3.54 ± 0.12	3.74 ± 0.12
Heptanal	1.15 ± 0.04	1.38 ± 0.03	1.01 ± 0.03	1.10 ± 0.02	1.14 ± 0	1 ± 0.05	1.01 ± 0.09	1.03 ± 0.06
(1*S*,5*S*)-(−)-α-Pinene[a]	25.95 ± 0.50	23.03 ± 0.3	23.98 ± 0.21	23.9 ± 0.32	23.30 ± 0.53	24.86 ± 0.13	24.78 ± 0.28	24.70 ± 0.31
6-Methyl-5-hepten-2-one[ab]	3.69 ± 0.25	7.58 ± 0.20	3.64 ± 0.12	3.69 ± 0.04	3.63 ± 0.19	3.73 ± 0.13	3.24 ± 0.30	3.79 ± 0.10
Hexyl acetate[ab]	3.28 ± 0.53	7.59 ± 0.23	3.46 ± 0.14	3.48 ± 0.17	3.58 ± 0.18	3.64 ± 0.16	3.13 ± 0.36	3.67 ± 0.11
(*R*)-(+)-Limonene[ab]	9.2 ± 0.04	15.46 ± 0.3	8.11 ± 0.28	8.01 ± 0.31	8.22 ± 0.25	8.48 ± 0.26	7.69 ± 0.70	8.59 ± 0.18
Undecane[a]	4.07 ± 0.23	6.32 ± 0.23	4.46± 0.49	3.87 ± 0.41	4.38 ± 0.44	3.65 ± 0.19	3.15 ± 0.18	3.79 ± 0.24

[a]Ratios of compounds showing significant differences ($P \leq 0.01$) between [a]syringe and SPME, and [b]SPME and polymers.

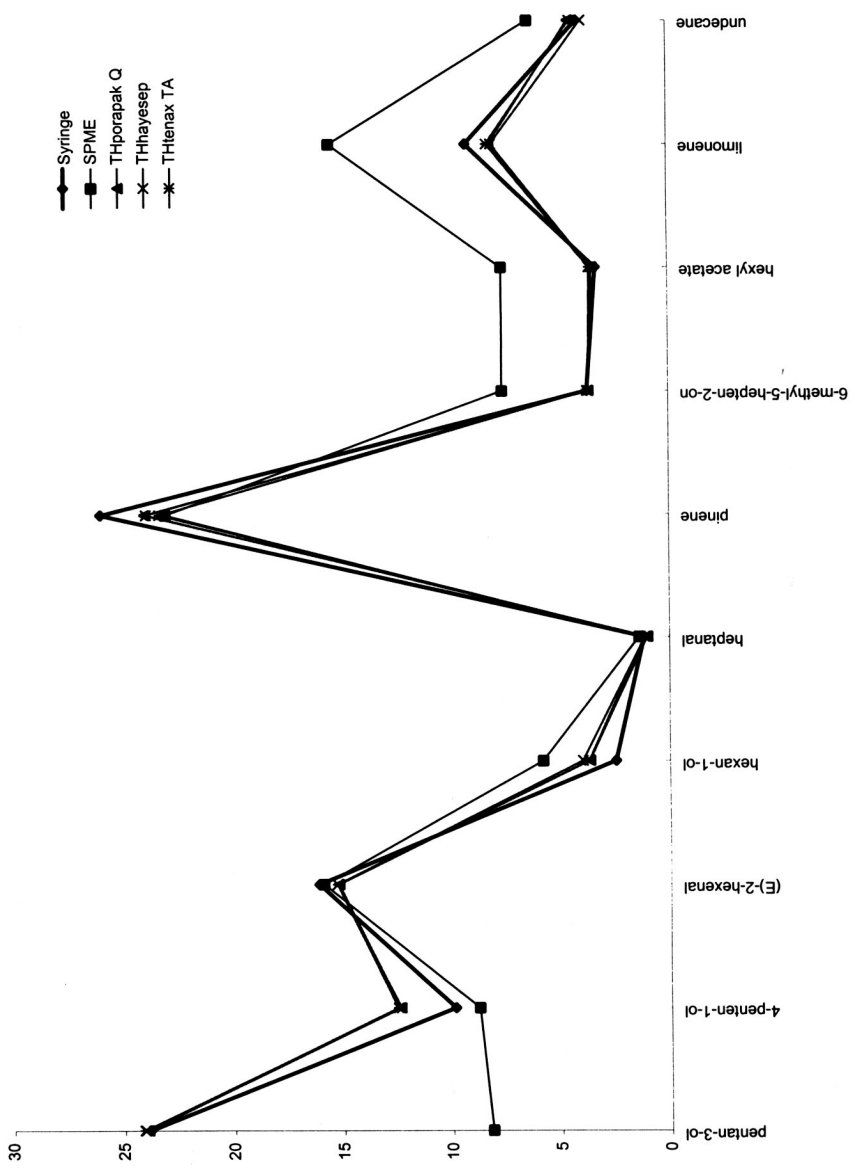

FIG. 1. Sampling by SPME deviated significantly from both sampling by syringe and porous polymers (TH = thermal desorption).

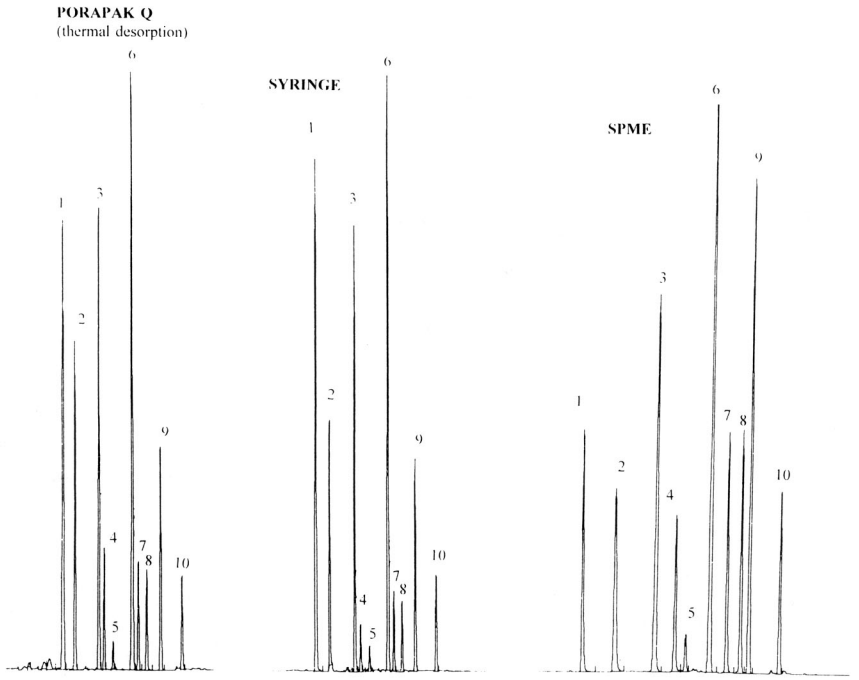

FIG. 2. Chromatograms (HP-1, 30 m) of headspace of the test mixture using Porapak Q, syringe, and SPME. Peak 1, pentan-3-ol; 2, 4-penten-1-ol; 3, (*E*)-2-hexenal; 4, hexan-1-ol; 5, heptanal; 6, (1*S*,5*S*)-(−)-α pinene; 7, 6-methyl-5-hepten-2-one; 8, hexyl acetate; 9, (*R*)-(+)-limonene; 10, undecane.

DISCUSSION

Headspace samples can be transferred to the GC injector in two ways. Direct transfer of a volume of the headspace is delivered to the injector by means of a syringe or a similar device. Indirectly, a volume of the headspace is transferred onto a cold trap or a medium with adsorbing properties (SPME, porous polymers, etc.) and the adsorbed analytes are released into the chromatographic column by either thermal or solvent desorption (for an overview see: Núñez et al., 1984). In our study we sampled the headspace of the test mixture as a static system by using a direct way, a syringe, and two indirect ways, the SPME and the porous polymers. Comparison of the three showed that the results from the syringe were very similar to that of the polymers and that SPME deviated markedly from both the syringe and porous polymers. Matich et al. (1996) recorded differences on the ratios of compounds present in

TABLE 2. EFFECTS OF DIFFERENT SAMPLING METHODS ON RATIOS (MEAN ± SE) OF COMPOUNDS PRESENT IN HEADSPACE OF RIPE BANANA

Compounds	SPME (N = 3)	Thermal desorption: Porapak Q (N = 3)	Thermal desorption: Tenax TA (N = 2)
2-Methylbutyl acetate[a]	15.37 ± 0.29	23.74 ± 0.30	25.53 ± 0.94
Pentyl acetate[a]	19.58 ± 0.43	25.21 ± 0.04	25.47 ± 0.06
2-Methylbutyl propanoate	15.43 ± 0.04	15.28 ± 0.17	15.02 ± 0.34
Butyl butanoate[a]	8.08 ± 0.28	5.10 ± 0.24	5.27 ± 0.65
Pentyl butanoate[a]	41.50 ± 0.47	30.77 ± 0.21	30.67 ± 0.69

[a]Ratios of compounds showing significant differences between SPME and the two polymers ($P \leq 0.01$).

the headspace of Granny Smith apples between sampling by SPME and the porous polymer Tenax TA. Field et al. (1996) also observed differences on the ratios of compounds present in the headspace of hops between sampling by SPME and a syringe.

SPME is an equilibrium process that relies on a three-way equilibrium between the sample, its vapors, and the fiber. The concentration of an analyte on the fiber is partly affected (Zhang and Pawliszyn, 1993; Yang and Peppard, 1994; Chai and Pawliszyn, 1995; Ai, 1997; Bartelt, 1997; Martos and Pawliszyn, 1997) by: (1) The chemical properties of the analytes—in addition to the usual absorption mechanism, the fiber has a number of binding sites with especially high affinity for nitrogen- and hydroxy-containing compounds and that affinity operates in addition to the usual absorption mechanism. (2) The equilibrium time—equilibrium time is the time needed for the concentration of an analyte in the headspace to be directly proportional to its concentration on the fiber. The equilibrium time depends on the nature of the compound, with longest equilibrium time exhibited for the highest molecular weight compounds. (3) The experimental conditions—SPME is sensitive to experimental conditions, and any changes may affect the distribution coefficient and adsorption rates of the analytes. (4) Concentration of the analytes—from theory, no concentration dependence is expected, but Bartelt (1997), in his study on calibration factors, found that for nitrogen- and hydroxy-containing compounds there was a concentration dependence.

The reasons for the observed differences between SPME and the other methods used in our experiment have not been established. The experiments were conducted for given sampling times, 30 sec for the standard mixture and

1 min for the banana sample, and those times may not have been long enough for the analytes in the headspace to equilibrate with the fiber. Sampling time by SPME is determined by the time needed for the compounds present in the headspace to equilibrate with the fiber, with different compounds showing different equilibrium times. Sampling times using the polymers and the syringe are mainly determined by the concentration of the analytes in the headspace. In highly concentrated headspaces the polymers and the syringe are able to operate in a very short time. SPME is also sensitive to experimental conditions, and 15°C, the temperature used in this study, may not have been the optimum temperature for SPME to be effective.

The most important question is what determines the sampling period and the experimental conditions under which the sampling is performed when we are dealing with a biological sample, e.g., living organisms. Obviously the sample should determine both parameters because organisms have certain temperature and humidity ranges and also release the chemicals of interest over particular time periods. In the case of SPME and because of the way that operates, SPME determines those very important parameters that should be determined by the sample and not by the method.

For the test mixture, sampling using a syringe came much closer to sampling using the polymers. Two compounds showed slightly different ratios: 4-penten-1-ol and hexan-1-ol when the syringe was compared to the porous polymers. These differences may be due to adsorption of some molecules of the analytes onto the glass walls of the syringe (Kolb, 1975). No differences were observed between the different polymers used and the different methods of release of the analytes from the polymers (solvent or thermal desorption). Advantages associated with solvent desorption are that it results in a liquid sample that can be stored in the freezer, sealed under nitrogen in glass ampules, and used a number of times when replication is required such as coupled runs of GC-EAD (Wadhams, 1990), peak enhancement coinjections (Pickett, 1990), and bioassays. Quantification is also facilitated by having a liquid sample in which an internal standard can be readily added. However, only a small volume of the sample can be injected at a time into a GC, and the solvent peak can mask compounds with short retention time. On the other hand, thermal desorption, although more sensitive since all sample goes through GC column, results in no sample at the end of the run. Two problems associated with thermal desorption are the difficulty of introducing an internal standard for quantification, a problem associated not only with thermal desorption of polymers but also when using a syringe or a SPME fiber, and the temperature of desorption may affect the thermal stability of some analytes. In our study, the maximum temperature used was 200°C for Tenax TA and 180°C for Porapak Q and Hayesep Q, with no obvious effects on the thermal stability of the compounds. Although polymers

of the Porapak series are not normally used in thermal desorption because of decomposition, if the temperature does not exceed the maximum described temperature of the manufacturers, polymers of the Porapak series operate well in thermal desorption (personal observations). As we have discussed above, all methods used in sampling the headspace of a sample have advantages and disadvantages and the results obtained are strongly related to the physical and chemical properties of the trapping agent, the concentration of the analytes in the headspace, the chemical properties of the analytes, and the distribution of the analytes between the sample, its headspace, and the adsorbent (Núñez et al., 1984).

In our study, for both the test mixture and bananas, all methods recorded the presence of compounds. SPME deviated markedly in recording the ratios of compounds from both the syringe and the porous polymers. Because SPME is a new sampling method and is still under study, we believe that it should be used in conjunction with another sampling method.

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CHEMICAL ECOLOGY OF THE DEFENSE OF TWO NYMPHALID BUTTERFLY LARVAE AGAINST ANTS

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Abstract—We analyzed the behavioral responses of the ants *Camponotus rufipes* and *Solenopsis geminata* towards all instars of *Dione junio* and *Abananote hylonome*. We also analyzed ant behavior towards hexane extracts of larvae and extracts of the spines and neck glands of the fifth instars of both species and identified the chemical compounds present. Larvae of both species were repellent to ants from the first instar onward. Later instars survived ant attacks better than earlier instars. The spines and neck glands of the larvae influenced the behavior of *C. rufipes*. The chemical compounds contained in the hexane extracts of whole first and fifth instars and in the spines and neck glands of fifth instars were principally carboxylic acids and terpenes. Further bioassays confirmed the repellent effect of some of these acids toward ants.

Key Words—Ants, *Camponotus rufipes*, *Solenopsis geminata*, *Dione junio*, *Abanotes hylonome*, Lepidoptera, Nymphalidae, repellency, defense.

INTRODUCTION

Ants are important predators of insects both in natural and agricultural ecosystems (Pavis et al., 1992; Way and Khoo, 1992). Lepidopteran larvae are an attractive prey, due to their relative immobility and soft cuticle. Nevertheless, many larvae possess an array of chemical defenses effective against both invertebrate and vertebrate predators (Brower et al., 1967; Weatherston et al., 1979; DeVries, 1987; Montllor et al., 1991; Attygalle et al., 1993; Deml and Dettner, 1993; Dyer, 1995). Although the study of larval chemical defenses has been

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directed mainly towards vertebrate predators, in recent years the importance of invertebrate predators (including ants) has been recognized. Several studies demonstrate that the chemistry of the larva is an important factor in their defense against invertebrates (Bernays, 1988; Bernays and Cornelius, 1989; Dyer and Floyd, 1993; Dyer, 1995). Defensive compounds that are effective against ants have been found in the integument of some larvae (Montllor et al., 1991; Nickisch-Rosenegk and Wink, 1993), in the hemolymph (Deml and Dettner, 1994, 1995), and released from specialized glands (Eisner et al., 1972; Honda, 1983; Deml and Dettner, 1993, 1994, 1995). Compounds identified in lepidopteran larvae that are an effective defense against ants include fatty acids (Honda, 1981, 1983; Howard et al., 1982), terpenes (Honda, 1983), ketones (Eisner et al., 1972), aldehydes (Deml and Dettner, 1994, 1995), sulfur alellochemicals (Nowbahari and Thibout, 1992), and proteins (Reimann, 1983).

METHODS AND MATERIALS

Species of Ants and Butterfly Larvae Used in This Study. Camponotus rufipes (Formicinae) is a common species of ant widely distributed in South America. Although it forms colonies on the ground, it is commonly found in the vegetation and thus probably regularly encounters lepidopteran larvae. *Solenopsis geminata* (Myrmecinae), one species of fire ants, is also common in South America. Although this species is found less often foraging in vegetation, it is aggressive and has been cited as a possible biological control for certain insect pests (Risch and Carroll, 1982a, b; Perfecto, 1991; Jaffe et al., 1991).

The butterfly larvae studied were *Dione junio* and *Abananote hylonome*, both in the family Nymphalidae, and belonging to subfamilies known to be highly cyanogenic (Heliconiinae and Acraeinae, respectively) (Brown and Francini, 1990). Cyanogenic compounds are probably more effective against birds and other vertebrate predators (Brower et al., 1963; Nahrstedt and Davis, 1982, 1985; Franzl and Naumann, 1985). Both *D. junio* and *A. hylonome* are spiny and possess an eversible neck gland situated ventrally in the prothorax, just in front of the first pair of legs. It is probable that they contain other defensive compounds that are secreted via the spines and/or neck gland when the larva is molested (personal observation).

A. hylonome larvae were collected from their host plants *Verbania* sp. (Compositae), which are commonly found in the surroundings of the Simón Bolívar University, Sartenejas, Caracas, Venezuela. *D. junio* larvae were collected from two passion fruit plantations (*Passiflora edulis*, Passifloraceae) located in Mesa de Urape, Dtto. Acevedo, and in Las Maravillas, Dtto. Brión, Miranda State, Venezuela. The larvae were transported to the laboratory in plastic containers in a cool box. The two species were maintained separately in

glass containers (20 cm × 20 cm × 50 cm) at a constant temperature of 25°C and a relative humidity of 70–80%. They were fed daily with fresh leaves from their respective host plants.

Three colonies of each of the two ant species, *C. rufipes* and *S. geminata*, were collected in the surroundings of the university and maintained in the laboratory in plastic bowls of 1 m diameter and 50 cm depth, at the same temperature and humidity as the butterfly larvae. A metal tripod 40 cm high was put in each bowl on which we placed a glass platform of 20 cm^2 as the foraging area. Two plastic lids of 5 cm diameter and 1 cm depth were placed on the glass platforms, and in these we put pieces of soft netting, one soaked in water and the other soaked in a 1 : 1 ratio of water and honey. In addition, every two days a larva of *Tenebrio molitor* (Coleoptera: Tenebrionidae) was placed on the foraging area. These beetles were raised in the laboratory in an eight-liter bucket, on a commercial diet of oatmeal and wheat-germ.

Bioassays with Live Butterfly Larvae (Bioassay I). The objective of this bioassay was to discover whether or not the live lepidopteran larvae possess chemical defenses against ants, to evaluate the effect of these defenses on each species of ant, and to investigate the development of the defenses in the different larval stages. We placed a larva (*D. junio* or *A. hylonome*) on the foraging area of one of the colonies of each species of ant and observed the behavior of the ants during the first 20 interactions. We compared the responses of the ants towards the butterfly larva with their responses towards a larva of *T. molitor* (control). We also noted whether the butterfly larva survived the bioassay. If the larva died during the bioassay, we noted whether the ants carried it to the nest or left it on the glass platform. The butterfly larva and the control larvae could not be placed simultaneously on the foraging area due to the difficulty of distinguishing which of the two was causing the behavior of the ants. For this reason, the butterfly larva and the control were tested separately, one after the other (the first to be tested was chosen randomly), with each pair of bioassays being considered an experiment. Five replicates were performed for each species of larva and for each instar, with alternation between colonies of ants so that no ant colony participated in more than two experiments per day.

The results of the five replicates were summed and analyzed with a chi-square test (Sokal and Rohlf, 1981). These data were further analyzed to obtain a Spearman correlation for each species of ant. In each case, the following variables were correlated: the responses of alarm, inspection, repellency, and attack by ants in the presence of the larva and the survival of the larva. The indices used for each variable are explained in greater detaill in the Results section.

Bioassays with Hexane Extracts (Bioassay II). In order to characterize the chemical nature of the defense systems of *D. junio* and *A. hylonome* and to evaluate the contribution of the spines and neck glands to their chemical de-

fenses, we repeated experiments with hexane washes of whole larvae and hexane extracts of the spines and the neck glands of each species. Preliminary tests showed that hexane washes produced better behavioral results compared to methanol washes, probably because they also extracted a lower number of compounds compared to methanol. The washes (or extracts) of whole larvae of each instar of the two species were prepared in the following way: 10 individuals of an instar were placed in glass vials (1.5 × 3 cm) and 1 ml of solvent was added. After a period of time, the larvae were removed and the solution was concentrated to 100 μl before each bioassay. The extracts of the spines were prepared by placing the spines from three individuals or the neck glands from five individuals in a glass vial, adding 1 ml of hexane, and subjecting the structures to ultrasonic waves in an ultrasonic bath for 7 min in order to improve extraction. The solvent volume later was evaporated with N_2 gas to 100 μl. The bioassays were similar to those already described by bioassay I, but in this case, extracts of *D. junio* and *A. hylonome* were applied to a *T. molitor* larva. Each *T. molitor* larva received 10 μl of the extract to be evaluated, and a control larva of *T. molitor* received the same quantity of pure hexane. The order in which the extracts were assayed was random, and 30 sec was allowed for the solvent to evaporate before presenting the treated or control larvae to the ants. The extracts and the control were tested separately, as before, and the behavior of the ants towards the treated and control *T. molitor* larva was noted as in bioassay I until they carried a larva to the nest, or for a maximum period of 4 min. If at the end of 4 min, the larva was not carried to the nest, it was left on the foraging tray until it was carried to the nest or for a maximum period of 30 min. Each bioassay was repeated six times, and the results were analyzed with a Friedman test (Siegel and Castellan, 1988).

Confirmation of Repellent Effect of Compounds Identified (Bioassay III). The five acids used in the experiments were obtained commercially. Solutions of each (10%) were prepared in 1 ml of hexane in clean glass vials. The solutions were tested as described for bioassays II, except that the two test materials were placed simultaneously on the foraging area, and experiments were undertaken only with *S. geminata.* Each bioassay was repeated 10 times, and the results were analyzed with a Wilcoxon test for each behavioral response.

Chemical Analysis. Hexane extracts of 20 individuals of the first instar and five individuals of the fifth instar of *D. junio* and *A. hylonome* were prepared in 1 ml in clean glass vials. Extracts of spines cut from five individuals of each species (approximately 50 spines per individual) or 20 neck glands were extracted in clean glass vials with 1 ml of hexane per vial. The dissections of spines and neck glands were made in distilled water with the aid of a stereoscopic microscope. The extracts were evaporated to 100 μl by blowing a gentle stream of N_2 over the extract, and 1 μl was used for analysis.

The standards used for coinjections were acetic, oleic, linoleic, palmitic,

and stearic acids at 2.5 ppm (2.5 μg/ml). One microliter of extract and/or standard was analyzed with gas chromatography (GC) coupled to mass spectrometry (MS). GC was performed with a Hewlett Packard 5 and a Perkin Elmer Autosystem, both with spitless injector, a fused silica capillary column (25 m $\times$ 0.18 mm ID, stationary phase 0.15 μm of 5% phenylmethylsilicone). The carrier gas was helium at a flow rate of 1 ml/min, and the oven was held at 40°C for 4 min, raised to 280°C at 6°C/min, and held for 30 min. Compounds were detected by the Perkin Elmer mass selective detector, QMASS-900. Mass spectra were identified by comparison of the retention times and mass spectra of synthetic standards and by means of the NIST library. The parameters for the mass spectrometer were the following: Scanning of masses, 20–450 units of atomic mass; electron ionization at 70 eV; electromultiplier voltage, 1100 V; interface temperature, 170°C.

RESULTS

Definition of Behavioral Responses of Ants. Preliminary experiments with live larva allowed us to define the following behavioral responses of the ants towards the larvae: (1) Antennation: ants wave their antennae in the direction of the larva but maintain a distance of >1 mm from it. (2) Explore: ants touch the larva with the tips of their antenna and immediately retire (≤ 1 sec). For statistical purposes these two categories were lumped together as "repellency." (3) Inspection: ants touch the larva with their antenna for a period of >1 sec. (4) Attack: ants bite the larva with their mandibles or sting the larva, sometimes dragging it across the foraging tray. The stinging behavior is only applicable to *S. geminata*. *C. rufipes* does not possess a stinging apparatus, although the ants curve the abdomen ventrally until the apex touches the body of the larva. (5) Ignore: ants do not respond to the presence of the larva. (6) Alarmed circling: ants open their mandibles (*C. rufipes*) or raise their abdomens (*S. geminata*) while running in irregular circles around the foraging area with a marked increase in the speed of their movements. (7) Alarmed trembling: ants vibrate rapidly with their mandibles open. They may be stationary or run in circles in the same way as for alarmed circling. This behavior was observed only in *C. rufipes*. For statistical purposes, alarmed circling and alarmed trembling were placed in the category "alarm." (8) Cleaning antennae or first pair of legs: ants repeatedly pass their antenna from the basal to the distal part of the first pair of legs or pass their legs through the mandibles.

Behavior Against Chemical Defenses. Chi-square analysis of bioassay I data from tests with live larvae are shown in Table 1. Both *D. junio* and *A. hylonome* were repellent to *C. rufipes*. Ants were more likely to become alarmed with later instars, and the survival rate of later instars was also higher. A similar

TABLE 1. BEHAVIORAL RESPONSES OF ANTS *Camponotus rufipes* AND *Solenopsis geminata* TOWARDS INSTARS OF *Dione junio* AND *Abananote hylonome*[a]

Ant spp. and instar	*Dione junio*				*Abananote hylonome*			
	Behavior		Survival index		Behavior		Survival index	
	Repel	Attack	Median	Range	Repel	Attack	Median	Range
C. rufipes								
1		+	2	1-3		+	2	1-3
2	−	+	1	1-3		+	2	2-3
3		+	2	1-3		+	3	3-3
4	−	+	3	2-3	−	+	3	2-3
5	−	+	3	2-3	−	+	3	3-3
ANOVA (*P*)		<0.05	<0.05			<0.05	<0.05	
S. geminata								
1		+	1	1-2	−	+	3	1-3
2	−	+	2	1-3		+	1	1-3
3	−		2	1-3	−	+	1	1-2
4	−		2	2-0		+	2	1-2
5	−	+	2.5	2-3		+	2	2-2
ANOVA (*P*)	<0.05		<0.05			<0.05	<0.05	

[a]Repel: the larva was repellent to the ants; attack: the larva was attacked by the ants. (−) response of the ants greater towards the larva; (+) response of the ants greater towards the control.

pattern was observed when *S. geminata* confronted *D. junio*, except that the third and fourth instars, although repellent to ants, were attacked as much as the control. The larvae generally caused alarm in the ants, and although the larvae rarely survived encounters with *S. geminata*, later instars were generally not taken to the nest when killed. Fifth instars were left on the tray if killed and the ants proceeded to cover them with bits of earth. First and second instars of *A. hylonome* were more repellent to *S. geminata* than third and fourth instars, which were more inspected. Although the butterfly larvae were always attacked less than the control, they seldom survived, except for the first instar.

For each type of behavior the ants showed towards larvae (repellency, alarm, inspection and attack), we calculated the difference (d_1) between the number of ants that showed a given behavioral response to the butterfly larva and the number that responded to the control. We constructed an index in which a value of 3 indicated that the larva survived. A value of 2 was given if it was killed but left on the tray, and 1 was assigned if it was killed and taken to the nest. We calculated the difference (d_2) between the survival values of the control and the larva for each experiment. The values of d were then correlated with

TABLE 2. CORRELATION COEFFICIENTS OF RELATIONS BETWEEN RESPONSES OF *Camponotus rufipes* AND *Solenopsis geminata* TOWARDS INSTARS OF *Dione junio* AND *Abananote hylonome* AND SURVIVAL OF THE LATTER[a]

Ant spp.	Behavior	Inspection	Repellent	Alarm	Attack
C. rufipes	Repellent	−0.535**			
	Alarm	0.072	0.016		
	Attack	−0.059	−0.354**	0.057	
	Survival	−0.086	0.125	0.321*	−0.209
S. geminata	Repellent	−0.453**			
	Alarm	−0.451**	0.091		
	Attack	−0.682**	−0.095	0.447**	
	Survival	0.158**	0.024	−0.092	−0.271*

[a] $*P \leq 0.05$, $**P \leq 0.01$, $N = 25$.

each other for each species of ant by a Spearman correlation (Table 2). The correlations show that for *C. rufipes*, the survival of the larva correlates positively with the state of alarm in the ants, and the attack and inspection behaviors are negatively correlated with repellency. For *S. geminata*, the attack rate is positively correlated with their state of alarm, and the survival of the larvae is negatively correlated with the attack rate. The inspection response is also negatively correlated with the repellency.

Experiments with Hexane Extracts. The results of experiments with bioassay II are presented in Table 3. For *A. hylonome*, extracts of a whole larva repel *C. rufipes* significantly more than the solvent, and the ants attacked the control more than a whole larva and extracts of spines. There was no significant difference between the extracts and the control with respect to the inspecting behavior of *C. rufipes*. The behavior of *S. geminata* towards the extracts of *A. hylonome* showed that the whole larvae repelled the ants more than the control, and they were inspected less. Hexane extracts of *D. junio* larvae, spines, and neck glands repelled *C. rufipes* more than the control, and the ants attacked the extracts of whole larvae and the neck gland less.

Chemical analyses revealed that the hexane extracts of both larvae contained mainly carboxylic acids. The compounds identified from the first and fifth instars and from the spines and neck glands of *D. junio* and *A. hylonome* are presented in Table 4. The chemical composition of the first and fifth instars of both species are quantitatively and qualitatively different.

Repellency of Acids Tested. Using bioassay III, we found that all the acids tested were repellent for *S. geminata*. The ants rarely approached the *Tenebrio* larvae treated with these compounds and always showed alarm behavior in their

TABLE 3. FREQUENCY OF BEHAVIORAL RESPONSES OF ANTS TOWARDS EXTRACTS OF LARVAE[a]

Extracts	Behavior					
	Inspection		Repellent		Attack	
	Median	Range	Median	Range	Median	Range
	C. rufipes vs. *A. hylonome*					
Whole larva			4*	1-4	1.5*	1-2
Spines	NS		2.25	1-3	1.5*	1-3
Neck gland			2.75	2-4	3	1.5-4
Pure hexane (Control)			1	1-3	4	3-4
	S. geminata vs. *A. hylonome*					
Whole larva	1*	1-1	4*	3-4		
Spines	3	2-4	3	1-3	NS	
Neck gland	3	2-4	2	2-4		
Pure hexane (Control)	3	2-4	1	1-2		
	C. rufipes vs. *D. junio*					
Whole larva			3*	2-4	2.5*	1-3.5
Spines	NS		3*	2-4	3	1-3
Neck gland			3*	2-4	2*	1-2
Pure hexane (Control)			1	1-1	4	3.5-4

[a]*Significant difference between the extract and the control, $P \leq 0.05$, $N = 6$.

presence (Table 5). Although both aleic and linoleic acid and the combination of the two caused alarm in the ants, there was no significant difference between the number of ants attacking a treated *Tenebrio* larva and those attacking the control. The combination of linoleic and oleic acid tended to cause less attack behavior than the separate acids. Acetic acid was the only one that did not cause alarm behavior in the ants, and it is notable that this was the only acid attacked significantly less than the control.

DISCUSSION

Both *D. junio* and *A. hylonome* repel and cause alarm in *C. rufipes* and *S. geminata*. When *C. rufipes* workers are alarmed, the larvae are more likely to survive, whereas an increased alarm in *S. geminata* results in an increased level of aggression of workers, at least if close to the nest. The bioassay were carried carried out close to the ant colonies in an area that may be territorial for the ants. It is likely then that the chemical defenses of these larvae will be more

TABLE 4. RELATIVE PROPORTION (IN PERCENTAGE RELATIVE TO MOST ABUNDANT COMPOUND) OF COMPOUNDS FOUND IN HEXANE EXTRACTS OF LARVAE OF *Dione junio* AND *Abananote hylonome*

Compound	First instar	Fifth instar	Spines	Neck gland
Dione junio				
1. Acetic acid	51	26	14	34
2. Tetradecanoic acid	<1	<1	<1	1
3. Methyl ester palmitic acid	18	<1	17	1
4. Palmitic acid	14	16	24	42
5. Methyl ester linoleic acid		11		
6. Methyl ester linolenic acid		11		
7. Methyl ester stearic acid	100	<1	<1	5
8. Linoleic acid	76	100	100	100
9. Oleic acid	92	100		
10. Stearic acid	7	3	14	12
Abananote hylonome				
2. Tetradecanoic acid	3	<1	7	<1
3. Methyl ester palmitic acid	20	<1	8	2
4. Palmitic acid	40	20	100	5
7. Methyl ester stearic acid	80	4	9	1
8. Linoleic acid	19	100	32	100
9. Oleic acid	100	48	75	
10. Stearic acid	40	2	20	<1
11. α-Farnesene		3		
12. Isomer of α-farnesene		2		
13. Cubenene		17		
14. Cadinene		19		
15. Copaene		31		

effective against ants foraging relatively far from their nest outside their test territory.

The fact that hexane extracts of both species produced repellency demonstrates that the repellent nature of *D. junio* and *A. hylonome* has a chemical basis. Extracts of whole larvae produced a more marked reaction in both species of ants than extracts of the spines and neck glands. The extracts of spines and neck glands were less concentrated (equivalent to 0.3 and 0.5 individuals, respectively) than those of whole larvae, due to the methods used. This difference in concentration could explain the difference results.

The repellent properties of *A. hylonome* and *D. junio* may be due to at least three acids: acetic, linoleic, and oleic acid. These compounds are repellent

TABLE 5. BEHAVIORAL RESPONSES OF *Solenopsis geminata* TOWARDS LISTED ACIDS[a]

	Acids							
	Acetic		Linoleic + oleic		Linoleic		Oleic	
Behavior	%	*N*	%	*N*	%	*N*	%	*N*
Repellency	98.2**	10	88.2*	10	100**	9	97.2**	8
Inspection	7.3**	10	34.5	10	2.2**	9	67.3	10
Attack	14.5**	10	19.4	8	71.4	6	70	10
Alarm	90	5	100**	8	100*	5	100**	7

[a] % refers to the percentage of replicates where the number of ants that responded to the presence of the acid was greater than the number of ants that responded to the presence of the control. * and ** indicate statistical significance in a chi-square test for $P < 0.01$ and 0.001, respectively.

to different species of ants (Howard et al., 1982; Honda, 1983). Dani et al. (1996) investigated the sternal gland secretion of adult females of *Polistes dominulus* and *P. sulcifer* (Vespidae) and discovered several long-chain carboxylic acids. Of these, oleic, linoleic, and palmitoleic acid (all unsaturated) had a repellent effect on the ants *Crematogaster scutellaris*, *Formica cunicularia*, and *Lasius* sp., while lauric, myristic, palmitic, and stearic acid (saturated) did not. Howard et al. (1982) found that oleic acid present in the eggs of *Gastrophysa cyanea* (Coleoptera: Chrysomelidae) was repellent to several species of ants including *Camponotus pennsylvanicus* and *Solenopsis invicta.* Workers of the ant, *Iridomyrmex humilis*, rapidly consumed controls but covered *Tenebrio* larvae treated with oleic acid with small pieces of earth. Honda (1983) investigated the effect on the ants *Lasius niger* and *Crematogaster matsumurai* of several compounds present in the defensive secretions of larvae in the genera *Papilio* and *Graphium.* Among the compounds tested, acetic acid was found to be repellent and extremely toxic, producing 100% mortality in both species tested.

Among the sesquiterpenes present in the extracts of whole larvae of *A. hylonome*, only α-farnesene has been reported as a component of the defensive secretions of butterfly larvae of *Papilio* spp. (Papilionidae) (Honda, 1981). Honda (1983) reports that α-farnesene has a limited effect on *L. niger* and *C. matsumurai.* This compound was repellent to both species of ant in the present study. β-Farnesene, which differs from α-farnesene only in the relative position of the substituent groups, has been reported as an alarm pheromone in three subfamilies of aphids (Dettner and Leipert, 1994) as well as being the principal component in the defensive secretion of *Atta* spp. (Hernández, 1996). It is possible that α-farnesene forms part of the defense system of *A. hylonome.*

Chemical Defense of Dione junio. In *D. junio*, acetic and linoleic acid were present in all extracts, while oleic acid was found only in extracts of whole larvae. *C. rufipes* was repelled by all the extracts of *D. junio*, and ants attacked the whole larvae and the neck gland less than the control. Acetic acid, present in the spines and neck gland of *D. junio*, is probably the most important component in the repellency of this species. The first instar was not repellent to either ant species. The lack of the methylesters of linoleic and oleic acids in this instar could be the cause of the lower repellency. However, these methyl esters were also absent from the extracts of the spines and neck glands, which were repellent to *C. rufipes*.

Chemical Defense of Abananote hylonome. In *A. hylonome*, we identified oleic, palmitic, linoleic, and stearic acid, and the methyl esters of palmitic and stearic acid in all extracts, except in the neck glands from which oleic acid was absent. Live *A. hylonome* larvae were repellent and survived several of the bioassays, especially with *C. rufipes*, although these larvae do not contain acetic acid in their defensive secretion. The extracts of the spines of *A. hylonome* were attacked less by *C. rufipes* than the control, whereas the neck gland extracts were attacked similarly to the control. The combination of linoleic and oleic acid caused fewer attacks than the separate acids. In the experiments with *S. geminata*, there was no significant difference between the control and extracts of *D. junio*, while in the experiments with *A. hylonome*, only washes of a whole larva showed significant differences with the control. This indicates that the aggressiveness of *S. geminata* can annul the repellent effect of the larval defensive compounds and that the combination of linoleic and oleic acid in *A. hylonome* represents an effective defense against these ants. This suggests that in *A. hylonome*, with no acetic acid, the combination of linoleic and oleic acid may be at least partially responsible for the chemical defense.

In *A. hylonome*, sesquiterpenes were absent in first instars but present in fourth and fifth instars, which also alarmed more *C. rufipes* workers. With *C. rufipes*, alarm of the ants is related to survival of the larvae, suggesting that the terpenes contribute to this survival.

First instars of *A. hylonome* did not induce aggression from *S. geminata* workers. The absence of sesquiterpenes could contribute to this lack of aggression. These terpenes are also absent from spines and neck gland extracts of *A. hylonome*. It was not possible, however, to prove the separate effect of the sesquiterpenes, but it is likely that they induce alarm response in *S. geminata* as well as in *C. rufipes*. In *S. geminata*, the alarm response results in increased aggression towards the larvae. Thus, the lack of terpenes in the first instar could account for the lack of aggression. This result emphasizes the fact that a chemical defense effective against one species of ant is not necessarily effective against other species.

There is more of each compound in fifth than in first instars, and there was

a tendency for later instars to survive more often than early ones. Thus, it is probable that in the first instars, the quantity and concentration of the defensive compounds in a single larva is not enough to be effective against ants. One bite by the ants can kill a first instar, whereas later instars are more resistant. In the experiments undertaken, only one larva was presented to the ants. Larvae of both species of butterfly are gregarious until the fifth instar, and the chemical defenses employed by the larvae may be more effective when they are aggregated. The benefits of gregarious behavior in insect defenses have been discussed by Vulinec (1990). Codella and Raffa (1995) found that groups of 40 larvae of Diprionidae (Hymenoptera) showed higher survival rates than groups of five. The survival of larvae of *Hemileuca lucina* (Saturnidae: Lepidoptera) was greater in groups of 20 individuals (80% survived) than in groups of five (35% survived).

The first and fifth instars of both species share many of the compounds identified and seem to be protected by chemical defenses from the start of the larval stage. This has been reported also for *Diaprepes abbreviatus* (Coleoptera), in which first instars are repellent to *S. geminata* (Pavis et al., 1992). First instars of *Lymantria dispar* (Lepidoptera) that had not eaten survived more attacks from *Formica* spp. than first instars that had fed (Weseloh, 1989).

The defensive carboxylic acids identified from *D. junio* and *A. hylonome* are common products of metabolism and widely distributed among insects (Rockstein, 1978). Attygalle et al. (1993) proposed that small quantities of acetic acid may be common in insect defensive secretions. This acid has been found in the secretion of the beetle, *Hellumorphoides clairvillei* (Attygalle et al., 1992), and in the defensive spray of the ant, *Camponotus floridanus* (Attygalle et al., 1993). It is also present as a major compound in the defensive secretion of ants of the genus *Atta* (Hernández, 1996). In Lepidoptera larvae, acetic acid forms part of the defensive secretion from the osmeteria of *Papilio helenus* (Papilionidae) (Honda, 1983) and the neck gland of *Schizura unicornis* and *S. badia* larvae (Notodontidae) (Weatherston et al., 1979).

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ESSENTIAL-OIL-MEDIATED INTERACTIONS BETWEEN OREGANO PLANTS AND HELICIDAE GRAZERS

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Abstract—Aromatic plants dominate in Mediterranean-type ecosystems. They often produce remarkable quantities of essential oils containing high amounts of phenolic isoprenoids, such as thymol and carvacrol. The purpose of this work was to examine the interactions between commonly occurring aromatic plants in the Mediterranean environment and their snail grazers. The aromatic plants used were two *Origanum vulgare* subspecies, subsp. *hirtum* and subsp. *vulgare*. They differ in the content and the qualitative composition of their essential oil; subsp. *hirtum* contains a much larger amount and is rich in phenolic compounds. Their effect on the foraging behavior of three snail species, native in Greece, was studied; the snail species were *Helix lucorum*, *H. aspersa*, and *Eobania vermiculata*. The snails' responses to different food sources, raw or processed, with or without essential oils, were evaluated during the different stages of the foraging cycle. During the encounter stage, snails were more repelled than attracted when close to phenol-rich foods. During the acceptance stage, all snail species tended to reject food types that contained high concentrations of subsp. *hirtum* essential oil. At the feeding stage, subsp. *hirtum* essential oil caused reduction of daily consumption rates. Overall, the essential oil of *O. vulgare* subsp. *vulgare* did not produce any marked change in the snails' behavior. In contrast, that of *O. vulgare* subsp. *hirtum* had a repellent effect, particularly when present in naturally occurring high concentrations. Among the snail species, *H. lucorum*, which does not share the same biotope with *O. vulgare* subsp. *hirtum*, was the least tolerant to its essential oil.

Key Words—Carvacrol, foraging cycle, Mediterranean-type ecosystems, *Origanum vulgare*, snails, terrestrial gastropods, *Helix*, *Eobania*.

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INTRODUCTION

The long summer drought that characterizes Mediterranean climatic zones places severe constraints on the photosynthetic potential of the resident plants (Mooney and da Silva, 1981). Phrygana (east Mediterranean ecosystems; syn. tomillares in Spain, batha in Israel, gariga in Italy), in particular, which mainly occur at the dry end of the precipitation gradient, are nonproductive during almost half of the year (Margaris, 1981). Another characteristic of phryganic ecosystems is the abundance of aromatic plants, both in species number and in biomass (Vokou, 1992).

Labiatae is one of the dominant families of aromatic plants in Mediterranean ecosystems (Kokkini et al., 1988). Its representatives produce essential oils that, frequently, are carvacrol-rich. Carvacrol is a C_{10} phenol, produced through the mevalonic acid pathway. Several aromatic plants, belonging to different species and genera, are referred to under the commercial name oregano if they are carvacrol-rich. Among them are *Thymus capitatus* (L.) Hoffmanns. and Link, *Satureja thymbra* L., *Origanum onites* L., and *O. vulgare* subsp. *hirtum* (Link) Ietswaart, all common components of Mediterranean ecosystems. The dominance of phenol-rich aromatic plants led us to undertake a multiaspect program of research aimed at understanding the function of essential oils in these ecosystems; we have assessed their allelopathic and autopathic effects (Vokou, 1992; Vokou and Margaris, 1986a), their effects on the native soil bacteria and fungi (Vokou et al., 1984; Vokou and Margaris, 1988), and their contribution to pollinator attraction (Petanidou and Vokou, 1993). In this context, the present work is our first attempt to examine the essential-oil-mediated interactions between common aromatic plants and their grazers in the Mediterranean environment.

Given the rather low primary production in these ecosystems, plant species endowed with defense mechanisms, such as secondary compounds, which deter grazing, would have a competitive advantage and predominate in zones of heavy herbivory. The question asked, therefore, is whether the possession of phenol-rich essential oils can be related to and explained in terms of chemical defense against herbivores.

Among the native grazers are snails. Very few works have been published concerning the selective herbivory on chemically differing aromatic plants by terrestrial mollusks (Gouyon et al., 1983; Lincoln and Langenheim, 1979; Linhart and Thompson, 1995; Rice et al., 1978). For these reasons, we decided to study the effect of two *O. vulgare* subspecies on native snail species, which cooccur with these aromatic plants, and particularly during the different stages of their foraging cycle. The two subspecies studied differ remarkably with respect to the content and composition of the essential oil that they produce.

The foraging cycle of a snail is a complex sequence of activities and inter-

actions, which can be divided into four stages: (1) encounter, (2) acceptance, (3) feeding, and (4) postfeeding. Each stage is influenced by various factors, including the previous stages. Encounter is influenced by the food density and attractiveness and also by the animal's actual activity (Hunter and Symonds, 1970). Food attractiveness is difficult to define; in addition, the distinction between gustatory phagostimulants and olfactory attractants has not always been clearly made (Bailey and Wedgwood, 1991). The stages of acceptance and feeding are both affected by the physical and chemical properties of the food that may be stimulatory or inhibitory. These stages are also affected by the animal's level of food arousal, and the net phagostimulatory properties of the food can do little to induce an uninterested animal to feed (Bailey and Wedgwood, 1991).

The specific questions asked in the present work were: (1) whether *Origanum vulgare* essential oils exert antifeeding effects against native snails and at what stage(s) of the foraging cycle; (2) if effects vary depending upon the content and the qualitative composition of the oil, as suggested by Gouyon et al. (1983) and Linhart and Thompson (1995); (3) if a high phenolic content in an aromatic plant consistently results in a more marked effect, irrespective of the plant species studied; and (4) if snail species of the same or different habitats respond differently to the same food sources. Three Helicidae snail species and two aromatic plants, closely related taxonomically but very different phytochemically, all native in Greece, were used.

METHODS AND MATERIALS

Aromatic Plants: Distribution, Features, and Analysis

The essential oils tested in these experiments were extracted from samples of two subspecies of *Origanum vulgare* L. *O. vulgare* is the commonest *Origanum* species in Greece and is characterized by a high morphological and chemical variability (Ietswaart, 1980; Kokkini et al., 1991). The two subspecies used in the experiments were *O. vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare*.

O. vulgare subsp. *hirtum* is very common in Mediterranean-type ecosystems. It grows in the Balkan Peninsula and Turkey (Ietswaart, 1980) and is the commonest *O. vulgare* subspecies in Greece; it occurs throughout the country except for the NE mountain ranges, at elevations between sea level and 1500 m, in dry, sunny places, near the coasts, usually on limestone. The plants have dense and conspicuous glands on their leaves and on their green colored bracts. They flower from May to September (Kokkini et al., 1991). Comparison of populations occurring in different areas of the country showed a large variation in essential oil content; at the flowering stage, it ranged from 1.8 to 8.2 ml/100

g of air-dried material. The main essential oil constituents are the phenols, carvacrol, and thymol, each ranging from traces up to 90% (Vokou et al., 1993) depending on the population studied. The samples used were collected from a site near Posidi (Halkidiki, Macedonia), 20 m from the coast of the Thermaikos Gulf.

O. vulgare subsp. *vulgare* is found all over Europe except for the Iberian Peninsula (Ietswaart, 1980). In Greece, it is found in the northern part of the country at altitudes between 600 and 2000 m, on limestone and schist, mainly in openings of deciduous forests. The plants have sparse and inconspicuous glands on their leaves and on their purple colored bracts. They flower from July to October (Kokkini et al., 1991). Their oil content is always very low (0.1–0.3 ml/100 g of air-dried material); it consists mainly of mono- and sesquiterpene hydrocarbons (80–90% of the oil) and less than 1% of the phenols, carvacrol, and thymol (Kokkini and Vokou, 1989; Maarse and van Os, 1973). The samples used were collected from a site on Mt. Chortiatis, near Thessaloniki, at 600 m.

The collected plant material was left to dry in air in the dark; the aerial parts were then submitted to distillation for 3 hr in a Clevenger apparatus (Wertheim, Germany). The oil content of *O. vulgare* subsp. *vulgare* sample was 0.32 ml/100 g dry weight (gdw); that of *O. vulgare* subsp. *hirtum* was 3.07 ml/100 g dw. Both the extracted oil and the residue of the distillation were kept at 10°C before they were used in the experiments.

The essential oils extracted were further analyzed by gas chromatography in a Varian 3700 apparatus under the following conditions: injection temperature, 170°C; detection temperature, 230°C; carrier gas, nitrogen with a flow rate of 30 ml/min; column, 2 m 10% Carbowax 20 M; column temperature program: 80°C for 3 min, followed by an increase of 3°C/min to 190°C, remaining for another 10 min at the final temperature.

Phenolic constituents were identified on the basis of retention times; we further cochromatographed authentic samples of compounds to cross-check their identity.

Snails: Distribution and Treatments

The snail species used, *Helix aspersa* Mull., *Eobania vermiculata* Mull., and *Helix lucorum* L., are all members of the Helicidae family. *H. aspersa* is found naturally all over the Mediterranean basin, western and central Europe, Asia Minor, and North Africa; it has been introduced to many other areas. Its habitat are forests, thickets, fields, and seaside dunes (Pfreger and Chatfield, 1988). In Greece, it occurs in the lowlands of the southern part of the country, mainly on the island of Crete and in Peloponnesus; the diameter of its shell is 25–40 mm (Lazaridou-Dimitriadou and Kattoulas, 1985). The samples tested were collected from a site near Chania (Crete), where many aromatic plants grow, *O. vulgare* subsp. *hirtum* included.

E. vermiculata is common in the Mediterranean countries, Asia Minor, and Crimea. It originally inhabited steppes but has subsequently colonized fields, thickets, gardens, vineyards, and roadsides (Pfreger and Chatfield, 1988). In Greece, it occurs on the islands and along the coastal zone of the continental part of the country; the diameter of its shell is 22–30 mm (Lazaridou-Dimitriadou and Kattoulas, 1985). The samples tested were collected from Posidi, the same site as *O. vulgare* subsp. *hirtum.*

H. lucorum is distributed in Italy, the Balkan Peninsula, Asia Minor, the Crimea, and the Caucasus. It occurs in both dry and damp habitats, slopes overgrown with vegetation, open woods, gardens, and thickets (Pfreger and Chatfield, 1988). In Greece, it occurs in the continental part, mainly in Macedonia and the Peloponnesus; the diameter of its shell is 35–55 mm (Lazaridou-Dimitriadou and Kattoulas, 1985). The samples tested were collected from the same site, Chortiatis, as *O. vulgare* subsp. *vulgare.*

All snails were kept in transparent plastic boxes (13.5 × 7.5 × 5.5 cm) at a temperature of 20–22°C and high humidity supplied by wet sponges and Petri dishes filled with water. Lights were on for 13 hr a day. The snails were deprived of food for two days before being used in an experiment. As far as possible, snails of the same species used in the experiments were matched by size.

The experiments were designed to describe the foraging behavior of snails in relation to olfactory and gustatory variability of the food, associated with different content and composition of essential oil. They were based on two food types; raw (pieces of various food types) or processed (pellets, soaked or not, in essential oil). The variables examined were related to the attractiveness, acceptance, palatability, and consumption of the various food sources.

Experiments with Raw Food Material

Time-Lapse Video Recordings. In this series of experiments, the olfactory attractiveness and the acceptability of a food source were investigated. Five food types were offered to each snail species: (1) slices of carrot, (2) the air-dried residue of *O. vulgare* subsp. *hirtum* leaves after distillation of the essential oil, (3) leaves of *O. vulgare* subsp. *hirtum*, (4) leaves of *O. vulgare* subsp. *vulgare*, and (5) the enriched residue, i.e., the air-dried residue sprayed with the same quantity of *O. vulgare* subsp. *hirtum* oil as is contained in an equivalent amount of air-dried plant material before distillation. The enriched residue was kept in an air-tight container in order to avoid evaporation of the essential oil.

Recordings of the movement of the snails were made overnight in a controlled temperature room (17.4–22.2°C), from 6:00 PM (when the lights were switched off) until 6:00 AM the following day (when the lights were switched on). Three snails (one of each species) were placed every night under a ceramic

tile, in the center of a box (93 × 93 × 5 cm) filled with peat and covered with sand, which was regularly sprayed with water in order to keep humidity at high levels. An electrical fence connected to a 9-V battery prevented the snails from escaping out of the box. The five food types, each duplicated, were placed each in one of the 10 segments of a circle with a radius of 36.5 cm so that neighboring foods were at a distance of about 22 cm. Foods of the same type were placed in diametrically opposite positions. This arrangement of food types gave snails equal opportunity to advance towards any type so that any distant food selection might be revealed. Results were based on six overnight replications with different individuals.

Recordings were made on a time-lapse video recorder, as described by Bailey and Wedgwood (1991). Data, such as time of activity, position of each snail, and distance traveled, were extracted from the video recording. The tracks followed by the snails were assessed for two variables; the time needed to travel them and their shape. Assessment of the track was made when the snails were close to the food sources, at a distance less than 10 cm. A snail was considered to be attracted by a certain food type if its track curved towards it; if it curved away from it, the snail was considered to be repelled, while a nondeviating track indicated a neutral response. For each food type and for each snail species the frequency of the different track types was recorded.

Consumption Experiments. In this series of experiments, food sources accepted by the snails were evaluated on the basis of their consumption level. The experiments were conducted in chambers and conditions similar to those used for keeping the snails. One snail at a time was placed in the transparent box containing only one of the four food types; carrot was used as a marker food before and after each experiment to determine the fecal production (Phillipson, 1960). The method used in these experiments is described by Lazaridou-Dimitriadou and Daguzan (1978). Each experiment lasted for six days. For each food type, there were five replicates for *H. aspersa* and *E. vermiculata*, and four for *H. lucorum*; different individuals were used in each replication.

The quantification of daily consumption rate was based on the formula used by Lazaridou-Dimitriadou and Daguzan (1978):

$$\text{Daily consumption rate} = \frac{\text{amount of food ingested (mg)}}{\text{snail's live body weight (g)}}$$

Experiments with Processed Food Material (Pellets)

In this series of experiments the snails' response, when in contact with the food, was investigated in order to provide information on the gustatory-related attractiveness of the various food sources.

The "acoustic pellet" technique was developed by Senseman (1978). It is used to record the individual bites in a single meal allowing precise measuring

of the meal characteristics of snails feeding on pellets. The technique is described in detail in Wedgwood and Bailey (1988) and Bailey and Wedgwood (1991). Each pellet is attached to a piezoelectric transducer; signals of a snail biting are simultaneously recorded on a tape-recorder, displayed on an oscilloscope, and fed into a BBC microcomputer. To avoid counting the same bite twice, no recordings were taken for 1.5 sec after an impulse. A meal was considered completed if there was no bite for 120 sec. The combination of the acoustic pellet technique and video-recordings can supply considerable information on foraging behavior of snails (Bailey and Wedgwood, 1991).

The pellets (plain pellets), 2 cm long × 3 mm wide, were made of maize flour and 5% gelatin, as described in Bailey et al. (1989). Some of these were further soaked (1) in 100% acetone solution (acetone pellets) as a control, (2) in *O. vulgare* subsp. *hirtum* oil, dissolved in acetone, in a quantity equivalent to that contained in an equal weight of *O. vulgare* subsp. *hirtum* plant material (100% pellets), and (3) with an oil quantity equivalent to 10% of that contained in the plant material (10% pellets). All pellets were left to dry before storing them in airtight polyethylene containers.

Five individuals of each snail species were tested on each food type except for *H. lucorum* feeding on plain pellets, when 10 individuals were used. Before taking any recordings, snails were tested for their readiness to feed on a plain pellet. A snail was considered to reject a pellet type only if it rejected three different pellets of the same type presented to it.

As estimates of the food palatability, examined through the acoustic pellet technique, the mean bite rate and the index of irregularity were used. The index of irregularity has been defined as the difference between the mean and the median bite intervals (Bailey and Wedgwood, 1991). Slow bite rates and high indices of irregularity indicate low palatability of a food. Both parameters are temperature-dependent and are affected by the hardness of the pellet; they are independent of the duration of a meal (Bailey and Wedgwood, 1991).

RESULTS

Stage One: Encounter (Response Before Contact with Food). The mean step length or the speed at which each snail species advanced when approaching a food type is shown in Table 1. *H. aspersa* snails moved significantly faster towards *O. vulgare* subsp. *hirtum* leaves than to other food types (including the enriched residue). *E. vermiculata* snails moved fastest towards the carrot slices; they never approached *O. vulgare* subsp. *hirtum* leaves. *H. lucorum* advanced at similar high speed towards the residue and the residue enriched with essential oils of subsp. *hirtum*, but not to the *O. vulgare* subsp. *hirtum* leaves.

The shape of the track traveled was assessed when snails were close to the food source, at a distance less than 10 cm. Replicated tests of goodness of fit

TABLE 1. SPEED OF SNAILS (ESTIMATED AFER MEAN STEP LENGTH) MOVING TOWARDS FOOD TYPE[a]

Snail species	Mean step length (cm/min, ±SE)				
	Carrot	Residue	Subsp. *vulgare* leaves	Subsp. *hirtum* leaves	Enriched residue
H. aspersa ($F = 7.01$, $P < 0.001$)	3.10 ± 0.158b	3.20 ± 0.171b	3.17 ± 0.215b	3.87 ± 0.195a	2.73 ± 0.115b
E. vermiculata ($F = 28.37$, $P < 0.001$)	4.01 ± 0.222a	2.15 ± 0.120b	2.69 ± 0.123c		3.35 ± 0.122d
H. lucorum ($F = 3.99$, $P < 0.01$)	2.97 ± 0.125a	3.71 ± 0.146b	3.69 ± 0.366bc	3.16 ± 0.150ac	3.66 ± 0.194b

[a] Data were analyzed by one-way ANOVA and LSD multiple comparisons within species. Means for a particular species are not significantly different when followed by the same letter. *E. vermiculata* never approached subsp. *hirtum* leaves offered to it.

(Sokal and Rohlf, 1981) allowed for the unification of the results for all three snail species (G for heterogeneity, $G_H < 7.33$ in all cases; $\chi^2_{0.05[4]} = 9.49$). Through the same test, significant differences were found in the response (positive, negative or neutral) of snails towards each food source (G for the pooled data, $G_p > 7.41$ in all cases; $\chi^2_{0.05[2]} = 5.99$) except for the residue ($G_p = 4.23$). In particular, snails were attracted by carrot slices and *O. vulgare* subsp. *vulgare* leaves more often than they were repelled, and they were repelled by *O. vulgare* subsp. *hirtum* leaves and the enriched residue more often than they were attracted (Figure 1).

Stage Two: Acceptance. Upon arrival at a certain food type, a snail may start feeding (acceptance) or may reject the food, as evidenced through video recordings. Figure 2 shows the number of times that a snail species accepted or rejected a food type. *O. vulgare* subsp. *hirtum* leaves were never accepted; the same holds true for the enriched residue, with the exception of *H. aspersa* that partially accepted this food type. In contrast to the other two snail species, *H. lucorum* never rejected the carrot food.

In the case of acoustic pellet experiments (Table 2), all snail species accepted the plain and the acetone pellets and all rejected the 100% pellets. As for the 10% pellets, only one of the five *H. aspersa* and three of the five *E. vermiculata* or *H. lucorum* rejected them. By both standards of observation (video-recordings and acoustic pellet), *H. aspersa* seems to be the least discriminating.

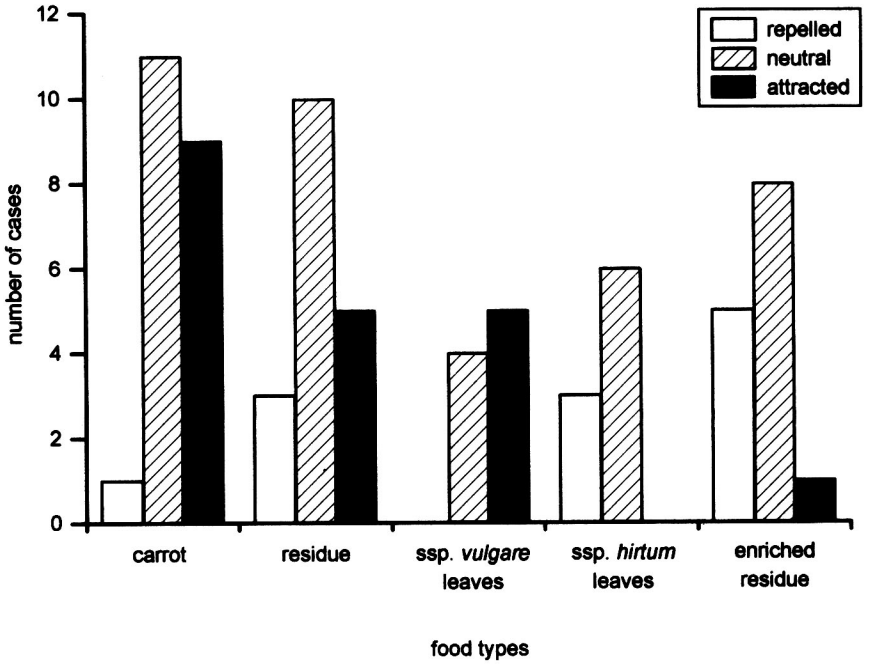

FIG. 1. Attractiveness of the five food types used, defined by the number of times a snail's response was negative, positive, or neutral. Data for the three snail species were pooled. Significant differences were found in the response of snails towards each food source (G for the pooled data, $G_p > 7.41$ in all cases; $\chi^2_{0.05|2|} = 5.99$) except for the residue ($G_p = 4.23$).

Stage Three: Feeding. At this stage, we assessed the palatability and the consumption rate of various food types with or without essential oils. Mean values of bite rates for snails that did accept the various pellet types are given in Table 2. Log-linear frequency analysis (Sokal and Rohlf, 1981) was performed to describe the effect of the food type and the snail species. The lower values associated with *H. lucorum* and bite rate in the case of the 10% pellets were not significantly different. Overall, the bite rate was constant, independent of the snail species or the food type.

Indices of bite irregularity are given in Table 3. With this measure of palatability, significant differences were found in the case of *H. lucorum* with the 10% pellets being less palatable (100% pellets are excluded from the analysis as no snail accepted them).

As far as consumption is concerned, Figure 3 shows the mean values of daily consumption rates of all food types (except for the carrot slices) by each

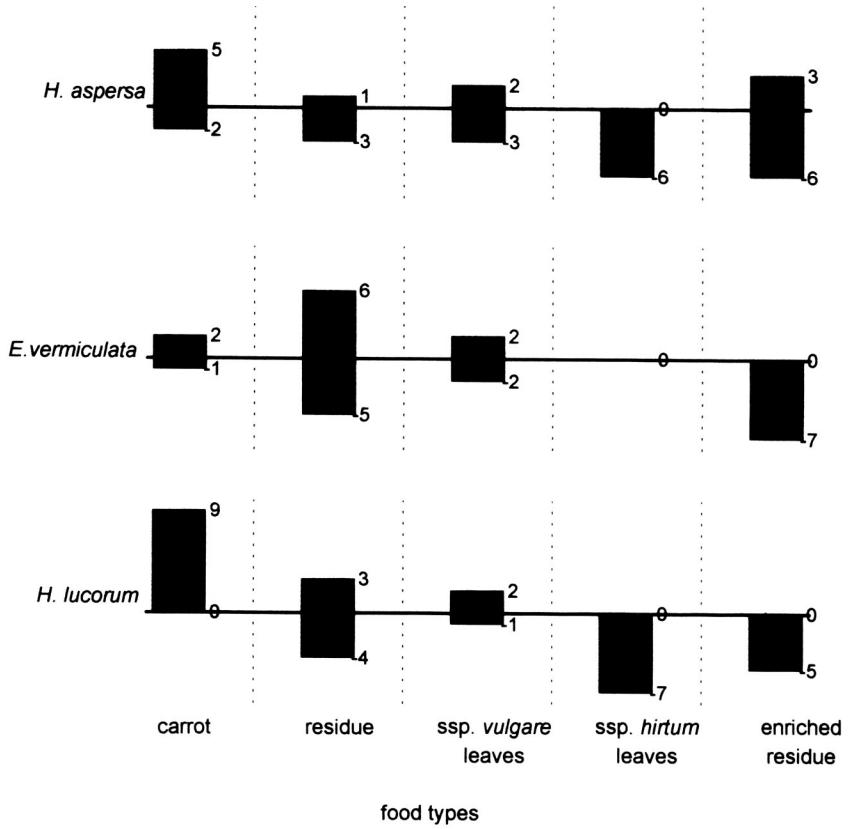

FIG. 2. Number of times each snail species accepted (positive values) or rejected (negative values) each of the five food types offered.

of the three snail species. Daily consumption rates varied significantly among snail species. Nevertheless, all snails presented a similar pattern of consumption magnitude; high values of daily consumption rate were recorded for snails feeding on the residue and *O. vulgare* subsp. *vulgare* leaves, and low values were recorded for those feeding on foods rich in *O. vulgare* subsp. *hirtum* oil, i.e., the enriched residue and the *O. vulgare* subsp. *hirtum* leaves.

DISCUSSION

The present study assesses the response of three species of snails with respect to the presence of essential oils (differing in content and composition)

TABLE 2. RATIO OF FEEDING INDIVIDUALS TO TOTAL NUMBER TESTED IN EACH TREATMENT (IN PARENTHESES) AND MEAN BITE RATE OF SNAILS FEEDING ON PELLETS WITH OR WITHOUT *O. vulgare* subsp. *hirtum* ESSENTIAL OIL[a]

	Mean bite rate (bites/min, ±SE)			
Snail species	Plain pellets	Acetone pellets	10% pellets	100% pellets
H. aspersa	27.6 ± 1.57 (5/5)	25.3 ± 1.11 (5/5)	24.0 ± 1.26 (4/5)	(0/5)
E. vermiculata	24.7 ± 2.08 (5/5)	26.2 ± 1.05 (5/5)	25.1 ± 4.85 (2/5)	(0/5)
H. lucorum	21.9 ± 0.65 (10/10)	19.4 ± 1.44 (5/5)	16.7 ± 0.50 (2/5)	(0/5)

[a]After log-linear frequency analysis, differences in bites rates were not significant.

TABLE 3. MEAN INDEX OF BITE IRREGULARITY, DEFINED AS DIFFERENCE BETWEEN MEAN AND MEDIAN BITE INTERVALS, FOR COMBINATIONS OF SNAIL SPECIES AND PELLET TYPES[a]

	Index of irregularity (sec, ±SE)			
Snail species	Plain pellets	Acetone pellets	10% pellets	100% pellets
H. aspersa ($F = 0.70$, NS)	0.07 ± 0.054	0.04 ± 0.011	0.19 ± 0.169	
E. vermiculata ($F = 0.22$, NS)	0.71 ± 0.248	0.52 ± 0.096	0.62 ± 0.328	
H. lucorum ($F = 28.72$, $P < 0.001$)	0.12 ± 0.036a	0.01 ± 0.093a	1.04 ± 0.232b	

[a]Data were analyzed by ANOVA and LSD multiple comparisons for *H. lucorum*. Means followed by the same letter are not significantly different.

in various food types. This response was examined: (1) when snails were not in contact with the food, implying olfactory mechanisms of attraction or repellency (stage of encounter); (2) when snails were in contact to the food, implying also gustatory mechanisms (stage of acceptance); and (3) when consuming the food (stage of feeding).

Encounter. The effect of phenol-rich essential oils at this stage is not clear-cut. Nevertheless, when snails are close to the phenol-rich foods, they are more

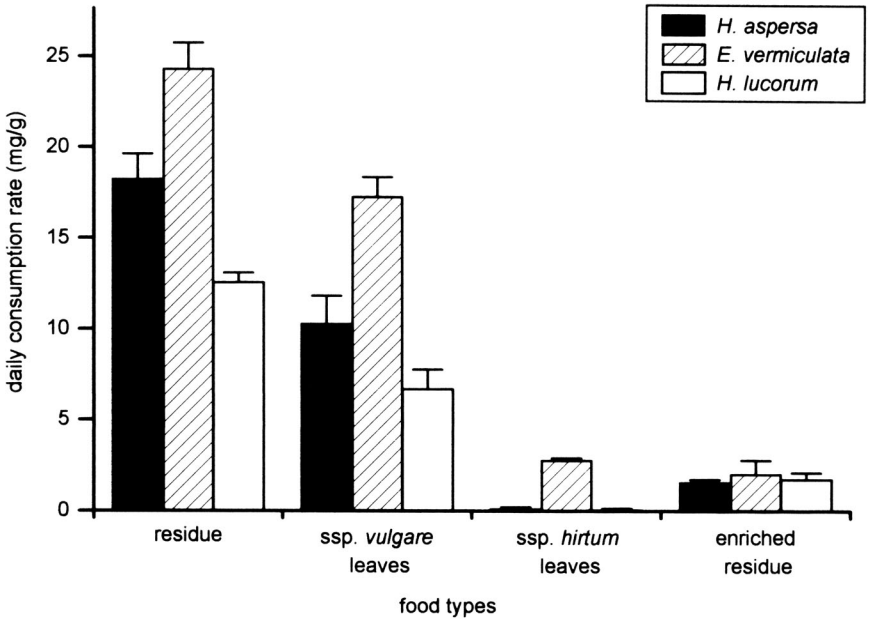

FIG. 3. Mean daily consumption rates (±SE) of each food type by the three snail species, expressed as amount of food ingested over snail's live body weight.

repelled than attracted. The two phenols, thymol and carvacrol, made up more than 60% of the essential oil of *O. vulgare* subsp. *hirtum* (26.2% and 34.5%, respectively). In contrast, only traces were found in the case of *O. vulgare* subsp. *vulgare* oil.

Differentiation between food types of the same or similar appearance (which applies to all the food types used except for the carrot) must be due to the presence of volatile compounds, detected by snails through olfaction. The discrimination made by *H. lucorum* between two food types that are similar in essential oil content and composition, i.e., between subsp. *hirtum* leaves and the residue impregnated with subsp. *hirtum* oil, may be due to other differences. For example, due to the distillation, the enriched residue is deprived of volatile water-soluble compounds and carries the essential oil not inside glandular trichomes, as do the leaves, but externally.

Acceptance. Acceptance of a food describes the snails' response when in contact to that food. It involves not only olfactory but also gustatory stimuli. The physicochemical characteristics of the food that determine acceptance may be, to some degree, independent of the factors involved in attraction (Bailey and Wedgwood, 1991). Acceptability of a food containing essential oils was

studied by use of pellets, which differed only in the oil content and raw food types, which differed also in oil composition and texture.

After contact with the food had been achieved, all snail species tended to reject food types that contained high concentrations of *O. vulgare* subsp. *hirtum* oil (*O. vulgare* subsp. *hirtum* leaves, enriched residue, 100% pellets), except for *H. aspersa* snails, which accepted the enriched residue. Foods containing this oil at much lower concentrations (10% pellets) were accepted by individuals of all three snail species.

Among the snail species, *H. lucorum* seems to be the most influenced by texture, as deduced from its lower acceptance of the residue and the *O. vulgare* subsp. *vulgare* leaves compared to carrot. Dirzo (1980) has also suggested that the acceptance of food by the slug *Deroceras caruanae* Pollonera is affected by its texture.

Overall, experiments assessing the acceptability of a food showed that the presence of *O. vulgare* subsp. *hirtum* essential oil in that food at high concentrations that can naturally occur in late spring-summer has a repellent effect on snails.

Feeding. All pellet types that were accepted were equally palatable to either *H. aspersa* or *E. vermiculata.* In contrast, either the plain or the acetone pellets were more palatable to *H. lucorum* than the 10% pellets (index of irregularity <0.15 sec for the plain and acetone pellets compared to 1.04 sec for the 10% pellets). This increase is comparable to that given by Bailey and Wedgwood (1991) for the slug *Deroceras reticulatum* (Mull), when metaldehyde or methiocarb, well-established poisons for land mollusks, were added to maize pellets, on which it fed.

The magnitude of food consumption by snails is affected by the season of the year and reaches its maximum in spring (Lazaridou-Dimitriadou and Kattoulas, 1981). Temperature and photoperiod conditions used in the experiments were similar to those prevailing during this season. From the daily consumption rates, we have shown that snails, regardless of species, consumed mainly the residue, less of the *O. vulgare* subsp. *vulgare* leaves, and even less of the two food types, rich in *O. vulgare* subsp. *hirtum* oil. In other words, the presence of *O. vulgare* subsp. *hirtum* oil caused the reduction of the daily consumption rates. However, when snails had no other choice, they grazed upon phenol-rich foods to satisfy their energy requirements, but at a low to very low rate. Given that the postfeeding stage has not been studied in this work, we cannot give information as to whether snails perform normally afterwards with respect to their physiology and reproduction.

In summarizing the snail's response at the different stages of the foraging cycle when provided with *O. vulgare*-related food sources, we can conclude that *O. vulgare* subsp. *vulgare* does not exert any effect. In contrast, *O. vulgare* subsp. *hirtum* possesses antifeeding properties.

After their experiments, Linhart and Thompson (1995) associated the antifeeding activity of *Thymus vulgaris* L. against *H. aspersa* snails with the phenolic constituents of its essential oil, thymol and carvacrol. The *O. vulgare* subsp. *vulgare* plants used in our study have a much lower essential oil content, and they are almost phenol-free. The absence of any clearly expressed antifeeding activity of this subspecies is consistent with the conclusions of these authors.

Considering the overall patterns of foraging behavior, there are no markedly pronounced differences among the three snail species studied concerning their response to the *O. vulgare* subsp. *hirtum* essential oil, although its deterrent effect seems somewhat more pronounced towards *H. lucorum.* The latter could be related to the lack of familiarity of *H. lucorum* with *O. vulgare* subsp. *hirtum* and, concomitantly, with its oil. This holds true since *H. lucorum* shares the same biotope with *O. vulgare* subsp. *vulgare* but not with *O. vulgare* subsp. *hirtum*, as the other two snail species do.

The response of *H. aspersa* and *E. vermiculata*, which cooccur with *O. vulgare* subsp. *hirtum*, to its oil, is concentration-dependent. The snails tended to avoid high concentrations, naturally occurring in the field. Essential oil concentrations are not constant throughout the year (Vokou and Margaris, 1986b) and are not the same in all individuals (Vokou et al., 1993). Snail grazers might act as a selective force, particularly during the seedling stage, favoring dominance of aromatic plants containing high amounts of essential oils, rich in phenols. Such herbivore repellent features would be extremely advantageous under the constraints of the Mediterranean environment, characterized by a limited growth period.

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GLUCOSINOLATE CONTENT AND SUSCEPTIBILITY FOR INSECT ATTACK OF THREE POPULATIONS OF *Sinapis alba*

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Abstract—*Sinapis alba* is less susceptible to damage by insect pests than *Brassica napus*. We investigated the composition and distribution of glucosinolates in different plant parts in three populations of *S. alba*; two populations selected for low-seed-glucosinolate content and one commercial cultivar. We have assessed the susceptibility of low-seed-glucosinolate content populations of *S. alba* to four insect pests, a flea beetle, a pollen beetle, and two species of aphids. Over 90% of the total glucosinolates in the cotyledons of the three populations of *S. alba* consisted of sinalbin. There was no difference in feeding damage by flea beetles on different *S. alba* populations at the cotyledon stage, nor was there a difference in sinalbin concentration of cotyledons. Total glucosinolate levels were highest in younger plant tissues. Sinalbin declined as a proportion of total glucosinolate content in later growth stages, especially in the "low" breeding lines. Reproduction by aphids was the same on all three populations despite differences in sinalbin content of the *S. alba* leaves at the growth stage tested. The specialist aphid, *Brevicoryne brassicae*, was found mainly on young tissues, while the generalist aphid, *Myzus persicae*, was found predominantly on older plant parts. There was no difference in oviposition by pollen beetles between the *S. alba* populations, despite the fact that on one population total glucosinolate concentration and the proportion of sinalbin found in the buds were much lower than on the other two populations.

Key Words—Insect–plant interactions, growth stages, white mustard, glucosinolates, sinalbin, *Sinapis alba*.

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INTRODUCTION

Glucosinolates and their breakdown products, present in plants of the family Brassicaceae, may protect plants from some generalist pests and pathogens (Giamoustaris and Mithen 1995) while also acting as stimulants and attractants for specialist organisms. However, the status of glucosinolates as semiochemicals remains ambiguous and glucosinolates do not always mediate crucifer-insect interactions (e.g., Chew, 1988; Roessingh et al., 1992; Huang and Renwick, 1993; Hopkins et al., 1997; Bodnaryk, 1997). Low-seed-glucosinolate cultivars of oilseed rape and turnip rape are widely grown and initial concern about increased susceptibility to pests and pathogens was not realized (Williams, 1989). Part of the explanation has been that glucosinolate content in the seed is not always correlated with that found in other plant parts and that glucosinolate composition and concentration changes throughout plant development (Clossais-Besnard and Larher, 1991; Fieldsend and Milford, 1994a, b).

White mustard, *Sinapis alba* L. is largely grown as a condiment crop and the seeds of *S. alba* usually contain high levels of glucosinolates (Hemingway, 1995). Recently, however, breeding lines of *S. alba* have been developed with low concentrations of glucosinolates in the seed (Krzymanski et al., 1991). Sinalbin (*p*-hydroxybenzyl glucosinolate), an aromatic glucosinolate, is the dominant glucosinolate in *S. alba*. Bodnaryk (1991) demonstrated that during early growth the concentration of sinalbin (per unit fresh weight) declines rapidly during the first few days of growth of both cotyledons and the first true leaf of a normal glucosinolate (i.e., not selected for a specific glucosinolate trait) genotype of *S. alba*. The relationship between *S. alba* seed glucosinolate content and that in other plant parts remains, as yet, unexplored.

S. alba is less suceptible to insect attack than the widely cultivated *Brassica napus* L. and *B. campestris* L. This has been shown for both crucifer specialists (Reed et al., 1989; Bodnaryk and Lamb, 1991; Dosdall et al., 1994; Ekbom and Borg, 1996) and generalist herbivorous insects (Bodnaryk, 1991; McCloskey and Isman, 1993; Bodnaryk, 1996). Palaniswamy and Lamb (1992) found that cotyledons of *S. alba* possessed antixenotic resistance to the feeding of the flea beetles *Phyllotreta cruciferae* (Goeze) and *P. striolata* (F.). Ekbom and Borg (1996) assessed a range of crucifer species in field, semifield, and glasshouse trials and demonstrated that *S. alba* was a poor oviposition host for the pollen beetle, *Meligethes aeneus* Fab. In addition, *M. aeneus* exposed to *S. alba* reduced egg production (Hopkins and Ekbom, 1996). Thompson (1963) considered *S. alba* to possess antixenotic resistance to *Brevicoryne brassicae* (L.) and attributed this to the nature of the leaf surface waxes.

Bodnaryk (1991) concluded that sinalbin was a component of insect anti-

xenosis and antibiosis for a flea beetle (*P. cruciferae*) and an armyworm (*Mamestra configurata* Walker). Using this information, we predicted that sinalbin might also be detrimental to herbivores in other insect–*S. alba* interactions. We therefore compare the responses of four insect species that attack different plant parts to three populations of *S. alba* that differ in seed total glucosinolate content. We chose flea beetles (Coleoptera: Chrysomelidae), which attack the plant during early growth stages, and pollen beetles (Coleoptera: Nitidulidae), which utilize buds and flowers during later growth stages (Lamb, 1989). All growth stages of crucifers are subject to aphid attack (Ekbom, 1995) and in temperate zones *B. brassicae*, an oligophagous pest of crucifers, and the extremely polyphagous *Myzus persicae* (Sulz.) are the two most common aphid pests.

This study had two aims: (1) to investigate the distribution of glucosinolates in different tissues of populations of *S. alba* with differing seed glucosinolate content, and (2) to assess the susceptibility of low-seed-glucosinolate lines of *S. alba* to a range of insect pest species.

METHODS AND MATERIALS

Plant Material

Three types of *Sinapis alba* (white mustard) were grown in the glasshouse in 5-cm pots in compost. These were the cultivar Mustang (Sv 94-67001) and two Svalöf-Weibull breeding populations that had been selected for low-seed-glucosinolate content, Sv 94-63188 and Sv 94-60006 (hereafter line 88 and line 06, respectively). The total seed glucosinolate content of Mustang was approximately 103 μM/g dry weight). The seed glucosinolate contents of line 88 and line 06 were approximately 13 and 14 μM/g dry weight, respectively (Åhman, personal communication). There was insufficient seed material to verify independently the seed glucosinolate content of the populations.

Glucosinolate Analysis

Plant material for glucosinolate analysis was sampled at three distinct growth stages: (1) cotyledons, when the cotyledons were fully formed and the first true leaf had just started to show; (2) six true leaves (6TL), when six true leaves had formed and the seventh true leaf had just started to show; and (3) buds, only bud samples were taken from these plants, the whole raceme was sampled before flowering and raceme elongation took place. Plant material for glucosinolate analysis was collected by cutting with scissors. All plant parts were weighed immediately prior to being frozen in liquid nitrogen. The material was

then freeze-dried and homogenized before extraction of the glucosinolates. Glucosinolate extraction and analysis were performed according to Bjergegaard et al. (1995). The internal standard used was sinigrin and tentative identification of individual glucosinolates was made using published data and chromatograms supplied by Dr. H. Sørensen of the Chemistry Department of the Royal Veterinary and Agricultural University, Copenhagen, Denmark. Relative response factors for individual glucosinolates were also obtained from the laboratory of Sørensen. Capillary electrophoresis was performed on a ABI HT-200 instrument connected to the Beckman system GOLD for data collection and analysis. Usually three samples (from different plant materials) were analyzed for each point, but sometimes only two samples were available.

Insects

Phyllotreta undulata. Individual yellow striped flea beetles, *Phyllotreta undulata* (Kuts), were collected from potted *B. napus* plants placed outdoors. They were thereafter kept for several days feeding freely on *B. napus* prior to being used to assess feeding on one of the *S. alba* lines. Both choice and no-choice bioassays were performed in the greenhouse (temperature 18–20°C, ambient light levels, 18-hr photoperiod). For choice bioassays, 12 sets of plants, each comprising three plants, one from each population potted together, were placed on a rotating turntable (diameter 1 m) for 24 hr with approximately 100 flea beetles. The number of bite marks on each plant was then counted. No-choice tests were also conducted over 24 hr. A single potted plant was placed in a cylindrical netted cage (height 30 cm, diameter 13 cm) with one flea beetle. After 24 hr the number and location of bite marks were recorded. For both choice and no-choice experiments the cotyledons were used when the first true leaf had just started to show.

Brevicoryne brassicae *and* Myzus persicae. Both species of aphids were kept in culture on *B. napus* plants. On the day of emergence from the last larval instar, a single alate individual was placed on the first true leaf of a 3TL plant. The plant, in the greenhouse (temperature 18–20°C, ambient light levels, 18-hr photoperiod), was then covered with a cylindrical net cage (height 30 cm, diameter 13 cm). Five days later the number and distribution of nymphs on each plant were recorded. Seven replicates for each *S. alba* population were carried out.

Meligethes aeneus. Individual *M. aeneus* were collected in the field using sweep nets and pooters. Gravid females were identified by placing insects individually in a 40-ml plastic vial with buds of *B. napus*. After 24–36 hr buds were dissected, and those individuals that had laid eggs were introduced to the experiment. Bioassays of oviposition by *M. aeneus* were performed based upon

the methods of Ekbom and Borg (1996). Individual females were kept in the greenhouse (temperature 18–20°C, ambient light levels, 18-hr photoperiod) in a cylindrical net cage (height 30 cm, diameter 13 cm) with a single raceme of the test plant population. The raceme stood in a plastic bottle filled with water and with cotton wool around the stem. Every two days the raceme was replaced. The number of eggs laid on exposed racemes was determined. Individual beetles were kept and monitored until they died. A smaller group of individuals was also kept on *B. napus* (highly susceptible to *M. aeneus* oviposition) to check that the environmental conditions were suitable for oviposition.

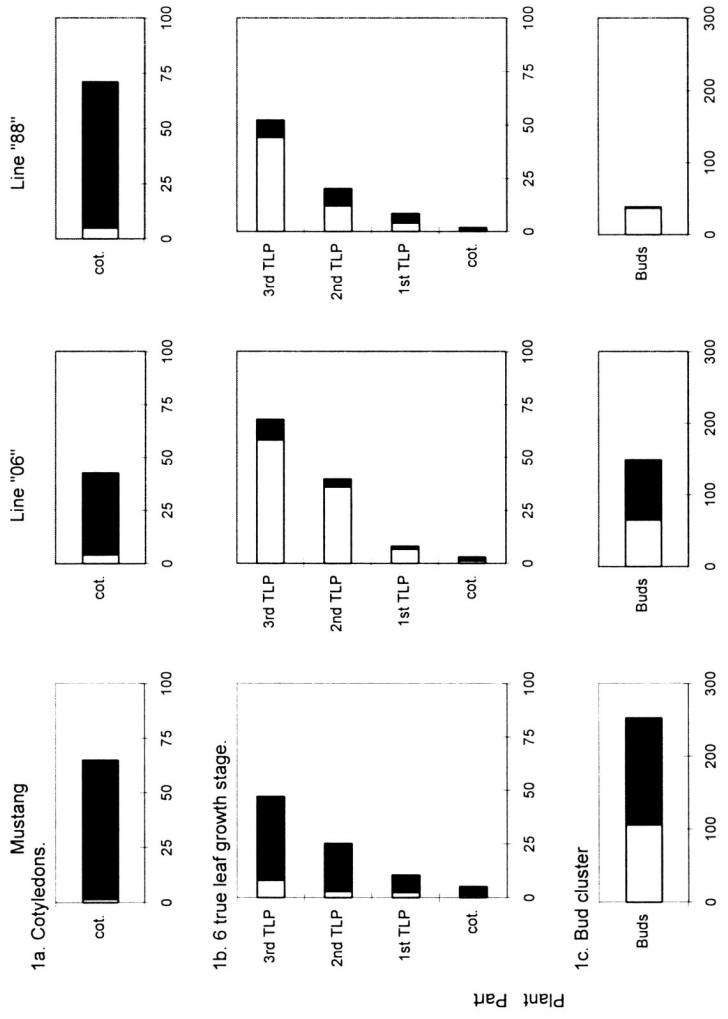
Statistics

For the glucosinolate data, GLM analyses were performed. At the cotyledon stage, a one-way ANOVA was used to test differences between populations. For the 6TL stage, a two-way ANOVA was used with populations and plant parts being the main effects. Differences in damage by flea beetles between populations were analyzed using a one-way ANOVA. Aphid reproduction was tested using an ANOVA. Differences in aphid distributions were analyzed using a χ^2 test. A χ^2 test was also used to determine if pollen beetle oviposition varied between populations. All data were analyzed using the statistical package Minitab.

RESULTS

Glucosinolate Content of Different Lines. Although the composition of the total glucosinolates was complex, most of the information is readily summarized. Six glucosinolates were consistently detected in all tissue from the three populations of *S. alba* tested. The glucosinolates detected were benzyl, *p*-hydroxybenzyl (sinalbin), 3-indolyl methyl, isobutyl, 4-hydroxy-3-indol methyl, and phenylethyl glucosinolate. Four other glucosinolates, which were not identified, were detected as minor components of the buds; each constituted a mean of 1–4% of the total glucosinolate content. Overall, the dominant individual glucosinolate was sinalbin (*p*-hydroxybenzyl glucosinolate), which averaged approximately 60% of all foliar samples and for cotyledons accounted for over 90% of the total glucosinolates. The sinalbin concentration showed a marked decline over time (Figure 1a–1c). The majority of the remaining glucosinolates detected were either the phenylethyl or benzyl forms. Benzyl glucosinolate was not detected in the cotyledons of Mustang, and the small concentrations found in the breeding lines were consequently significantly higher ($F_{2,24} = 5.04$; $P = 0.02$).

Cotyledons. Analyses of the cotyledons of the three populations of *S. alba*



Note, scale differs in figure 1c.

FIG. 1. The total glucosinolate concentration of different tissues of three populations of *S. alba*, and the proportion of total glucosinolate content that is sinalbin (the filled portion of the bar). Statistical differences are discussed in the text.

at the cotyledon stage found no difference ($F_{2,8} = 0.78$; $P = 0.5$) between the three populations (Figure 1a). There was no variation in sinalbin ($F_{2,8} = 0.78$; $P = 0.5$), which made up over 90% of the glucosinolates at this early growth stage. In cotyledons, levels of benzyl glucosinolate and isobutyl glucosinolate (not shown) were approximately 1 and 2 μM/g dry weight, respectively. Benzyl glucosinolate was not detected in the cotyledons of Mustang at any growth stage, and isobutyl was only ever found at low levels (0.02–0.20 μM/g dry weight). Cotyledons were still present on plants at the 6TL stage and overall analyses found very clear differences ($F_{2,24} = 18.54$; $P < 0.001$) between the total glucosinolate concentration found in cotyledons of the different growth stages, the total concentration falling to about one twentieth of initial levels by the time samples were taken from plants with six true leaves (Figure 1a and 1b).

6 True-Leaf Stage. At the 6TL growth stage there was no significant difference in total glucosinolate content between the three populations ($F_{2,34} = 0.41$; $P = 0.67$). However, the concentration of sinalbin did differ between populations ($F_{2,34} = 9.22$; $P < 0.001$), the sinalbin content being much lower in the two breeding lines than in Mustang. There were clear differences in the total glucosinolate concentration ($F_{3,34} = 7.53$; $P < 0.001$) and sinalbin concentration ($F_{3,34} = 6.32$; $P = 0.003$) found in different plant parts; both were higher in younger parts (Figure 1b). The proportion of sinalbin was lowest in the second and third true leaf pair of line 88 and line 06 at the 6TL growth stage (Figure 1b). In these samples the total glucosinolate concentration was maintained by a rise in the concentration of benzyl and phenylethyl glucosinolates. In Mustang, benzyl and phenylethyl glucosinolates constituted no more than 10% and 5%, respectively, of the total glucosinolate content of foliage. The percentages of benzyl and phenylethyl glucosinolates in the second and third true leaf pair of line 88 and line 06 at the 6TL growth stage were approximately 50% and 15%, respectively, of the total glucosinolates.

Flower Bud Clusters. Analysis of the glucosinolate content of bud clusters found considerable variation in the total glucosinolate concentration both between and within *S. alba* populations. Glucosinolate concentration, particularly sinalbin, was lower in buds of line 88 (Figure 1c). This difference was not significant for either total glucosinolate concentration ($F_{2,5} = 0.81$; $P = 0.524$) or sinalbin concentration ($F_{2,5} = 1.12$; $P = 0.432$). However, the proportion of sinalbin in line 88 was significantly lower ($F_{2,5} = 67.15$; $P = 0.003$) than in the other two populations.

Phyllotetra undulata. In both choice and no-choice experiments, there were no discernible feeding preferences of *P. undulata* for any of the populations (Figure 2). In both sets of tests there were marginally more bite marks on cv. Mustang, but for both choice ($F_{2,105} = 2.72$; $P = 0.07$) and no-choice ($F_{2,89} = 1.49$; $P = 0.231$) there were no statistically significant differences, despite

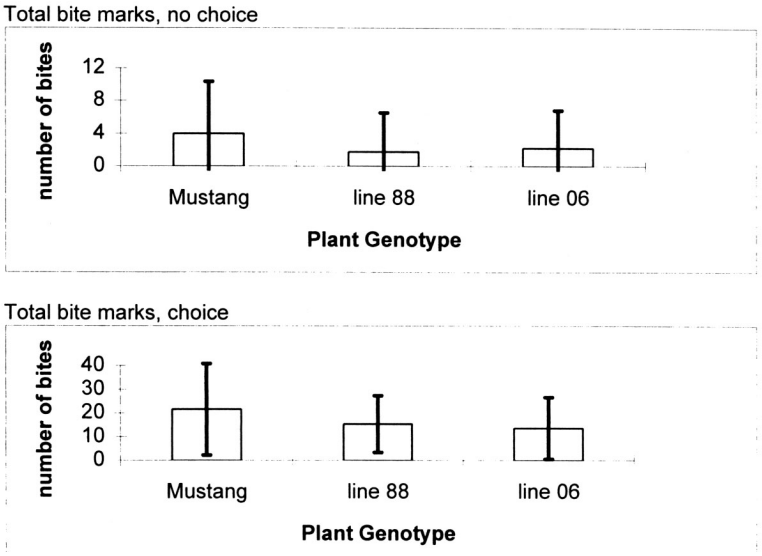


FIG. 2. Mean and standard deviation of bite marks made by *Phyllotreta undulata* on three populations of *S. alba* during choice and no-choice experiments.

fairly high replications ($N = 36$ for choice tests and $N = 29$ to 31 for no-choice tests).

Aphids. There was no significant difference ($F_{2,41} = 0.65$; $P = 0.527$) in the number of nymphs produced by either of the aphid species on the three populations of *S. alba*. Overall *B. brassicae* was marginally more fecund ($F_{1,41} = 4.90$; $P = 0.033$) than *M. persicae*, the former produced a mean of 13.7 ($\pm$ 0.87 SD) nymphs in five days and the latter only 11.0 ($\pm$ 0.87). Analyses of the distribution of aphid nymphs over the three populations (Figure 3) showed interesting and significant (Table 1) variations in the positions where nymphs were found. *B. brassicae* tended to concentrate at the growing point of the plant, and this was particularly marked for the two low glucosinolate populations. *M. persicae* was more evenly distributed over the plant.

Meligethes aeneus. On average, fewer than 25% of the pollen beetles on *S. alba* plants laid any eggs at all, while 16 of 17 individuals kept on *B. napus* oviposited at least once (Table 2). χ^2 analysis showed no significant difference in the proportion of *M. aeneus* ovipositing on *S. alba* populations ($\chi^2 = 3.088$, $df = 2$, $P = 0.214$), while analysis of the proportion of insects laying eggs when *B. napus* was included was significantly different ($\chi^2 = 42.356$, $df = 3$, $P < 0.001$).

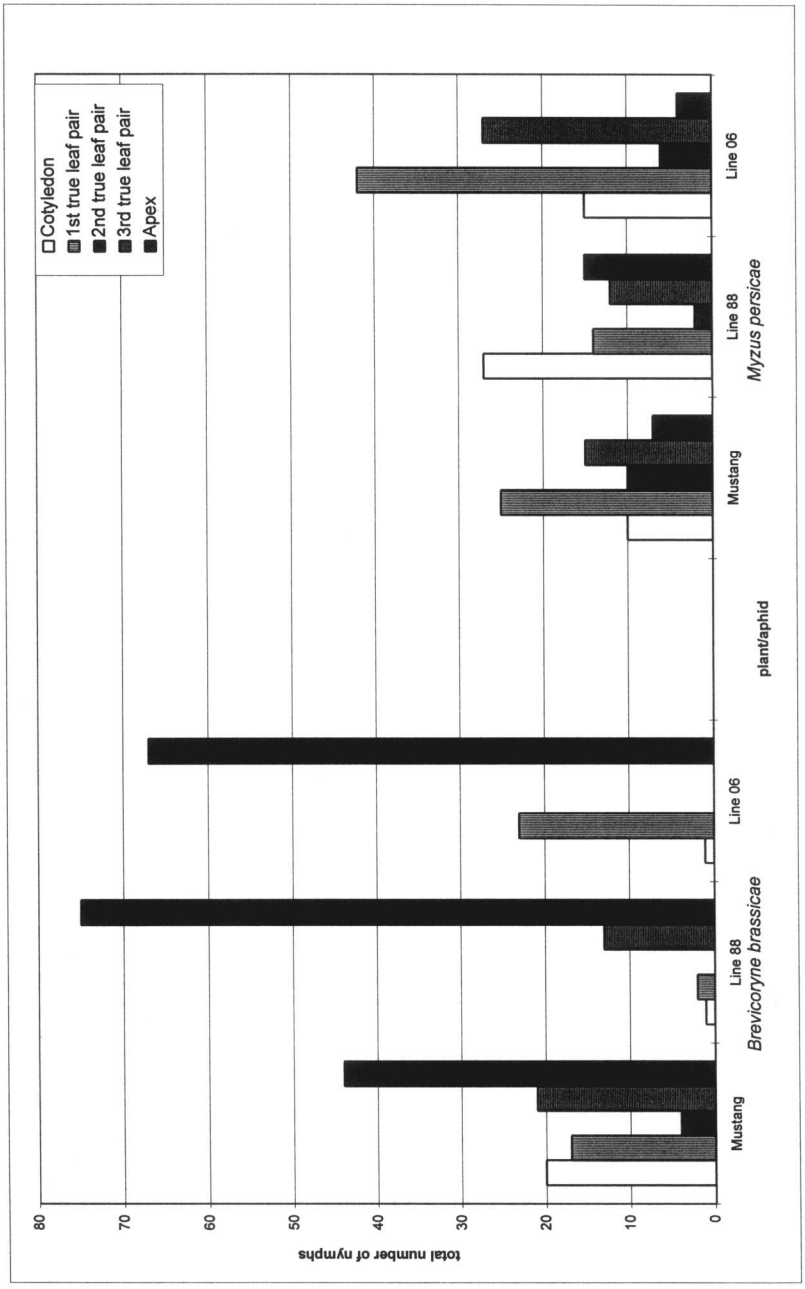


FIG. 3. The distribution of aphid nymphs of *Myzus persicae* (generalist) and *Brevicoryne brassicae* (crucifer specialist) on three populations of *Sinapis alba*. The total number of nymphs on different plant parts for seven replicates of each combination of plant population and aphid species is shown.

TABLE 1. STATISTICAL ANALYSES OF APHID DISTRIBUTION ON THREE *Sinapis alba* POPULATIONS[a]

Analysis	χ^2	*df*	Significance (*P*)
B. brassicae on 3 populations	82.76	8	<0.001
M. persicae on 3 populations	38.34	8	<0.001
2 aphid species on Mustang	27.90	4	<0.001
2 aphid species on line 88	73.70	4	<0.001
2 aphid species on line 06	106.67	4	<0.001

[a]The total number of nymphs (*Brevicoryne brassicae* and *Myzus persicae*) on cotyledons, first true leaf, second true leaf, third true leaf, and plant apex were recorded. See Figure 1.

TABLE 2. NUMBER OF POLLEN BEETLES, *Meligethes aeneus*, OVIPOSITING ON THREE *Sinapis alba* POPULATIONS AND *Brassica napus*[a]

Plant species	Plant genotype or population	Individuals laying eggs	Individuals not laying eggs
S. alba	Line 06	7	30
S. alba	Line 88	10	28
S. alba	Mustang	3	28
B. napus	Paroll	16	1

[a]Each individual female was exposed to buds for a minimum of six days.

DISCUSSION

The concentration of total and individual glucosinolates of plant parts at different growth stages could not be predicted from the glucosinolate concentration of the seed. Cotyledons of all three populations contained similar levels of total glucosinolates and over 90% was sinalbin. Total glucosinolate content at the 6TL growth stage was also similar, although sinalbin comprised a much larger proportion of the total glucosinolates in the normal seed glucosinolate population (Mustang) than in the low seed glucosinolate lines. This suggests that a reduction in sinalbin has led to a compensating increase in the other glucosinolates, such as phenylethyl and benzyl glucosinolate. For the buds, glucosinolate concentration and composition was similar for Mustang and one low glucosinolate population (line 06), while the other low glucosinolate population (line 88) contained much lower levels of total glucosinolates as well as

a lower proportion of sinalbin. There was an overall trend towards a reduced proportion of sinalbin in older plant parts, and this was particularly marked for the two populations with low seed glucosinolate content.

Generally, insect response to the three populations of white mustard was similar for all four insect species tested. There were no differences in feeding damage by flea beetles on cotyledons of the three populations. Pollen beetles rejected buds as oviposition sites to the same extent in all three populations. Aphid reproduction was similar on high- and low-glucosinolate populations. However, aphids of the crucifer specialist, *B. brassicae*, aggregated in the upper parts of the plants, where glucosinolate levels were highest, while the generalist aphids, *M. persicae*, tended to be found on plant parts with lower glucosinolate levels.

Our hypothesis that levels of sinalbin mediate the behavior of the insects tested can be partially rejected. Two populations of *S. alba* contained high levels of sinalbin in the buds while the third did not. However, pollen beetles did not show differential oviposition behavior. Sinalbin levels in the leaves of Mustang were over three times greater than those in the two low glucosinolate populations, but there was no difference in aphid reproduction. We cannot say anything conclusive about the influence of glucosinolates on the feeding damage caused by flea beetles as sinalbin and total glucosinolates were not markedly different in the three populations. In both choice and no-choice tests, feeding damage by *P. undulata* was similar on all *S. alba* populations. However, recently published results have found no variation in the feeding of the flea beetle *P. cruciferae* when exposed to populations of *S. alba* with differing foliage glucosinolate levels (Bodnaryk, 1997). Bodnaryk (1997) tested cotyledons with differences of greater than 1000-fold in their glucosinolate concentration and found no significant difference in the feeding rates of either specialist or generalist insect pests.

That glucosinolate concentration and composition is part of a dynamic process during growth that has been well documented. For *B. napus* (Clossais-Besnard and Larher, 1991; Fieldsend and Milford, 1994a) and for *S. alba* (Bodnaryk 1991) concentrations of glucosinolates are highest in young tissues and then decline, partially by dilution, as the tissues grow. For *B. napus* it has also been shown that knowing seed glucosinolate content does not necessarily yield predictive information about the glucosinolate concentrations in plant parts during vegetative growth stages (Clossais-Besnard and Larher, 1991). The profiles of glucosinolates expressed as relative proportions of individual glucosinolates also change throughout growth of *B. napus* (Clossais-Besnard and Larher, 1991; Fieldsend and Milford, 1994b), and this was also found to be the case for the cotyledons of *S. alba* in this study (Figure 1a and b). The absence of a significant difference between the total glucosinolate content of cotyledons of different populations of *S. alba* found in this study may be a result of the time of sampling. Clossais-Besnard and Larher (1991) found that high- and low-seed glucosinolate

lines of *B. napus* germinated with nearly threefold differences in cotyledon glucosinolate concentration. However, as the tissues grew, the differences decreased, until after 10 days the concentrations were similar. Because the cotyledons used in these experiments were fully expanded, it may be that any initial differences in cotyledon glucosinolate content had already disappeared.

Lower proportions of sinalbin were found in the low-seed-glucosinolate populations of *S. alba* when compared to the high-glucosinolate genotype (Figure 1). As in a previous study (McCloskey and Isman, 1993), we found moderate concentrations of another aromatic glucosinolate, benzyl glucosinolate (glucotropaeolin), in *S. alba* tissues. Glucotropaeolin in its degraded form, benzyl isothiocyanate, has been shown to be toxic for some insects (Bartelt and Mikolajczak, 1989). However, intact glucotropaeolin stimulates flea beetle feeding (Nielsen, 1978) and *Pieris rapae* L. oviposition (Huang and Renwick, 1994).

Brevicoryne brassicae were predominantly found in the apex of the plant, while *Myzus persicae* were more evenly distributed over the whole plant. It can be hypothesized that *M. persicae* mitigates possible negative effects of the secondary compounds by utilizing plant parts where the concentration of glucosinolates is lower. However, the specialist *B. brassicae*, appears to be more tolerant of high glucosinolate concentrations than the generalist, *M. persicae*, and therefore the specialist aggregates at points of high food quality, i.e., in rapidly growing tissues (Larsson, 1985). Further work will be required, but there are strong indications that the behavioral mechanisms of host utilization are different in the two species of aphid. Little is known about the variation in within-plant feeding site location in different crucifer-feeding aphids. However, Weber et al. (1986) found variations in the reproductive performance of adult *M. persicae* and *B. brassicae* on different parts of oilseed rape (*B. napus*) plants. *Brevicoryne brassicae* reproduced equally well on both new and old oilseed rape leaves, while *M. persicae* reproduced far better on older leaves than on young leaves (Weber et al., 1986).

It appears that sinalbin is not the only reason for white mustard's low susceptibility to insect attack. Low insect preference for *S. alba* is retained in the low-seed-glucosinolate populations tested in these experiments, even in the tissues with lower sinalbin content. The complex variations in glucosinolate concentration during plant growth are, however, independent of the seed glucosinolate level.

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ATTRACTION OF A FLYING NITIDULID (*Carpophilus humeralis*) TO VOLATILES PRODUCED BY YEASTS GROWN ON SWEET CORN AND A CORN-BASED MEDIUM

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Abstract—Attraction to microbial volatiles was examined for the sap beetle, *Carpophilus humeralis*, which is a pest of maize. Using 54 pure yeast and bacterial cultures, we evaluated differences in volatile emissions among species of microorganisms and whether these differences were associated with insect attraction. On a sterile corn-based medium, both yeasts and bacteria generally multiplied well and produced detectable volatile metabolites. The yeasts ranged from inactive to highly attractive, but no bacterial cultures attracted beetles above control levels. A variety of alcohols, esters, ketones, acids, and phenolic compounds were identified from the headspace above yeast cultures. Growth, volatile production, and, ultimately, attractiveness to beetles depend strongly on the ability of the yeasts to assimilate and/or ferment the carbohydrates present. Abundant volatile production on sweet corn was observed only with yeasts that are able to ferment sucrose and/or maltrose. *Saccharomyces cerevisiae* (ferments glucose, sucrose, and maltose) and *Candida shehatae* (ferments glucose and maltose) produced considerably more attractive volatiles than *Candida guilliermondii*, which only ferments glucose. Yeast volatiles important for beetle attraction included typical fermentation-associated substances (ethanol, acetaldehyde, 2-methyl-1-propanol, 1-propanol, ethyl acetate, 3-methyl-1-butanol and 2-methyl-1-butanol), and also 3-hydroxy-2-butanone, whose presence was not correlated with the occurrence of fermentation. Using aqueous mixtures of synthetic components that pro-

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duced headspace compositions simulating those of attractive yeasts, it was shown that the typical fermentation volatiles are attractive but not essential for attractiveness. 3-Hydroxy-2-butanone is sufficient but not necessary, although its attractiveness is enhanced by the presence of fermentation volatiles such as ethanol and 2-methyl-1-propanol. In nature, the beetles could take advantage of a variety of different microbial metabolic processes to locate hosts. The laboratory bioassays in this study involved flight and therefore were particularly relevant to host-finding behavior in the field.

Key Words—Sap beetle, attraction, yeast, volatile, headspace, fermentation, glucose, maltose, sucrose, maize, sweet corn.

INTRODUCTION

Sap beetles (Coleoptera: Nitidulidae) are pests of a wide variety of crops and commodities and are known to be especially attracted to plant materials that are damaged, fermenting, or otherwise decomposing. One example of such attraction is the ability of sap beetles to locate maize (*Zea mays* L.) ears that have been wounded by lepidopterous larvae or by birds. *Glischrochilus quadrisignatus* (Say) and *G. fasciatus* (Olivier) are two nitidulids that respond to damaged corn kernels (Alm et al., 1985). Beetles of the genus *Carpophilus* are also attracted to wounded ears and are particularly important because they are capable of introducing mycotoxin-producing fungi at the wound sites (Lussenhop and Wicklow, 1990). A likely factor contributing to the initial attraction of these beetles is that yeasts present on the ears proliferate in the wounds and emit attractive volatiles.

Attractive microbial and fruit-derived volatiles have been identified and have even been used to bait field traps for sap beetles. Smilanick et al. (1978) used a mixture of ethanol, acetaldehyde, and ethyl acetate for *Carpophilus* spp. Alm et al. (1985, 1989) employed butyl acetate and other esters to attract *Glischrochilus* spp. Lin and Phelan (1991a, b, 1992) and Phelan and Lin (1991) analyzed the volatiles from a number of representative yeasts and fungi grown on natural and prepared substrates, and they studied their attractiveness to nitidulid beetles. Their work was especially germane to understanding natural systems because their synthetic volatile blends closely simulated attractive microbial volatiles both qualitatively and quantitatively. Their insect bioassays monitored activity primarily by observing walking behavior. Active compounds included ethanol, acetaldehyde, ethyl acetate, 2-methyl-1-propanol, and other alcohols, esters, and ketones. Having appropriate mixtures of compounds was crucial for maximum activity, but the interactions of component volatiles were complex, and it was not always necessary to include all natural components to achieve full attractiveness.

Attraction of insects by microbial volatiles was examined further in this

study, using the nitidulid *Carpophilus humeralis* (F), which is a pest of maize. A wide range of yeast species and strains found on maize (Nout et al., 1997) and other plants (Starmer et al., 1992) was studied for their production of volatiles. One objective was to evaluate, using pure cultures, any differences in volatile emissions among species of microorganisms and to determine whether these differences were associated with the degree of insect attraction. The results of Lin and Phelan (1991a) for the yeast *Saccharomyces cerevisiae* provided valuable guidance for recognizing volatile compounds of potential behavioral importance. For several yeasts, the performance on sterile simulation media was compared to that on fresh sweet corn over time. Growth, volatile production, and beetle attraction are discussed in relation to yeast physiology (ability to assimilate and/or ferment various carbohydrates). The laboratory bioassays in this study involved flight, and therefore were particularly relevant to host-finding behavior in the field. Solid-phase microextraction (SPME) was used to sample volatiles for GC and GC-MS analysis; this greatly simplified the analytical procedures and offered particularly good sensitivity for volatiles of higher molecular weight.

METHODS AND MATERIALS

Microbial Strains. Tables 1 and 3 below list all strains of yeasts and bacteria tested. Yeasts isolated from cacti, fruits, insects, and other natural habitats were supplied by W. T. Starmer, whereas some killer yeasts were provided by L. Polonelli and Wageningen Agricultural University, and flavor producing yeasts and bacteria by the National Center of Agricultural Utilization Research. Other strains of yeasts and bacteria were isolated by us from maize (Nout et al., 1997).

Yeasts were grown on yeast malt agar (YM, containing 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, and 15 g agar per liter of distilled water) at 28°C, and maintained at 4°C. Bacteria were grown on Plate Count Agar (PCA, Difco 0479 containing 5 g tryptone, 2.5 g yeast extract, 1 g glucose, and 15 g agar per liter of distilled water) or on De Man, Rogosa, and Sharpe agar (MRS, containing 10 g peptone, 10 g meat extract, 5 g yeast extract, 20 g glucose, 1 g Tween 80, 2 g K_2HPO_4, 5 g Na acetate, 2 g $(NH_4)_3$ citrate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$, and 15 g agar per liter of distilled water) at 30°C, and maintained at 4°C.

Tests for Assimilation and Fermentation Abilities. Assimilation of glucose and maltose was assessed at 25°C after 7, 21, and 28 days in broth, and fermentation of glucose, sucrose, and maltose was assessed at 28°C after 1, 2, 4, 6, 8, 12, 16, 20, and 24 days in broth, according to Wickerham (1951) and Kreger-Van Rij (1984). Ability to ferment was assessed by measuring production of carbon dioxide.

Corn Medium. A mixture containing 1 g $(NH_4)_2HPO_4$, 0.2 g KCl, 0.2 g

$MgSO_4 \cdot 7H_2O$, 0.12 g $MnSO_4$, 0.034 g $FeSO_4$, 0.2 g yeast extract, 20 g of the carbohydrate to be tested (glucose, sucrose, or maltose), 150 g sifted corn meal (Quaker), and 1 liter of tapwater was cooked in a microwave oven. The paste was stirred and dispensed into glass wide-neck screw-capped jars, and sterilized for 30 min at 121°C.

Sweet Corn Chunks. Freshly purchased cobs of sweet corn were sliced and quartered. Damaged kernels were removed. Until inoculation, the chunks were kept in sterile Petri dishes in a laminar flow hood under UV for 1 hr. The inoculation of a chunk of sweet corn was carried out by jabbing 10 kernels on each chunk with a straight inoculation needle carrying the designated microorganism. Previously, glass jars (2-oz size), which were covered with a layer of textile gauze kept in place by a copper wire, had been sterilized for 15 min at 121°C. Each inoculated chunk was transferred into a sterile jar, which was covered with aluminum foil to prevent excessive loss of volatiles. Prepared jars were placed in a high humidity box to avoid dehydration and were incubated at 28°C.

Monitoring of Growth of Yeasts and Bacteria. For sweet corn chunks, the entire chunk was weighed in a sterilized beaker of a Stein mill (Seedboro Equipment Co., Chicago, IL). A ninefold weight of sterile physiological salt solution (PPS, containing 1.0 g peptone and 8.5 g NaCl per liter of distilled water) was added and the mixture was homogenized (giving a 10-fold dilution of the sample). For corn medium, a representative subsample of about 10 g was weighed and treated similarly. Consecutive decimal dilutions were prepared using PPS as diluant.

Appropriate decimal dilutions were pour-plated in YM agar duplicate plates with tetracycline (Sigma T-3383, 37.5 mg/liter) for yeast counts. Bacteria were counted in duplicate pour-plates of PCA with nystatin (Sigma N-3503, 50 mg/l). Microbial colonies were counted in plates containing 20–250 colonies. Numbers of colony forming units (CFU, N/g = number of colony forming units per gram of sample) were calculated by multiplying average numbers of counted colonies by dilution factors used and were reported as $\log_{10} N$/g.

Insects. The *C. humeralis* culture was originally collected near Fresno, California. The beetles were reared on the artificial diet described by Dowd (1987), except that the pinto beans were replaced with additional brewers yeast. The species is prolific and easily reared.

Bioassays to Measure Insect Attraction. The wind tunnel used for bioassays was as described previously (Bartelt et al., 1990). For bioassays, about 500–1000 adult beetles (less than 1 week old) were removed from rearing cups and released into the wind tunnel. The inlet air was heated to 29 ± 1°C. After 2–3 hr, the beetles became active and began to fly readily, so that about 100 were in the air at any instant. Test samples were suspended from a wire stretched between

the sides of the wind tunnel near the upwind end, 40 cm above the wind tunnel floor, and perpendicular to the air flow. Samples were normally tested in pairs, separated by 30 cm. Bioassays were of 1.5 or 2 min duration, with the time being constant within any experiment. Total counts of beetles alighting on the samples during the test period were recorded. The initial study that screened cultures for attractiveness was not analyzed statistically, but subsequent studies (with replicated treatments) were analyzed in the variance-stabilizing $\log(x + 1)$ scale as described previously (Bartelt et al., 1990).

The 30-ml vials containing the microbial cultures on prepared medium were covered with thin cloth to prevent beetle entry; this was held in place by a rubber band. The cloth was white in color to provide contrast for counting beetles. A wire hook, slid under the rubber band, allowed the bottles to be suspended in the wind tunnel. The relatively bulky sweet corn samples (about 10 g) were held in larger bottles (70 ml). Between tests the bottles were covered with aluminum foil to reduce diffusion of volatiles.

Collection of Headspace Volatiles, Chromatography, and Mass Spectrometry. Solid-phase microextraction (SPME) was used for collection of headspace samples. The fiber type was 100-μm polydimethylsiloxane (Supelco, Bellefonte, Pennsylvania). The fiber–needle assembly was inserted through the cotton plug (or through the aluminum foil cover) into the vial to be sampled. The fiber was extended and left in place for about 30 min. Sample desorption in the injector of the gas chromatograph (GC) was for 0.5 min, and the fiber was conditioned for an additional 2 min at 200°C in another GC injector before collecting the next sample.

Sample analysis was by GC on a Hewlett-Packard 5890 Series II instrument, equipped with cool on-column injector, flame ionization detector, electronic pressure control, and Hewlett-Packard 3396A integrator. The column was a 30-m DB-1 capillary (J&W Scientific, Folsom, California), with an inside diameter of 0.32 mm and a film thickness of 5 μm. Carrier gas was helium and injector head pressure was 16.5 psig. Oven temperature program began with a 1-min hold at 50°C, increased at 10°C/min to 250°C, and ended with a final 6-min hold at 250°C. Injector temperature was 200°C, and detector temperature was 250°C.

A Hewlett-Packard 5970 MSD was used to obtain mass spectra. Scanning range was normally 40–300 amu, but additional runs under different conditions were sometimes beneficial for compound identification (e.g., starting mass of 20 amu). Parameters for the GC inlet were as above except that the injector was of the split/splitless type, operated in splitless mode, and the head pressure was 6 psig. Again, samples were introduced by SPME.

Tentative compound identifications were based on searches of the mass spectral library. Most identifications were confirmed by demonstrating that

authentic standards had the same GC retentions and mass spectra as the unknown compounds. Quantitation of headspace concentration by SPME and GC was as described by Bartelt (1997).

SPME offers significant advantages over the thermal desorption procedure described by Phelan and Lin (1991). First, it is not necessary to handle the volatile sample with a gas-tight syringe, so that there is no chance of polar or less volatile components being lost to adsorption onto syringe surfaces. Second, a single sampling scheme is appropriate for analyzing all volatiles, regardless of quantity or volatility, and the very volatile and abundant compounds such as ethanol and acetaldehyde do not have to be sampled in a special way (Phelan and Lin, 1991). Third, SPME analyses are extremely simple to conduct and require no special sampling valves or injector hardware on the GC. Fourth, the sensitivity of SPME is sufficient to detect the concentrations and compounds reported by Lin and Phelan (1991a), and SPME has superior sensitivity for compounds of still higher molecular weight (by as much as three or four orders of magnitude) (Bartelt, 1997).

The measured headspace concentrations represent a balance between diffusion through the cotton plug (or around the foil covering) and volatile production by the microbial culture. While we use the values for comparing cultures, they do not represent an absolute rate of chemical emission by the cultures.

Screening, Attractiveness, and Volatile Production Tests. An initial screening of pure cultures of yeasts and bacteria for their attractiveness towards *C. humeralis* was performed. Pure cultures were inoculated on sterile corn medium with glucose (CM-G), incubated for two days at 28°C and tested for attractiveness by wind-tunnel bioassay. Subsequently, the volatiles produced by selected attractive as well as nonattractive cultures on CM-G were compared after a two-day incubation at 28°C.

Relations between the ability to assimilate or ferment glucose and maltose and the production of volatiles were tested using CM-G and CM-M (maltose) after two days of incubation at 28°C.

Using a representative attractive yeast (*C. guilliermondii* Y17861), growth and volatile production were compared when inoculated on CM-G and sweet corn.

Test of Hypothesis Relating Carbohydrate Fermentation, Production of Volatiles, and Attraction of Beetles. To test the hypothesis that the ability of yeasts to ferment the predominant sugars in sweet corn will result in significant headspace volatiles and attraction of *C. humeralis*, we inoculated chunks of sweet corn with yeast cultures able to ferment glucose, sucrose, and maltose (*Sacch. cerevisiae* Y17898); glucose and maltose (*C. shehatae* Y17880); and glucose only (*C. guilliermondii* Y17861). Volatile production, attractiveness to beetles, and the number of colony-forming units present were measured for the corn chunks. All three measurements were made on the same, specific sweet

corn chunks (rather than on replicates) so that variation among chunks would not affect correlations among these measurements. This was done in two consecutive experiments: in experiment 1, Y17861 was grown on sweet corn and compared with uninoculated sweet corn. Volatiles were analyzed every day (0–7); bioassays and cell counts were conducted on days 0, 2, 4, and 6. For the bioassays, Y17861 grown on CM-G at peak fermentation level served as sample with maximum attraction (a high control). In experiment 2, Y17861, Y17898, and Y17880 were grown on sweet corn and compared with uninoculated sweet corn. Volatile analyses, bioassays, and cell counts were conducted on each of days 0, 1, 2, 3, and 4. For these bioassays, baker's yeast on bread dough served as the high control sample with which to verify beetle responsiveness.

Test of Synthetic Mixtures. The attractiveness of a few mixtures of volatile synthetic compounds was assessed. Mixtures of synthetic volatiles were prepared by dissolving small amounts in water. Headspace concentrations were measured by GC-SPME and adjustments were made to solutions until the desired headspace concentrations were achieved. Ten milliliters of the solutions in 70-ml wide-mouth bottles were covered with cloth and bioassayed in the wind tunnel in the same way as the chunks of sweet corn. The mixtures were chosen to simulate two natural samples that were attractive in the wind tunnel (Y17861 and Y17898, both on sweet corn on day 3). One sample was composed primarily of typical fermentation volatiles, and the other contained a compound (3-hydroxy-2-butanone) that was not correlated to fermentation.

RESULTS AND DISCUSSION

An initial screening of microbial cultures grown on corn medium with glucose (CM-G) for two days at 28°C for their attractiveness to *C. humeralis* (Table 1) revealed that the uninoculated corn medium remained sterile ($\log_{10}N/g < 1.7$) and had a very low attraction level. There was a wide range of attractiveness among microbial cultures, ranging from none to very attractive; none of the bacteria were attractive, but they grew well and also developed a distinct odor (as noted by human olfaction; no objective data available). All yeasts grew well in CM-G as confirmed visually and by smelling for typical yeastly volatiles. Typically, yeast cultures achieved $\text{Log}_{10}N/g$ ranging from 6.8 to 7.2 (data not shown). We considered a culture attractive if it showed >5% of the attractiveness of the reference strain Y17861. All of the attractive yeasts are able to ferment glucose. On the other hand, 17 of the 25 nonattractive strains are not able to ferment glucose. These findings indicate that CM-G is a suitable medium for studies of beetle attraction because its baseline attractiveness is very low. The data also suggest that the unattractiveness of some yeasts is not caused by an inability to grow on CM-G, but by their inability to ferment glucose.

TABLE 1. FERMENTATIVE PROPERTIES OF YEASTS AND BACTERIA, LISTED IN DECREASING ORDER OF ATTRACTIVENESS TO *C. humeralis* WHEN GROWN ON CORN MEDIUM WITH GLUCOSE (CM-G) AT 28°C FOR 2 DAYS

Strain	Supplied as		Corresponding collection No.	Ferment[d]			Wind tunnel[e]		
	Collection and No.[a]	Origin[b]		G	S	M	Attr. 1	Attr. 2	Attr. 3
Pichia kluyveri var. *kluyveri*	WTS; 88-330.1	F	NRRL Y17716	+[f]	−	−			124[g] (185%)[h]
Kluyveromyces lactis var. *drosophilarum*	WTS; 85-272.1	T	NRRL Y17756	+	ND	ND			120 (179%)
Williopsis californica	WTS; 85-756.1	C	NRRL Y17720	+	ND	ND			108 (161%)
Pichia pastoris	WTS; 85-263.1	NS	NRRL Y17741	+	−	−			103 (154%)
Saccharomyces cerevisiae	WAU; LU340	NS	NRRL Y17898	+	+	+	125 (145%)		
Candida shehatae A	NPW; NRRL Y17869	M	LU024	+	−	−	123 (143%)		
Pichia kluyveri var. *cephalocereana*	WTS; 82-555B-13	NS	NRRL Y17739	±	−	−			92 (137%)
Debaryomyces hansenii	NPW; NRRL Y17873	M	LU182	+	−	−	107 (124%)		
Candida zeylanoides B	NPW; NRRL Y17863	M	LU027	+	−	−	99 (115%)		
Candida guilliemondii C	NPW; NRRL Y17881	M	LU031	+	−	−	96 (112%)		
Pichia anomala	LP; K3 UM(2)	NS	NRRL Y17901	ND	ND	ND		143 (104%)	
Candida guilliermondii A	NPW; NRRL Y17861	M	LU021	+	−	−	86 (100%)	137 (100%)	67 (100%)
Pichia kluyveri var. *cephalocereana*	WTS; 82-556D-30	C	NRRL Y17738	±	−	−			63 (94%)
Kluyveromyces lactis var. *lactis*	WAU; LU270	NS	NRRL Y17889	ND	ND	ND		113 (82%)	

Saccharomycopsis fibuligera	NCAUR; NRRL Y2388	FF		ND	ND	ND	69 (80%)		
Pichia anomala	WTS; 85-256.2	T	NRRL Y17777	+	ND	ND		108 (79%)	
Williopsis mucosca	NCAUR; NRRL YB1344	NS		ND	ND	ND	58 (67%)		
Pichia kluyveri var. *kluyveri*	WTS; 86-927.3	C	NRRL Y17750	+	–	–			37 (55%)
Candida guilliermondii	LP; K22 UCSC	NS	NRRL Y17887	+	–	–	43 (50%)		
Pichia fermentans	WTS; 83-379.2	F	NRRL Y17755	+	–	–			23 (34%)
Pichia fermentans	WTS; 88-387.2	F	NRRL Y17717	+	–	–			20 (30%)
Williopsis saturnus var. *mrakii*	NCAUR; NRRL Y1364	NS	ATCC 10743	ND	ND	ND	19 (22%)		
Candida zeylanoides E	NPW; NRRL Y17886	M	LU029	±	–	–	5.5 (6%)		
Candida zeylanoides B	NPW; NRRL Y17878	M		±	–	–	3.5 (4%)		
Pichia kluyveri	WTS; 87-498.2	NS	NRRL Y17743	+	–	–			2 (3%)
Pichia thermotolerans	WTS; 83-510.1	IN	NRRL Y17745	–	–	–			2 (3%)
Pichia membranifaciens	WTS; 85-753.4	C	NRRL Y17736	–	–	–			1.5 (2.2%)
Saccharomycopsis crataegensis	NCAUR; NRRL Y5902	FF		ND	ND	ND	1.5 (1.7%)		
Bacillus natto	NCAUR; NRRL B14206	FF		±	–	–	1.5 (1.7%)		
Pichia opuntiae var. *opuntiae* "hem"	WTS; 84-860.2	C	NRRL Y17721	–	–	–			1 (1.5%)
Pichia cactophila	WTS; 85-201.2	C	NRRL Y17722	–	–	–			1 (1.5%)

TABLE 1. Continued

Strain	Supplied as: Collection and No.[a]	Supplied as: Origin[b]	Corresponding collection No.	Ferment[d]: G	Ferment[d]: S	Ferment[d]: M	Wind tunnel[c]: Attr. 1	Wind tunnel[c]: Attr. 2	Wind tunnel[c]: Attr. 3
Pichia kluyveri var. *kluyveri*	WTS; 84-670.2B	C	NRRL Y17734	+	−	−			1 (1.5%)
Pichia antillensis	WTS; 82-550A	C	NRRL Y17719	−	−	−			1 (1.5%)
Pichia antillensis	WTS; 82-604B	NS	NRRL Y17740	−	−	−			1 (1.5%)
Pichia thermotolerans	WTS; 83-505.1	IN	NRRL Y17744	−	−	−			1 (1.5%)
Pichia opuntiae var. *opuntiae*	WTS; 84-677.2	C	NRRL Y17746	−	−	−			1 (1.5%)
Pichia opuntiae var. "hem"	WTS; 84-853.3	C	NRRL Y17748	−	−	−		2 (1.5%)	
Trichosporon cutaneum	NPW; NRRL Y17874	M	LU696	−	−	−		1 (0.7%)	
Pichia kluyveri	WTS; 88-370.2F	F	NRRL Y17715	+	−	−			0.5 (0.7%)
Pichia kluyveri var. *eremophila*	WTS; 83-466.1B	C	NRRL Y17735	±	−	−			0.5 (0.7%)
Pichia opuntiae var. *opuntiae*	WTS; 84-821.2	C	NRRL Y17737	−	−	−			0.5 (0.7%)
Pichia amethionina var. *pachycereana*	WTS; 80-314.1	IN	NRRL Y17779	−	−	−			0.5 (0.7%)
Trichosporon cutaneum	NPW; NRRL Y17868	M	LU697	−	−	−	0.5 (0.6%)		
Trichosporon cutaneum	NPW; NRRL Y17875	M		−	−	−	0.5 (0.6%)		
Pichia kluyveri var. *eremophila*	WTS; 86-541.5A	C	NRRL Y17751	±	−	−	0.5 (0.6%)		

Bacillus subtilis	NPW; NRRL B23190	M		±	−	−		0.5 (0.4%)		
Bacillus subtilis	NPW; NRRL B23189	M		±	−	−		0.2 (0.15%)		
Pichia kluyveri var. *cephalocereana*	WTS; 82-555B-17	C	NRRL Y17718	±	−	−				0 (0%)
Pichia opuntiae var. "hem"	WTS; 84-833.1	C	NRRL Y17747	−	−	−				0 (0%)
Pichia fermentans	WTS; 88-379.2	F	NRRL Y17749	+	−	−				0 (0%)
Lactobacillus maltaromicus	NCAUR; NRRL B14852	NS	DSM 20342	+	+	+	0 (0%)			
Bacillus natto	NCAUR; NRRL B14204	FF		±	−	−	0 (0%)			
Candida mucilagina	WTS; 84-201.7	C	NRRL Y17780	−	−	−				0 (0%)
Crytococcus cereanus	WTS; 83-1112.2	C	NRRL Y17778	−	−	−				0 (0%)
Control CM-G							0 (0%)	0.13 (0.1%)		0.4 (0.6%)

[a] LP = L. Polonelli, NCAUR = National Center for Agricultural Utilization Research, NPW = Nout et al. (1997), WAU = Wageningen Agricultural University, WTS = W. T. Starmer.

[b] C = cacti, F = fruits, FF = fermented food, IN = insects, M = maize, NS = not specified, T = trees.

[c] ATCC = American Type Culture Collection, DSM = Deutsche Sammlung fuer Mikroorganismen, LU = Department of Food Technology and Nutritional Sciences, Wageningen Agricultural University, NRRL = collection at NCAUR.

[d] Ability to ferment glucose (G), sucrose (S), maltose (M).

[e] Three independent single wind-tunnel bioassays.

[f] +, able; ±, weakly able; −, unable; ND, not determined.

[g] Average number of landings per 1.5 min.

[h] Relative to *C. guilliermondii* NRRL Y17861.

Headspace volatiles of representative attractive and nonattractive cultures (grown on CM-G at 28°C for two days) were separated and identified by GC and MS. Table 2 shows that the less-attractive yeasts produced considerably less volatile matter. Specific volatiles that occurred only in attractive samples

TABLE 2. HEADSPACE VOLATILES FORMED ON CM-G AT 28°C[a]

	Volatile (ng/ml gas)					
Identity	*P. kluyveri kluyveri* Y17716	*C. shehatae* Y17869	*D. hansenii* Y17873	*C. zeyla-noides* B Y17863	*C. guillier-mondii* C Y17881	*C. guillier-mondii* A Y17861
Relative attractivity index (*C. guilliermondii* A Y17861 = 100%)	185	143	124	115	112	100
Acetaldehyde	6.2	5.2	3.9	0.55	1.2	11.5
Ethanol	790	120	200	120	210	790
Formic acid	16	4.7	1.7	0	1.9	14
1-Propanol	0	0	0	0	0	2.0
Acetic acid	3.2	0	0	3.3	0	0
Ethyl acetate	1000	0	2.2	0	0	1.1
2-Methyl-1-Propanol	7.9	2.9	7.1	16	8.9	13
3-Hydroxy-2-Butanone	0	0.54	0.72	2.4	0	0.41
Ethyl propanoate	2.4	0.19	0	0	0	0.15
Propyl Acetate	0	0	0	0	0	0
3-Methyl-1-Butanol	4.7	6.0	6.7	42	5.7	6.9
2-Methyl-1-Butanol	1.9	1.5	2.3	6.4	3.1	3.1
2,3-Butanediol (chiral)	0	0	0.079	0	0.05	0.05
2-Methylpropyl acetate	2.6	0	0	0	0	0
2,3-Butanediol (*meso*)	0	0.066	0.015	0	0	0.04
Butyl acetate	0.20	0	0	0	0	0
3-Methylbutanoic acid	0.025	0.018	0.060	0.034	0.033	0
2-Methylbutanoic acid	0	0.036	0.062	0.11	0.074	0.04
3-Methylbutyl acetate	110	0	0.093	0	0	0
Pentyl acetate	0.25	0	0	0	0	0
2-Octanone	0.055	0	0	0	0	0
Ethyl hexanoate	0.0090	0	0	0	0	0
Hexyl acetate	0.51	0	0	0	0	0
Heptyl acetate	0.074	0	0	0	0	0
2-Phenylethanol	0.0077	0.040	0.054	0.094	0.055	0.037
4-Ethylphenol	0.0017	0	0	0	0.022	0.020
Benzyl acetate	0	0	0	0	0	0
Ethyl octanoate	0.011	0	0	0	0	0
2-Phenylethyl acetate	1.62	0	0	0.0034	0	0
4-Ethyl-2-methoxyphenol	0	0	0	0	0.032	0.035
2-Phenylethyl propanoate	0	0	0	0	0	0
Ethyl decanoate	0	0	0	0	0	0

[a]Headspace concentration of 0 indicates that the analyte was not detected.

were alcohols [ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2,3-butanediol (chiral and *meso*)], ketones (3-hydroxy-2-butanone), esters (ethyl acetate, ethyl propanoate), and phenolic derivatives (2-phenylethanol, 4-ethylphenol, 2-phenylethyl acetate, 4-ethyl-2-methoxyphenol). Some

TABLE 2. Continued

Volatile (ng/ml gas) Continued							
P. kluyveri kluyveri Y17750	*C. zeylanoides* E Y17886	*C. zeylanoide* B Y17878	*T. cutaneum* Y17874	*T. cutaneum* Y17875	*T. cutaneum* Y17868	*P. kluyveri cephalocereana* Y17718	CM-G uninoculated control
55	6	4	0.7	0.6	0.6	0	
1.8	9.4	0	1.7	0.48	1.1	0	0
420	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
4.8	0	0	0	0	2.2	0	0
340	0	0	0	0	0	0	0
1.4	4.2	0.90	0	0	0	0	0
0	8.5	0.88	0	0	0	0	0
0	0	0	0	0	0	0	0
2.1	0	0	0	0	0	0	0
0.17	32	13	0	0	0	0	0
0.53	4.2	2.0	0	0	0	0	0
0	0.16	0	0	0	0	0	0
5.7	0	0	0	0	0	0	0
0	0.70	0.027	0	0	0	0	0
0.49	0	0	0	0	0	0	0
0	2.8	2.9	0	0	0.11	0	0
0	0.50	0.44	0	0	0	0	0
44	0	0	0	0	0	0	0
0.32	0	0	0	0	0	0	0
0.041	0	0	0	0	0	0	0
0.0072	0	0	0	0	0	0	0
0.45	0	0	0	0	0	0	0
0.056		0	0	0	0	0	0
0.0054	0.13	0.081	0	0	0	0	0
0	0	0	0	0	0	0	0
0.015	0	0	0	0	0	0	0
0.0068	0	0	0	0	0	0	0
0.44	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0.0012	0	0	0	0	0	0	0
0.0032	0	0	0	0	0	0	0

volatiles such as acetaldehyde and acetic acid, as well as minute amounts of other, unidentified compounds occurred throughout, including less attractive samples. This would suggest that if a culture is not able to ferment a carbon source, low levels of volatile production result. It appears that such nonfermenting cultures would only produce some volatiles such as acetaldehyde and acetic acid. On the other hand, fermentation yields much larger quantities of volatiles, especially alcohols (predominantly ethanol), ketones, esters, and phenolic derivatives and also higher amounts of acetaldehyde. The phenolic compounds presumably originate from metabolism of aromatic amino acids.

The effect of carbohydrate assimilation and fermentation on volatile production by selected yeasts was measured using CM-G or CM-M (maltose) (incubated at 28°C, for two to four days) and analysis by GC and MS (Table 3).

TABLE 3. MAJOR HEADSPACE VOLATILES FORMED ON CM-G AND CM-M AT 28°C (MAXIMUM VALUES ACHIEVED AT 2–4 DAYS)[a]

	Candida shehatae B NPW; NRRL Y17880; M		*D. hansenii* Y17873		*C. guilliermondii* Y17861	
Identity	G	M	G	M	G	M
Assimilation	+	+	+	+	+	+
Fermentation	+	+	+	−	+	−
Acetaldehyde	2.9	5.9	6.8	0[b]	4.5	0
Ethanol	365	214	410	0	410	0
Formic acid	1.5	0	4.9	0	5.3	0
1-Propanol	11.6	0	1.5	0	2.1	0
Acetic acid	24.4	49.4	3.7	5.8	3.5	6.5
Ethyl acetate	0	0	0.9	0	0	0
2-Methyl-1-Propanol	3.2	1.3	4.9	0.3	8.4	0.2
3-Hydroxy-2-Butanone	6.7	3.2	0.6	0	0.7	0
Ethyl propanoate	0	0	0	0	0	0
3-Methyl-1-Butanol	3.2	2.4	1.7	0.3	2.9	0.5
2-Methyl-1-Butanol	1.4	0.8	0.7	0.3	1.2	1.0
2-Methylpropyl acetate	0	0	0	0	0	0
2,3-Butanediol (meso)	0.5	0.2	0	0	0	0
2-Phenylethanol	0.02	0.01	0.02	0.01	0.01	0.008
4-Ethylphenol	0.009	0.009	0	0.007	0.01	0.02
2-Phenylethyl acetate	0	0.008	0	0	0	0
4-Ethyl-2-methoxyphenol	0	0	0	0	0.007	0.03

[a]For abbreviations see Table 1. G, corn medium with glucose; M, corn medium with maltose. Assimilation, ability to assimilate; Fermentation, ability to ferment +, able; ±, weakly able; − unable.
[b]Headspace concentration of 0 indicates that the analyte was not detected.

The data indicate that the production of volatiles is determined by two factors: (1) the ability to assimilate a carbon source (compare Y17873 and Y17861 with Y17871 on CM-M) is responsible for some (trace) volatiles such as acetic acid, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-phenylethanol; and (2) the ability to ferment a carbon source (compare Y17880 with Y17873 and Y17861 on CM-M; compare Y17880, Y17873, Y17861, Y17886 and Y17871 with Y17876 and Y17874 on CM-G) is responsible for abundant amounts of ethanol and acetaldehyde and increased amounts of the volatiles associated with assimilation, such as 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol. In addition, 3-hydroxy-2-butanone, *meso*-2,3-butanediol, and several esters occurred sporadically in the cultures displaying fermentation.

TABLE 3. Continued

C. zeylanoides Y17886		*Pichia membranifaciens* NPW; NRRL Y17871; M		*Cryptococcus albidus* var. *aerius* NPW; NRRL Y17876; M		*T. cutaneum* Y17874	
G	M	G	M	G	M	G	M
+	±	+	−	+	+	+	+
±	−	±	−	−	−	−	−
5.5	0	0.9	0	0	0	0	0
569	0	269	0	1.8	2.7	0	0
0.5	0	2.0	0	0	0	0	0
3.2	0	0	0	0	0	0	0
5.1	3.7	6.7	0	0	3.3	0	0
0	0	1528	0	0	0	0	0
6.1	0	6.0	0	0	0	0	0
1.5	0	0	0	0	0	0	0
0	0	6.6	0	0	0	0	0
8.5	0.3	12.7	0.1	0	0	0	0
2.1	0.2	2.2	0.3	0	0	0	0
0	0	0.3	0	0	0	0	0
0	0	1.6	0	0	0	0	0
0.02	0.005	3.7	0	0	0	0	0
0	0.01	0.2	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0.01	0	0	0	0	0	0

To translate results obtained with the CM-medium to a more practical situation, the performance of *Candida guilliermondii* Y17861 on sweet corn and CM-G was tested. Table 4 summarizes volatile production (as indicated by ethanol) and growth, as a function of incubation time. The data indicate that ethanol production on CM-G was much stronger than on sweet corn; the fermentation on CM-G lasted longer as well (up to eight days, data not shown). No ethanol formation was observed on sterile uninoculated CM-G. From the uninoculated (nonsterile) sweet corn, a small amount of ethanol was detected, especially shortly after wounding. It is not known whether this is from corn itself or from other microbes present. In any case, the volatile production from *C. guilliermondii* Y17861 on sweet corn was relatively poor, and this supports a conclusion that even though it grows well on corn, this yeast can not ferment corn sugars (primarily maltose and sucrose) very well.

The studies with sweet corn were expanded to include yeasts that can ferment corn sugars and also to monitor attractiveness to *C. humeralis*. *Sacch. cerevisiae* Y17898 (which ferments glucose, maltose, and sucrose), *C. shehatae* Y17880 (which ferments glucose and maltose but not sucrose), and *C. guilliermondii* Y17861 (which ferments only glucose) all grew comparably well on sweet corn (Table 5). However, the ability to ferment the corn sugars dramat-

TABLE 4. CONCENTRATION OF ETHANOL (ng/ml OF HEADSPACE ± SD) ABOVE *C. guilliermondii* A (Y17861) CULTURES AND UNINOCULATED SUBSTRATES[a]

Volatile source	Days after inoculation				
	0	1	2	3	4
CM-G with Y17861					
Ethanol (ng/ml headspace)	0.5 ± 0.7	1.0 ± 1.4	760 ± 890	1130 ± 40	740 ± 360
Biomass (Log_{10} N/g)	3.0	4.68	8.86	8.3	8.89
CM-G sterile, uninoculated					
Ethanol (ng/ml headspace)	ND	ND	ND	ND	ND
Sweet corn with Y 17861					
Ethanol (ng/ml headspace)	5.3 ± 4.0	7.1 ± 6.4	53 ± 50	10 ± 11	7.0 ± 9.1
Biomass (Log_{10}N/g)	6.47		7.7		8.48
Sweet corn non-sterile, uninoculated					
Ethanol (ng/ml headspace)	6.2 ± 4.0	7.2 ± 8.9	2.3 ± 3.2	2.5 ± 4.2	0.9 ± 1.1

[a] Ethanol concentration is reported here as an indicator for amount of fermentation. There were two replications for CM-G (corn medium with glucose) and four for sweet corn. ND indicates that ethanol was not detected.

TABLE 5. NUMBERS OF YEAST AND BACTERIA COLONY FORMING UNITS PRESENT ON SWEET CORN INOCULATED WITH THREE YEAST STRAINS AT VARIOUS TIMES AFTER INOCULATION

Inoculum	Exp[a]	Days after inoculation					
		0	1	2	3	4	6
Yeasts (Log_{10} N/g)							
Uninoculated	1	1.74	ND[b]	5.76	ND	6.48	4.95
sweet corn	2	ND	5.26	5.26	6.85	6.70	ND
C. guilliermondii A	1	6.47	ND	7.70	ND	8.48	8.51
Y17861	2	5.43	8.13	8.26	8.68	8.38	ND
S. cerevisiae Y17898	2	4.13	8.02	8.09	8.25	8.22	ND
C. shehatae B Y17880	2	4.73	8.27	8.49	8.44	8.66	ND
Bacteria (Log_{10} N/g)							
Uninoculated	1	3.40	ND	7.53	ND	8.28	7.76
sweet corn	2	4.02	8.89	8.93	9.20	9.06	ND
C. guilliermondii A	1	3.93	ND	8.72	ND	8.71	7.82
Y17861	2	4.31	9.06	8.35	8.93	9.15	ND
S. cerevisiae Y17898	2	4.67	8.58	8.61	7.91	9.05	ND
C. shehatae B Y17880	2	4.51	7.13	7.99	9.59	9.03	ND

[a] In experiment 1, only Y17861 and uninoculated corn were analyzed. Experiment 1 corresponds to Figure 1, and experiment 2 corresponds to Figure 2.
[b] ND = not determined.

ically affected volatile production (Figures 1 and 2, Table 6) and attraction of *C. humeralis* (Figures 1 and 2).

The seven compounds reported from the headspace above yeast cultures by Lin and Phelan (1991a) (acetaldehyde, ethanol, 1-propanol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol) were detected above most of the attractive yeast cultures (Table 2), and for a given yeast strain and culture substrate, ratios of headspace concentrations among these compounds tended to be stable. Strains Y17861, Y17898, and Y17880 all had similar relative profiles (if greatly different absolute amounts) for these compounds when grown on sweet corn (Table 6). The correlations among the concentrations were used to simplify the graphs (Figures 1 and 2): only the most abundant of these seven volatiles, ethanol, was included in the graphs, but it served as an approximate indicator for trends in the other six. It is recognized that mixtures of the volatiles are important for insect attraction (Lin and Phelan, 1991a). However, the importance of individual components among the seven was not assessed for

TABLE 6. RELATIVE ABUNDANCE OF EIGHT VOLATILES FROM *SWEET* CORN INOCULATED WITH YEASTS AND CORRELATIONS AMONG CONCENTRATIONS OF THESE VOLATILES[a]

Compound	Relative abundance[b]	SD
Ethanol (EtOH)	100	
Acetaldehyde (AcALD)	19	26
2-Methyl-1-propanol (2MePrOH)	2.5	3.1
1-Propanol (PrOH)	0.07	0.1
Ethyl acetate (EtAC)	0.05	0.1
3-Methyl-1-butanol (3MeBuOH)	0.47	1.0
2-Methyl-1-butanol (2MeBuOH)	0.31	0.5
3-Hydroxy-2-butanone (OHBuKET)	5.2	15

Correlations:[c]

	EtOH	AcALD	2MePrOH	PrOH	EtAC	3MeBuOH	2MeBuOH
AcALD	0.93***						
2MePrOH	0.83***	0.75***					
PrOH	0.90***	0.85***	0.80***				
EtAC	0.72***	0.80***	0.70***	0.82***			
3MeBuOH	0.50**	0.42*	0.71***	0.49**	0.58**		
2MeBuOH	0.76***	0.67***	0.95***	0.75***	0.71***	0.86***	
OHBuKET	−0.23	−0.30	−0.06	−0.18	−0.08	0.04	−0.03

[a]Data combined for yeast strains *C. guilliermondii* A Y17861, *S. cerevisiae* Y17898, and *C. shehatae* B Y17880.

[b]Relative abundance expressed as percentage of headspace concentration of ethanol (26 observations, for which total concentration of the eight compounds exceeded 10 ng/ml). Standard deviation (SD) is in the same scale.

[c]Based on 26 observations for which total headspace concentration >10 ng/ml. Significance of correlation coefficients at the 0.05, 0.01, and 0.001 levels denoted by *, **, and ***, respectively.

C. humeralis. Rather, this seven-compound blend was considered primarily as a single unit when evaluating attractiveness.

On the other hand, 3-hydroxy-2-butanone, which was detected from many of the sweet corn samples, was not correlated with ethanol or the other fermentation-related volatiles (Table 6). Therefore, 3-hydroxy-2-butanone is graphed separately in Figure 2. (The compound was not detected in the experiment shown in Figure 1).

The results of the first experiment (Table 5, Figure 1) indicate that the growth of Y17861 on sweet corn was good and clearly different from the uninoculated corn. Values in Table 5 are logarithms; numbers of yeasts on uninoculated corn were only about 1% of those on inoculated corn. The bioassay beetles were responsive: there were, overall, 35 landings per test on the attractive, high control, but for Y17861 (which cannot ferment the major corn sugars)

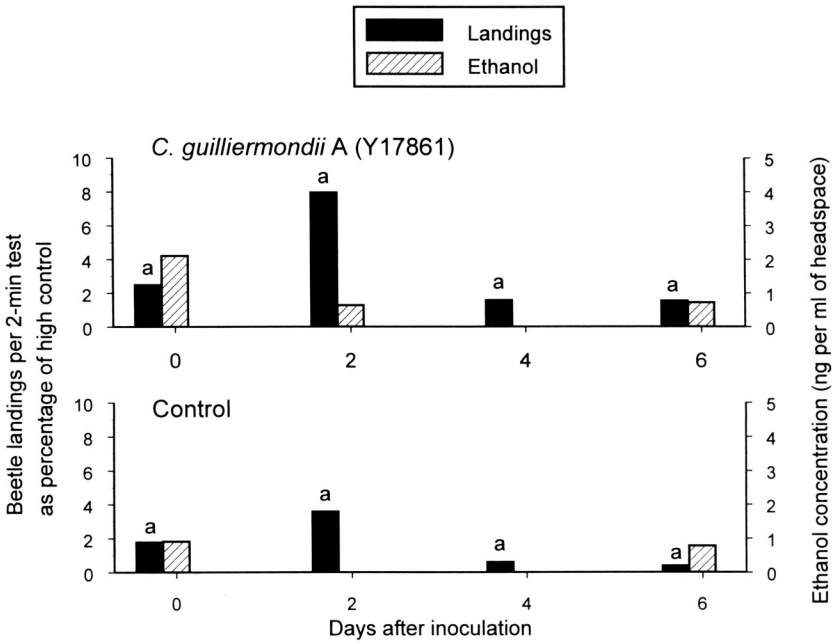

FIG. 1. Insect attraction and ethanol production for pieces of sweet corn inoculated with YM17861 and nonsterile, uninoculated wounded sweet corn. Within each day, bioassay values (landings) accompanied by the same letter were not significantly different (LSD, 0.05, $N = 7$). Means ($N = 7$) for samples with maximum attraction (high controls) on days 0, 2, 4, and 6 were 38.2, 25.2, 56.5, and 26.1, respectively.

and uninoculated corn, landings and ethanol production were low (essentially background), and there was no difference between Y17861 and uninoculated corn.

The results of the second experiment (Table 5, Figure 2) indicate that all yeasts grew equally well compared with the uninoculated corn; in addition, there was a consistent and equal background of bacterial growth in all treatments including the uninoculated corn. The attractiveness to *C. humeralis* was suitably low in the uninoculated corn, indicating (and supporting our earlier findings) that bacteria do not play a significant role in attraction of this particular insect.

Yeasts that can ferment sucrose and/or maltose (*S. cerevisiae* Y17898 and *C. shehatae* Y17880) were significantly more attractive, and this supports the hypothesis that it is the ability to ferment the naturally occurring C source that favors the attractiveness of a particular yeast. Although Y17861 was a little more attractive than previously, it was still not as active as the yeasts that can ferment corn sugars. As a result of using a different batch of corn, production

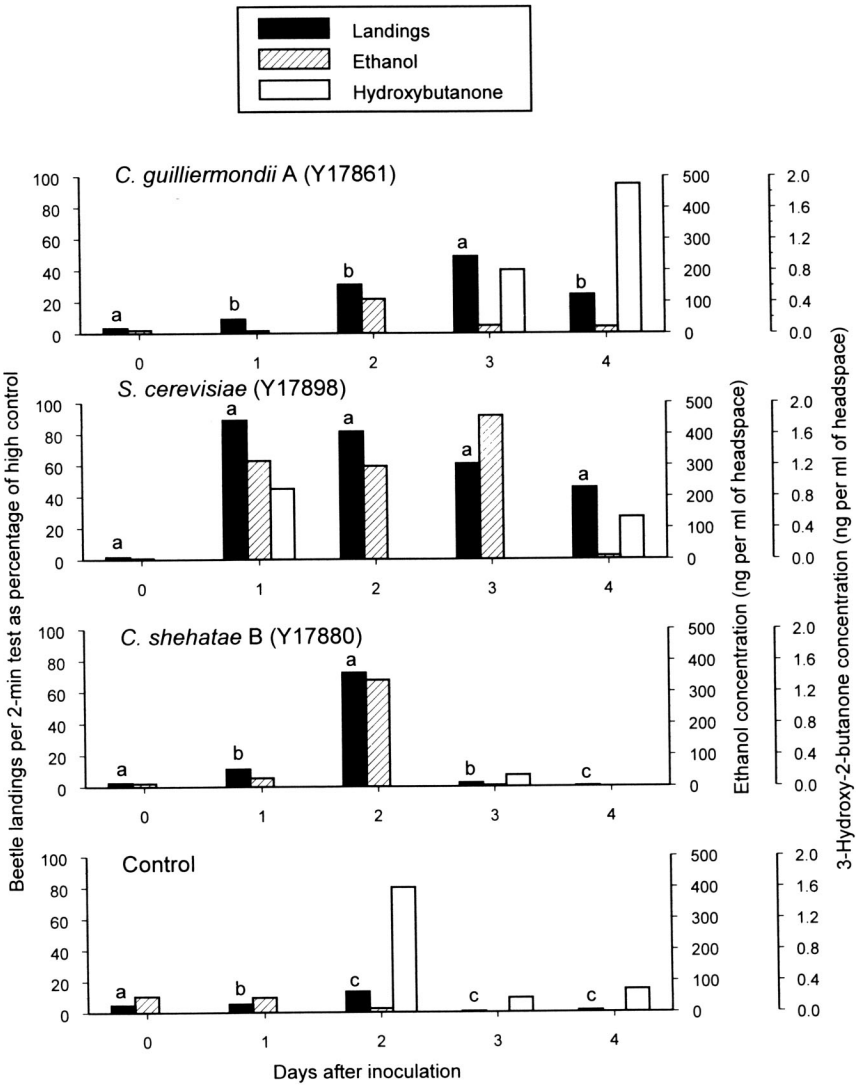

FIG. 2. Insect attraction and production of ethanol and 3-hydroxy-2-butanone from sweet corn pieces inoculated with three yeast strains, and nonsterile, uninoculated wounded sweet corn. Within each day, bioassay values (landings) accompanied by the same letter were not significantly different (LSD, 0.05, $N = 4$). Means ($N = 4$) for the sample with maximum attraction (high control) on days 0–4 were 166.0, 295.0, 171.5, 216.0, and 210.0, respectively.

of ethanol by Y17861 during experiment 1 was lower than in experiment 2. The relatively low levels of ethanol and other volatiles produced by Y17861 at day 2 may have originated from limited levels of glucose, the only carbohydrates that can be fermented by this strain.

Attraction measured by bioassay generally followed the amounts of fermentation volatiles (indicated in Figure 2 by ethanol), but several data points were higher than expected (the ethanol group could not account for the attraction). These were Y17861 on days 3 and 4, Y17898 on day 4, and control on day 2. This extra attraction coincided with presence of a small amount of 3-hydroxy-2-butanone, whose concentration did not correlate with the ethanol group. 3-Hydroxy-2-butanone was not detected in the first experiment with Y17861 on sweet corn. From these data, we conclude that the ability to ferment maltose and/or sucrose increases the potential attraction significantly as these are the main fermentable C sources in sweet corn. Low levels of glucose can cause low levels of attraction that by themselves can be sufficient to initiate insect visits.

We attempted to confirm the attractiveness of a few mixtures of synthetic volatiles (Table 7) and to demonstrate the attraction by 3-hydroxy-2-butanone.

TABLE 7. INSECT ATTRACTION BY MIXTURES OF VOLATILE SYNTHETIC COMPOUNDS DISSOLVED IN WATER[a]

	Mixtures				
Attractiveness[b]	A	B	C	D	Control
Experiment 1 (N = 4)	264.0a				1.7b
Experiment 2 (N = 4)		203.5a			1.5b
Experiment 3 (N = 3)		157.2a	5.8b	75.0a	3.6b
Composition (ng/ml of headspace)					
Ethanol	390	24	21	0	0
Acetaldehyde	39	0	0	0	0
2-Methyl-1-propanol	2.8	0.57	0.41	0	0
1-Propanol	2.0	0	0	0	0
Ethyl acetate	1.2	0	0	0	0
2-Methyl-1-butanol	0.33	0	0	0	0
3-Hydroxy-2-butanone	0	1.1	0	1.0	0

[a]Mixture A simulated the *S. cerevisiae* Y17898 inoculated fermentation of sweet corn on day 3, and mixture B simulated the *C. guilliermondii* Y17861 inoculated fermentation of sweet corn on day 3 (Figure 2). Mixtures C and D were subsets of B. The control was water only. Within a row, treatments accompanied by the same letter were not significantly different (LSD, 0.05).

[b]Average of number of landings per 2 min.

The mixtures were chosen to simulate two natural samples that were attractive in the wind tunnel (Y17861 and Y17898, both on sweet corn on day 3). One sample was composed primarily of typical fermentation volatiles, and the other contained a compound (3-hydroxy-2-butanone) that was not correlated with fermentation.

Bioassays were conducted to determine whether the mixtures (or mixtures minus certain components) were sufficient to attract the beetles. We did not attempt to compare synthetic mixtures with live yeast cultures because of their dynamic nature; by the time we analyzed them and made a good synthetic version, the culture volatile pattern would have changed, making the comparison invalid. The data in Table 7 indicate that the six-volatile mix (mixture A) was sufficient to elicit excellent bioassay response and that 3-hydroxy-2-butanone was not required for attraction. The mixture of ethanol, 2-methyl-1-propanol, and 3-hydroxy-2-butanone (mixture B) was also sufficient for attraction. In fact, the ethanol/2-methyl-1-propanol part of the mix (mixture C) was not attractive, whereas 3-hydroxy-2-butanone alone (mixture D) was, but the attractiveness is enhanced by the presence of ethanol/2-methyl-1-butanol.

It is important to note that the typical fermentation volatiles can account for attraction of the beetles, but it appears that these compounds are not essential. We therefore presume the existence of other attractants not related to fermentation. The beetles, being polyphagous, are very flexible in what they can use to direct them to a new food source.

We found 3-hydroxy-2-butanone at very low levels in many yeast cultures grown in the model medium CM-G and also on sweet corn when fermentation could take place (data not shown). 3-Hydroxy-2-butanone was noted earlier in the volatiles from *S. cerevisiae* by Phelan and Lin (1991), but this compound did not make much difference in the responses of *C. hemipterus* (L.); this may have been because of low amounts of the 3-hydroxy-2-butanone or because complex blends were being tested that could have masked the effect of the compound, or perhaps *C. hemipterus* responds in a different manner than *C. humeralis*.

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BEHAVIORAL AND PHYSIOLOGICAL EFFECTS OF NEEM (*Azadirachta indica*) SEED KERNEL EXTRACTS ON LARVAL PARASITOID, *Bracon hebetor*

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Abstract—An aqueous suspension and an ethanolic extract of neem seed kernel (NSK) at 0.3, 0.6, 1.2, 2.5, and 5.0% concentrations were tested for ovipositional deterrency, feeding deterrency, toxicity, sterility, and insect growth regulatory effects on a larval parasitoid, *Bracon hebetor*. Neither NSK extracts delivered in the food or by contact influenced the *B. hebetor* oviposition (parasitization). They also did not cause parasitoid sterility through feeding, but they showed feeding deterrent effects for a limited period. Parasitoid eggs and pupae also were unaffected by the extracts tested. The parasitoid larvae, however, were killed by feeding contaminated host larvae and also through contact with neem extracts. Thus, a minimum safety period is suggested for inundative releases of *B. hebetor* in integrated pest management.

Key Words—*Azadirachta indica*, *Bracon hebetor*, feeding deterrent, ovipositional deterrent, pest management, toxicity.

INTRODUCTION

The neem tree, *Azadirachta indica*, has an array of complex chemicals, the liminoids, which cause diverse biological effects on insects. Among several options for naturally occurring insecticides of plant origin, neem has emerged as the single most important, available, renewable, and effective source (Jacobson, 1986; Raguraman, 1987; Saxena, 1989, Ascher, 1993; Singh, 1993;

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Schmutterer, 1995; Isman, 1996). While sufficient data are available on the bioactivity of neem against insect pests, the information on neem's effect on insect parasitoids and predators is scanty. We studied whether the neem extracts that are most commonly used are safe for a polyphagous lepidopteran larval parasitoid, *Bracon hebetor* Say.

METHODS AND MATERIALS

Extraction and Formulation. Ripe neem fruits that had dropped on the ground were collected at New Delhi. Fruits were depulped, and the seeds were shade-dried for three days. The seeds were decorticated and the kernels were ground in an electric grinder. The powdered neem seed kernel (NSK) thus obtained was used for extraction. For preparing aqueous suspension, a weighed amount of NSK powder was put in a muslin cloth bag and soaked in a measured quantity of water in a beaker for 12 hr. The NSK powder in the bag was then squeezed in the water until the milky suspension stopped oozing. The milky suspension thus obtained was emulsified with 0.2% Triton X-100.

For making ethanolic NSK extract, 100 g of ground NSK was stirred with 500 ml of ethanol for 3 hr with a magnetic stirrer and filtered through Whatman No. 1 filter paper. The marc was restirred with 500 ml ethanol for 1 hr and filtered. The combined filtrate was freed of ethanol in a distillation unit at 50°C under reduced pressure. The extract was formulated by maintaining the solvent (ethanol) and the emulsifier (Triton X-100) at 10% and 5%, respectively, in the final volume of water.

Insect Culture. The nucleus cultures of the larval parasitoid, *B. hebetor*, and its host, the rice moth, *Corcyra cephalonica* (Stainton), were procured from the Parasitology Section of the Division of Entomology, IARI, New Delhi. *C. cephalonica* was reared at 28 ± 1°C and 65 ± 5% relative humidity. Freshly emerged moths were collected from the rearing jars with a mechanical aspirator and released into an oviposition cage. Eggs were collected every 24 hr. Approximately 3000 eggs (0.2 ml) were sown in about 1 kg of crushed sorghum grains contained in a glass jar (20 cm high, 15 cm diam) and covered with a white muslin cloth. The culture jars were kept undisturbed on racks for *C. cephalonica* develoment. The development of eggs, larvae, and pupae ranged from 4 to 5 days, 35 to 45 days, and 10 to 15 days, respectively.

B. hebetor was reared on 25- to 30-day-old *Corcyra* larvae at 27°C and 65 ± 5% relative humidity. The parasitization of host larvae was carried out in a specially designed (oviposition cage consisting of a glass jar (5 × 10 cm), white cloth, and a glass plate. Freshly emerged parasitoids were introduced into the glass jar and covered with the white muslin cloth. The *Corcyra* larvae were kept over the muslin cloth and the glass plate was fastened to the glass jar with

rubber bands. Raisins were provided as food for parasitoid adults. After 24 hr of exposure to parasitoids, the parasitized larvae were removed and kept on paper discs (6 × 4 cm) inside glass vials for further development. The parasitoid adults emerged 11 days after parasitization.

Bioassays. Both the aqueous suspension and the ethanolic extract of NSK were tested at 5.0, 2.5, 1.2, 0.6, and 0.3% concentrations for different biological effects, including oviposition and feeding deterrency, toxicity, sterility, and insect growth regulatory (IGR) activity against the larval parasitold, *B. hebetor*.

Oviposition Deterrency Test. The different effects of NSK extracts on *B. hebetor* oviposition were evaluated under choice and no-choice conditions. Ten 25- to 30-day-old host larvae were treated with 1 ml of the extract in Potter's tower (Potter, 1941) at 340 g/cm^3 pressure and 24 cm Hg. After shade drying, the larvae were offered for parasitization in the oviposition cage, as described above. The muslin cloth used in the oviposition cage was also treated. Five replications were maintained at each concentration with 10 host larvae and five pairs of 1-day-old *B. hebetor* adults in each replicate. Untreated *Corcyra* larvae treated with solvent served as controls. In the choice test, 10 treated and 10 untreated *Corcyra* larvae were offered for parasitization in a cage, while in the no-choice test only 10 treated larvae were exposed to parasitization. After 24 hr of exposure to the parasitoid adults, the *Corcyra* larvae were removed. Data on the number of eggs laid on *Corcyra* larvae in treatments and in controls were recorded. Mortality of parasitoid adults in 24 hr also was recorded.

Test of Settling/Feeding Response. The tests were conducted under choice and no-choice conditions. Raisins were split and dipped in both neem extracts at each concentration for 30 sec. After shade drying, the raisins were provided as food for the parasitoid adults. Controls consisted of solvent treated and untreated raisins. In the choice test, five pairs of newly emerged parasitoids were transferred to a jar (20 × 10 cm) containing two treated and two untreated raisins. Each jar comprised a replicate, with five such replicates. Since the parasitoids fed little on the raisins, it was not possible to determine the food intake. Consequently, the settling response, test probing, and the duration of stay on treated and untreated raisins by the parasitoid adults during 3-hr observation periods were used to evaluate feeding deterrency. In the no-choice test, the same number of parasitoids were introduced into the jars containing only treated raisins. Comparisons in this case were made with emulsified solvent-treated and untreated controls kept in separate jars. The number of replicates and method of evaluation were similar to the choice test.

Fertility Test. To determine the effect of feeding the parasitoid on raisins treated with extracts, five pairs of freshly emerged adults were placed in the oviposition jar (20 × 10 cm) containing treated raisins. Each jar formed a replicate with five such replicates for each treatment. Ten 25- to 30-day-old *Corcyra* larvae were offered in a cage for parasitization. After 24 hr of exposure,

the larvae were replaced with a fresh group of larvae. The process was continued for 10 days. Mortality of the parasitoid adults and the number of eggs laid on *Corcyra* larvae were recorded over a 10-day period. To preclude the effect of possible superparasitism, only two to three *Bracon* eggs were allowed to develop on a host larva. Ten host larvae along with 20 parasitoid eggs formed a replication. Data were collected on percentage of egg hatching, larvae reaching the pupal stage, and pupae reaching the adult stage and on longevity of emerged adults. Data were subjected to factorial completely randomized design, as given by Gomez and Gomez (1984).

Insect Growth Regulatory (IGR) Activity. The aqueous suspension and ethanolic extract of NSK were evaluated for IGR effects on the parasitoid in two ways. In the first procedure, host larvae were treated with the NSK extracts, and then the parasitoids were allowed to lay eggs on them. This was called the pretreatment procedure. In the second method, called the posttreatment procedure, 20 2-day-old *Bracon* along with the host larvae were sprayed with one of the two NSK extracts. Twenty *Bracon* larvae were allowed to develop on 10 host larvae in a glass vial (10 × 4 cm), which forms a replicate, with five such replicates.

Data on egg hatch, percentage of larvae reaching the pupal stage, deformed pupae, and adults emerging from the initial number of eggs and on average longevity of adults were recorded.

Statistical Analyses. Numerical data and percentages were transformed into square root and arc sin values, respectively, and analyzed by analysis of variance (ANOVA) in a completely randomized block design (Gomez and Gomez, 1984). Duncan's (1951) multiple range test (DMRT) was applied for comparing treatment means.

RESULTS

Oviposition Deterrent Effect. No significant oviposition deterrent effect was noticed with neem extracts at any concentration in choice and no-choice tests when compared to untreated control.

Settling/Feeding Response. In the choice test, irrespective of concentrations, both sexes of adult parasitoids were deterred from feeding on neem-treated raisins. Adults that settled on treated raisins characteristically raised the abdomen between the wings, and frequently cleaned their antennae, wings, and hind legs, did not feed, and were restless. These behavioral symptoms were less pronounced in adults that settled on untreated raisins, and feeding started immediately. In the no-choice test the behavioral effects were similar to those in the choice test on treated raisins. At the end of the observation period, none of the adults was resting on the treated raisins. Parasitoids in the untreated control and solvent treatment control fed normally on the raisins.

Toxicity. There was negligible mortality of male and female parasitoids at any concentration in 24 hr when the parasitoids were released on the neem treated surface of the host larvae. Feeding toxicity tests also showed that mortality of both sexes of parasitoid adults were least affected, and the mortality data between treatment and control did not vary significantly. No effect of NSK extracts was found in progeny of *B. hebetor* in fecundity, egg hatchability, or on larval and pupal development. Adults of both sexes that emerged lived as long as the controls.

IGR Activity. Pretreatment of the host larvae before parasitization revealed that the aqueous suspension had no affect on parasitoid egg hatching at any concentrations tested, but at or above 0.6% concentration the development of the parasitoid was severely affected and resulted in no adult emergence (Table 1). Even at 0.3% concentration, there was only 29% adult emergence. Larvae, prepupae, and pupae in the treatments failed to develop and showed different degrees of malformation. Ethanolic extract of NSK also showed effects on parasitoid growth and development similar to the aqueous suspension (Table 2). Only 5% of the larvae could reach adult stage at a 0.3% concentration of NSK extract.

Treatment of host larvae and parasitoid larvae (posttreatment) showed that

TABLE 1. EFFECT OF PRETREATMENT OF HOST LARVAE WITH AQUEOUS NEEM SEED KERNEL SUSPENSION (NSKS) ON EGG HATCHABILITY, DEVELOPMENT, AND ADULT LONGEVITY OF *B. hebetor*

Treatment	Eggs (no.) observed (*N*)[a]	Eggs hatched (%)[b]	Larvae reaching pupal stage (%)	Deformed pupae (%)[c]	Adults emerged from initial number of eggs (%)	Adult longevity (days)[d]	
						Male	Female
NSKS 5.0%	100	100	5a[e]	100a	0a		
NSKS 2.5%	100	100	14b	100a	0a		
NSKS 1.2%	100	100	29c	100a	0a		
NSKS 0.6%	100	100	39c	100a	0a		
NSKS 0.3%	100	100	66d	54b	29b	4.5	9.0
Emulsified water	100	100	98e	0c	98e	4.5	9.0
Untreated control	100	100	100e	0c	100c	4.5	10.5

[a] Total of five replications, 20 eggs/replicate.
[b] Mean of five replications.
[c] Deformed pupae failed to emerge.
[d] Not analyzed statistically.
[e] In a column, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's (1951) multiple range test.

TABLE 2. EFFECT OF PRETREATMENT OF HOST LARVAE WITH ETHANOLIC EXTRACT OF NEEM SEED KERNEL (ENSK) ON EGG HATCHABILITY, DEVELOPMENT, AND ADULT LONGEVITY OF *B. hebetor*

Treatment	Eggs observed (*N*)	Eggs hatched (%)[b]	Larvae reaching pupal stage (%)	Deformed pupae (%)[c]	Adults emerged from initial number of eggs (%)	Adult longevity (days)[d]	
						Male	Female
ENSK 5.0%	100	100	0a[e]		0a		
ENSK 2.5%	100	100	0a		0a		
ENSK 1.2%	100	100	0a		0a		
ENSK 0.6%	100	100	14b	100a	0a		
ENSK 0.3%	100	100	46c	89b	5b	4.0	9.0
Ethanol emulsified water	100	100	100d	0c	100c	4.5	9.5
Untreated control	100	100	100d	0c	100c	4.5	9.5

[a]Total of five replications, 20 eggs/replicate.
[b]Mean of five replications.
[c]Deformed pupae failed to emerge.
[d]Not analyzed statistically.
[e]In a column, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's (1951) multiple range test.

both the NSK extracts affected growth and development of the parasitoid larvae at all tested concentrations (Tables 3 and 4). Even the lowest concentrations (0.3% and 0.6%) of both extracts severely affected larval development and adult emergence. Unemerged parasitoid pupae in the neem treatments showed signs of malformations.

DISCUSSION

NSK extracts and pure compounds of NSK have been reported to possess oviposition deterrent effects on many crop pests (Schmutterer, 1990). Little information exists on the effect of neem extracts on ovipositional behavior of parasitoids. We found that an aqueous suspension and an ethanolic extract of NSK had no significant effect on oviposition of *B. hebetor*. This study supports the findings of Saxena et al. (1981), who reported that 25% and 50% neem oil sprayed on rice plants was safe for braconid and ichneumonid parasitoids of *Cnaphalocrocis medinalis*. Jhansi and Sundara Babu (1987) reported that 5% neem oil spray did not affect the parasitization of the pod borer, *Maruca tes-*

TABLE 3. EFFECT OF AQUEOUS NEEM SEED KERNEL SUSPENSION (NSKS) ON DEVELOPMENT OF PARASITOID (*B. hebetor*) LARVAE TREATED WITH HOST LARVAE

Treatment	Larvae observed (*N*)[a]	Larvae reaching pupal stage (%)[b]	Deformed pupae (%)[c]	Adults emerged from initial number of eggs (%)	Adult longevity (days)	
					Male	Female
NSKS 5.0%	100	22a[d]	100a	0a		
NSKS 2.5%	100	46b	100a	0a		
NSKS 1.2%	100	50b	100a	0a		
NSKS 0.6%	100	55b	63c	21b	4.2a	9.2a
NSKS 0.3%	100	92c	76b	22b	4.2a	9.4a
Emulsified water	100	100d	0d	100c	4.6a	9.7a
Untreated control	100	100d	0d	100c	4.6a	9.7a

[a]Two-day-old parasitoid larvae were used; a total of five replications, five larvae per replicate.
[b]Mean of five replications.
[c]Deformed pupae failed to emerge.
[d]In a column, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's (1951) multiple range test.

TABLE 4. EFFECT OF ETHANOLIC EXTRACT OF NEEM SEED KERNEL (ENSK) ON DEVELOPMENT OF PARASITOID (*B. hebetor*) LARVAE TREATED WITH HOST LARVAE

Treatment	Larvae observed (*N*)[a]	Larvae reaching pupal stage (%)[b]	Deformed pupae (%)[c]	Adults emerged from initial number of eggs (%)	Adult longevity (days)	
					Male	Female
ENSK 5.0%	100	0a[d]		0a		
ENSK 2.5%	100	0a		0a		
ENSK 1.2%	100	6b	90a	0a		
ENSK 0.6%	100	11c	63a	3ab		
ENSK 0.3%	100	45d	87a	6b		
Ethanol emulsified water	100	100e	0b	100c	4.5	9.6
Untreated control	100	100e	0b	100c	4.5	9.8

[a]Two-day-old parasitoid larvae were used; there was a total of five replications, five larvae per replicate.
[b]Mean of five replications.
[c]Defomed pupae failed to emerge.
[d]In a column, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's (1951) multiple range test.

tulalis by *B. hebetor*. Raguraman (1994) reported that NSK extracts had an oviposition deterrent effect on an egg parasitoid, *Trichogramma chilonis*. The difference in oviposition deterrent effect of neem extracts on *T. chilonis* and *B. hebetor* may be due to their differential sensitivity to neem compounds.

B. hebetor adults that settled on raisins treated with neem extracts showed several abnormal behavioral responses and did not prefer to feed. Similar effects were noticed by Raguraman (1994) when NSK-treated honey was offered for food to *T. chilonis*. The feeding deterrent effect of NSK extracts or compounds has not been reported in detail on parasitoids or predators.

Both contact toxicity and feeding toxicity studies showed negligible mortality of *B. hebetor* adults. Since the parasitoid lays eggs externally on the host larva, the possibility of acquiring a lethal dose might be low.

No sterilizing effects of the aqueous suspension or the ethanolic extract of NSK were noticed on *B. hebetor*. The results agree with the findings of Raguraman (1994), who reported that both an aqueous suspension and an ethanolic extract did not affect fertility of *T. chilonis*.

Both the aqueous suspension and the ethanolic extract of NSK showed IGR activity on *B. hebetor*. Even at a 0.3% concentration, less than 50% of the parasitoid larvae reached the adult stage. Since the parasitoid larvae suffered in our pretreatment and posttreatment tests, larval and pupal mortalities through malformations (IGR effects) were mainly due to ingestion as well as contact effects of NSK extracts. Reports on adverse effects of NSK extracts on parasitoids are scarce in the literature. Sharma et al. (1984) reported that an active neem fraction of NSK had adverse effects on *Apanteles rufricrus*, a larval parasitoid of the Oriental army worm, *Mythimna seperata*. Beckage et al. (1988) found that injection of 2.5–10 μg of azadirachtin in host larvae partially inhibited or totally suppressed first-instar ecdysis of the braconid, *Cotesia* (*Apanteles*) *congregata*, an internal larval parasitoid of tobacco hornworm, *Manduca sexta*. Lowery and Isman (1995) reported that dipping mummies of aphids in solutions of neem oil affected survival and adult emergence of the parasitoid, *Diaeretiella rapae* at 2.15% and 5.0% concentrations. Recent laboratory and field trials conducted with two commercial neem-based insecticides showed that while lower concentrations were safer, high concentrations adversely affected the development of *B. hebetor* (Srinivasa Babu et al., 1996). Srivastava et al. (1997) reported that an alcoholic extract of neem seed was more toxic to larval development of *Bracon brevecornis* than neem oil, which agrees with our study on the IGR effects of NSK to *B. hebetor*.

CONCLUSIONS

In the present study, neem seed kernel extracts neither influenced the oviposition of adult *B. hebetor* nor caused mortality through feeding and contact.

The extracts had no effect on parasitoid fertility through feeding, but the extracts showed a feeding deterrent effect for a limited period. Parasitoid eggs and pupae also were unaffected by the extracts. Parasitoid larvae, however, succumbed when feeding on neem-treated hosts and when exposed to contact with a treated surface. For inundative releases of *B. hebetor* in integrated pest management, a minimum safety period should be followed after spraying NSK extract in the field to avoid contamination of host and parasitoid larvae.

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INFLUENCE OF STIMULI FROM *Camellia japonica* ON OVIPOSITIONAL BEHAVIOR OF GENERALIST HERBIVORE *Epiphyas postvittana*

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Abstract—Stimuli from the plant *Camellia japonica* that influence the host-finding and acceptance behaviors of adult females of the generalist herbivore *Epiphyas postvittana* were investigated. In a binary choice test in a small (35-cm-diam.) arena, females discriminated between a plant and a laminated card model, laying many more eggs on the plant. Observations of females showed that the greater number of eggs laid on the plant were primarily due to on-plant behaviors, with females spending significantly more time per visit on the plant than on the model. Interestingly, females landed a similar number of times on the plant as on the model, suggesting that volatile chemicals from the plant did not influence host-finding. This observation was further supported by wind-tunnel studies, in which females showed little or no upwind flight activity in response to plants and laid similar numbers of eggs on upwind and downwind plants. Leaf surface texture, a combination of smoothness and fine structure (consisting of the midvein and other raised leaf veins), was found to stimulate egg laying by females. Methanol, dichloromethane, and pentane extracts were made of the waxes on the leaf surface. Of these three extracts, only the nonpolar (pentane) one stimulated egg laying by females. Although we did not find a role for volatile plant chemicals in host finding, they appeared to stimulate increased egg laying when the female was on the plant. It appears likely that these chemical and tactile stimuli in *C. japonica* are general stimuli, which may be found in a large number of plants and, in combination with the feeding preferences of the larva, may explain the generalist herbivorous nature of this pest.

Key Words—Lepidoptera, Tortricidae, chemical stimuli, tactile stimuli, light-brown apple moth.

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INTRODUCTION

For many species of insects, the finding and accepting of a plant for oviposition is a key step in determining what plant the larva will feed upon (Renwick and Chew, 1994). Much of the work concerning the selection of plants for oviposition by insects has been carried out on specialist herbivores, i.e., those insects whose host range is restricted to a single or relatively few plant species, often in a single genus or family. In these cases, it is thought that host selection by the female is influenced predominantly by stimuli that are peculiar to the host plant(s). Considerably less is known about host selection by females of generalist herbivores (i.e., insects whose host range includes a large number of plant species from a number of families). For generalists, host selection is thought to be influenced by general and relatively nonspecific stimuli, common to many different hosts, or by stimuli that inhibit host finding and/or acceptance in nonhosts (Thorsteinson, 1960; Renwick, 1983, 1989; Chew and Robbins, 1984).

The selection of a plant for oviposition by a female insect is commonly divided into three stages: host finding, which involves the finding of a plant over a distance; host contact, in which the insect first contacts the plant; and host assessment, in which the insect assesses the plant's suitability for oviposition, generally while on the plant. Throughout each of these stages, a female may perceive plant stimuli, and the distance from the plant may limit the type of stimulus involved. For instance, during host finding only stimuli that can be perceived at a distance, such as visual or volatile chemical stimuli, may influence the female's behavior, while during host assessment, contact stimuli, such as tactile or involatile chemical stimuli, can come into play and influence the female's behavior (Renwick, 1989).

Originally from Australia, the lightbrown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae) was introduced accidentally into New Zealand in the late nineteenth century (Wearing et al., 1991). Since its introduction, it has become a major pest of most fruit crops in New Zealand. The larvae of this pest are leafrollers and are highly polyphagous, with a known host range in excess of 250 species in more than 50 families (Wearing et al., 1991). In spite of its pest status, relatively little is known about host selection by this generalist. Female *E. postvittana* are influenced by tactile stimuli, preferring to lay eggs on smooth rather than rough leaf surfaces (Danthanarayana, 1975; Foster et al., 1997), as well as preferring to lay eggs on the smoother adaxial side of leaves rather than on the more hairy, abaxial side (Tomkins et al., 1991). Suckling et al. (1996) found that certain volatile chemicals released by plants elicited electroantennogram responses by female *E. postvittana*. However, in parallel ovipositional tests in small arenas, most of these compounds resulted in reduced numbers of eggs being laid by females relative to controls.

In order to understand the process of host selection by *E. postvittana*, and

thereby develop new approaches for controlling this important pest through manipulating its behavior (Foster and Harris, 1997), we have begun to study the external and internal stimuli that influence the finding and accepting of a plant by females. Female *E. postvittana* oviposit readily on the ornamental plant *Camellia japonica* (Foster et al., 1997). This plant, because leaves are available throughout the year, is convenient for characterizing some of the plant stimuli that influence the host-finding and acceptance behaviors of female *E. postvittana*. We report the types of stimuli from *C. japonica* that influence host selection by female *E. postvittana* herein.

METHODS AND MATERIALS

Insects

E. postvittana were from a laboratory colony maintained at Mt. Albert Research Centre. The original insects for this colony were collected from Nelson in 1971, with new wild material introduced into the colony in 1988 and 1989. The larvae were fed on a semisynthetic diet (Singh, 1974) and reared in groups. After pupation, the insects were sexed and the two sexes maintained in small plastic containers in separate incubators at 19 $\pm$ 0.5°C under a 16 : 8 light–dark photoperiod. Adults were collected each day and placed in 750-ml plastic containers with vermiculite on the bottom, and with a 10% sugar solution absorbed onto a cotton wick for food.

Mated females used in the experiments were obtained by placing 2-day-old females with an excess of 2- to 4-day-old males at the beginning of the scotophase. Pairs in copula were removed and placed in separate containers to finish copulation. Approximately 2–3 hr after copulation, the females were separated from the males and held in plastic containers until used in the experiments, usually commencing at the beginning of the photophase after mating.

Bioassay Arena

In most cases, oviposition experiments were carried out as binary choices in a standardized bioassay arena. The arena used in most of the choice tests in this study has been described in detail elsewhere (Foster et al., 1997). Essentially, it consisted of a cylindrical arena, 35 cm diam. $\times$ 22.8 cm high, constructed of garnet paper (60 grit, 3M, St. Paul, Minnesota) walls, with a cheesecloth top, and a bottom surface covered with vermiculite. This arena was used because the rough surfaces of the walls, top, and floor of the arena largely precluded oviposition by *E. postvittana* females on surfaces other than the ones being tested. A 10% sugar solution absorbed on a small cotton wick was placed

on the vermiculite for the moths to feed upon. The assays were conducted at 19 ± 0.5°C under a light regime of 16 : 8 LD. Ten mated females were put into the arena at the end of the scotophase in which they had mated. After three days, the females were removed and the numbers of eggs laid on the test substrates counted. At least 12 replicates of each experiment were conducted.

Experiments

C. japonica *versus Other Plants.* Before using *C. japonica* in these studies, we first compared it in choice tests against three other plant species—apple (*Malus domestica*, var. Royal Gala), kiwifruit (*Actinidia deliciosa*, var. Hayworth), and rose (*Rosa* X var. Noaschnee)—that are economically important hosts of *E. postvittana* (Wearing et al., 1991). Branchlets approximately 15–20 cm high, consisting of six mature leaves of *C. japonica* and the other species to be tested were placed in small containers filled with water, approximately 15 cm from each other in the arenas, and the number of eggs laid after three days on each of the plants were determined in choice tests.

Old versus New Leaves and Intact versus Broken Leaves. Roughly equal sized old leaves open on *C. japonica* for at least one year and new ones open for < 1 month were picked on December 4 and 11, 1995. Six whole leaves of either type were glued in pairs onto three 5-cm pieces of wire, which in turn were glued onto a 13-cm-high thin (0.1-cm-diam.) glass rod, such that the pairs of leaves were positioned horizontally at 13, 11.5 and 9 cm above the floor of the arena, and tested in a binary choice test lasting for 24 hr only. The leaves maintained their fresh appearance and had not wilted by the end of the bioassay.

Branchlets with six leaves each of *C. japonica*, either with leaves intact or broken by folding and creasing the middle of the leaf perpendicular to the leaf vein, were tested in the arena in a binary choice test over two days.

C. japonica *versus Model.* In the bioassay arena, females were given a choice between six mature leaves of *C. japonica*, glued in pairs onto a glass rod (as in the experiment with various leaves), and a model consisting of three pairs of green card model leaves (3.5 × 6 cm, roughly the same area as a *C. japonica* leaf) laminated between two polyester/polyethylene sheets and glued onto a glass rod. On the upper surface of each of the model leaves was a semicircular, 3-mm-diam. paraffin wax (Wolfe's Preserving Wax, C&R Packers Ltd, Auckland, NZ; melting point 48–50°C) ridge running the length of the model leaf. This model leaf had proven to be suitable for oviposition by *E. postvittana* females in previous experiments (Foster et al., 1997).

Behavioral Observations

The behavior of mated *E. postvittana* females inside a cage 60 × 60 × 60 cm constructed of wood and nylon mesh was recorded on video tape with a

black and white CCD video camera (Panasonic model WV-BL200, Matsushita Electric Co. Ltd., Osaka, Japan). Vermiculite was placed on the floor of the cage along with a sugar solution soaked onto a cotton wick. Inside the cage were a synthetic model, constructed of a single pair of laminated card model leaves, and a plant model with a single pair of *C. japonica* leaves, on the top position of the respective glass rods. Ten females were placed inside the cage at the end of the scotophase in which they had mated and were left for two days. The general light and temperature conditions were the same as the other experiments, except a 50-W infrared light source (Denard CCTV Products) was used during the scotophase to enhance the image detected by the video camera. During review of the recording, the following were noted: the number of times females landed on the model or plant leaves, the time a female spent on the synthetic model or plant leaves before taking flight, and the number of times the insect moved during each visit (i.e., discrete bouts of movement punctuated by being stationery for 30 s or more in the same position) to the model or plant leaves. The numbers of eggs laid by the females on the synthetic model or plant leaves were counted at the conclusion of the experiment (two days). The experiment was conducted five times, each time with different females and new plants and models.

Extracts of Surface Waxes of C. japonica

Pentane, dichloromethane, and methanol extracts of the epicuticular waxes of *C. japonica* leaves were made by dipping freshly picked leaves into solvent at 4°C for 45 seconds. The extracts were concentrated to 100 leaf equivalents (LE)/ml by rotary evaporation.

The bioassay consisted of a binary choice between models treated with a particular extract and models treated only with the appropriate solvent. The models consisted of three pairs of laminated card model leaves. One hundred μl (10 LE) of a particular extract or control solvent was applied with a hand-held micropipetter to cover the upper surface of each laminated card model leaf of the models as evenly as possible. The solvent was left to evaporate from the model leaves for at least 1 hr before use. The number of eggs laid after three days on the upper surface of the extract-treated model was compared to the number of eggs laid on the control.

Tactile and Chemical Stimuli

Molds of the preferred upper adaxial side of mature *C. japonica* leaves were made with a silicone mold-making compound (Silastic 3481, Dow Corning, Wiesbaden, Germany). Wax casts of these adaxial sides of leaves were made by pouring molten paraffin wax (Wolfe's Preserving Wax, melting point

48–50°C) into the molds, allowing the wax to harden, and then removing the casts. Because the outer surface of the molds may have removed some of the epicuticular waxes from the *C. japonica* leaves, which may in turn have been transferred to the wax casts, the first three casts made from each of the molds were discarded. Control wax leaves were made by pouring molten paraffin wax into a mold shaped like a leaf, but with a smooth laminated card bottom. Models, with two pairs of the leaf and control casts at the top and middle positions of the respective glass rods, were placed in the arenas, and the number of eggs laid on the upper side of the models compared in a binary choice test.

In a further experiment, a model consisting of two pairs of wax casts of the adaxial side of *C. japonica* leaves were constructed as in the previous experiment. The number of eggs laid on the upper side of this model was compared with the number of eggs laid on the upper side of a model with two pairs of real *C. japonica* leaves in a binary choice test. In a third binary choice experiment, the number of eggs laid on a model consisting of two pairs of wax casts of the adaxial side of *C. japonica* leaves treated with 10 LE of a pentane extract of the foliar chemicals of *C. japonica* leaves was compared with the number of eggs laid on a similar model treated with pentane.

Effect of Volatile Chemicals

Orientational Responses. In a preliminary experiment, the responses of mated female *E. postvittana* to a branchlet (approximately 20 cm high, with eight leaves) of *C. japonica* were tested in a large (2 m long × 1 m wide × 0.7 m high) wind tunnel based on the design of Miller and Roelofs (1978). The air velocity through the tunnel was 20 cm/sec. The floor of the tunnel was a random pattern of red circles (10 cm diam.) on a white background. A light level inside the tunnel of 1.0 lux was provided by four overhead voltage-regulated, 15-W tungsten lamps (Osram Co., Auckland, New Zealand). Thirty each of two types of females, recently mated and mated approximately 16 hr previously and kept inside a cheesecloth bag to prevent oviposition, were introduced individually into the tunnel, at either the 7th hour of the scotophase (for recently mated females) or at the beginning of the scotophase (for females mated ca. 16 hr earlier), and given 5 min to take flight. None of the 60 females tested flew upwind in a manner that suggested odor-mediated flight (Haynes, and Baker, 1989). On the rationale that the insects' responses to plant odors may be more readily observed if given more time and released at closer initial distances from the plant, we next tested females to either a branchlet of *C. japonica* or of rose (*Rosa* X var. Noaschnee) in a smaller, cylindrical (0.55 m diam. × 1.0 m) wind tunnel with similar light and floor conditions as for the larger tunnel. Females that had mated ca. 16 hr previously were introduced into the tunnel in groups of five during the first 3 hr of the scotophase and left for 1 hr

during which their responses were recorded on video tape. After an hour, the females were removed from the tunnel, a new group of females was placed inside the tunnel, and the experiment was repeated. No more than two sets of females were tested in a single day. During review of the video tape, the following were recorded: (1) the number of insects that took flight out of the container; (2) the number of insects that exhibited any apparent upwind flight responses typical of odor-mediated flight (Haynes and Baker, 1989); and (3) the number of insects that landed on the plant.

In a further test of the possibility of wind-oriented movement, 10 mated female *E. postvittana* inside an open-ended, wire mesh cylinder (5 cm diam. × 7.5 cm long) were placed gently in the middle of a nylon mesh cage 60 × 60 × 120 cm long inside the large wind tunnel at the end of the scotophase in which they had mated. Two *C. japonica* branchlets, approximately 20 cm high and with 12 leaves each, were at the upwind and downwind ends of the cage. The females were left until 2 hr into the next scotophase (18 hr in total), after which the experiment was stopped, and the positions of the females in the cage and any eggs laid on the plants were recorded. The final positions of the females were recorded according to whether they were on the upwind or downwind plant, or if not on the plant, the quarter of the cage where they were located: most upwind (U1), next downwind (U2), the quarter just downwind of the release point (U3), and the most downwind (U4). The experiment was replicated six times. The proportion of the total eggs laid on the upwind plant was compared to a random distribution, which assumed equal proportions of eggs laid on the two plants, by a Tukey-Kramer test after first transforming the proportions to their arcsin values. The proportion of the females on the upwind plant relative to the total number of females on the two plants was compared against a random distribution in a similar fashion.

Nonorientational Responses. In order to test whether plant volatiles elicited a response from females other than an orientational one, three mated females were placed in a small cylindrical arena (10 cm diam. × 13 cm high), constructed of garnet paper with a cheesecloth top, at the end of the scotophase in which they had mated. Inside the arena a circular (5 cm diam.) laminated card model leaf was suspended horizontally, approximately halfway down from the cheesecloth, by a thin glass rod. These arenas were placed on a nylon-mesh-covered surface with a regular array of 0.5-cm holes every 1.5 cm. Air was drawn through the holes and through the bottom of the arenas at a velocity of approximately 20 cm/sec by a 1100-W vacuum cleaner and expelled outside of the room through an exhaust fan. Either four whole leaves of *C. japonica* or four laminated card leaves were placed on top of an arena in a randomized complete block design with a total of 31 replicates for each treatment. In this way, either volatile chemicals released by the *C. japonica* leaves or plain air were drawn through an arena. The arenas were left for 18 hr, after which the

airflow was stopped, the females removed, and the number of eggs laid on the laminated card compared between treatments.

As plant leaves also release water, it was possible that effects seen in the previous experiment may have been due to differences in humidity between the two treatments. With the same setup, females were placed in arenas with either four wet or four dry balls (2 cm diam.) of cotton wool atop the cheesecloth, and air was drawn through the arenas for 18 hr. At the end of the experiment, the moist cotton wool balls were still wet. The numbers of eggs laid on the laminated card inside the arenas with moist or dry cotton wool balls above them were compared in a randomized complete block design, with 22 replicates of each treatment.

Two sets of arenas with both a single *C. japonica* leaf and a laminated card model leaf suspended vertically from two glass rods (5 cm apart) were set up, with one set of arenas having four *C. japonica* leaves on top, and the other four laminated card model leaves on top. After 18 hr, the airflow was stopped and the numbers of eggs on the *C. japonica* and the model leaf were determined for both treatments. The experiment was conducted in a split-plot design, with the effect of airstream as the whole plot, and the effect of ovipositional substrate as the split plot. The data were analyzed with the statistical program JMP (1991).

Effect of Antennectomy

To test the effect of antennectomy on egg laying by female *E. postvittana*, mated females were narcotized with carbon dioxide, antennectomized, and placed in the 35-cm-diam. bioassay arena at the end of the scotophase in which they had mated. Ten females were placed in each arena and given the choice of either a model with three pairs of *C. japonica* leaves or a model with three pairs of laminated card model leaves. For a sham control, mated females were narcotized with carbon dioxide, their antennae touched with scissors, and placed in an arena with the same choice. The females were left for two days, after which the numbers of eggs laid on the plant and on the model were determined. The experiment was conducted in a split-plot design, with the effect of antennectomy as the whole plot and the effect of ovipositional substrate as the split-plot, and analyzed by using the statistical program JMP (1991).

Statistical Analyses

In the binary choice tests, significant differences between treatments were determined by the nonparametric Wilcoxon signed-rank test. For the behavioral data and the no-choice tests (i.e., with airflow), treatments were compared by ANOVA after first checking that the data were distributed normally and that there was homogeneity of variances by using O'Brien's test of equal variance between treatments. Where the data were not distributed normally, they were

either transformed to their $\sqrt{(x + 0.5)}$ values before being analyzed by ANOVA, or they were analyzed by the Wilcoxon signed-rank test. Unless otherwise stated, all significant differences are reported at a probability level of $P < 0.05$.

RESULTS

C. japonica *versus Plants*

Females laid significantly more eggs in total on the *C. japonica* than on the apple (Figure 1a). This difference was due predominantly to the much greater number of eggs laid on the abaxial side of the *C. japonica* leaves than on the abaxial side of the apple leaves; the mean numbers of eggs laid on the adaxial side of leaves were not significantly different between the two types of plants. Although the average surface area of the kiwifruit leaves was nearly five times that of the *C. japonica* leaves, females laid similar numbers of total eggs and eggs on the upper side on the two types of leaves (Figure 1b). However, females laid significantly more eggs on the underside of the *C. japonica* leaves than on the kiwifruit leaves; no eggs were laid on the underside of the kiwifruit leaves. There were no significant differences in total eggs laid, eggs laid on the upper-side, or eggs laid on the underside of leaves between *C. japonica* and rose (Figure 1c).

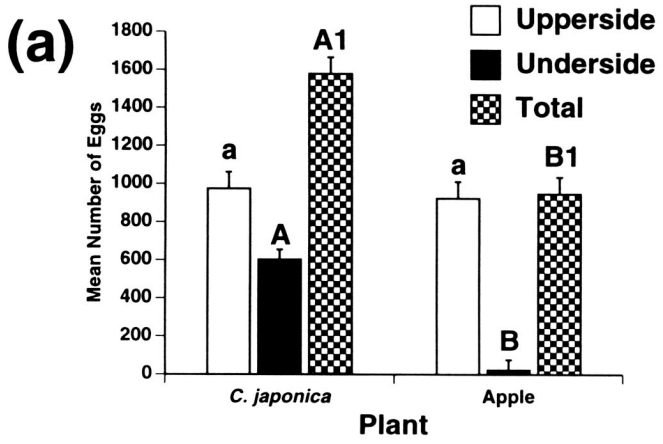
Old versus New and Intact versus Broken Leaves

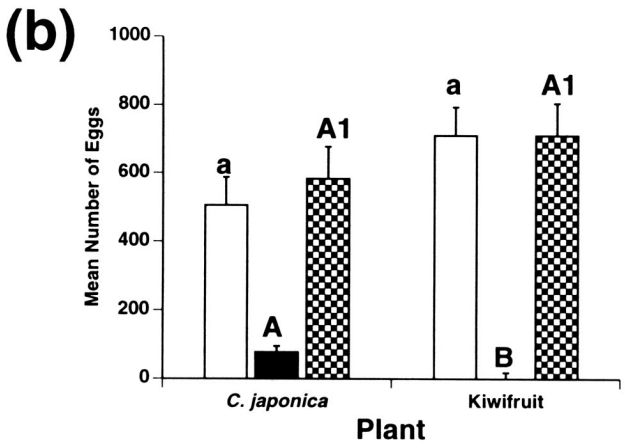
There was no significant difference between the mean numbers of eggs laid on older and younger leaves of *C. japonica* (Figure 2a). Similarly, there were no significant differences between the means of numbers of eggs laid on intact or broken *C. japonica* leaves (Figure 2b).

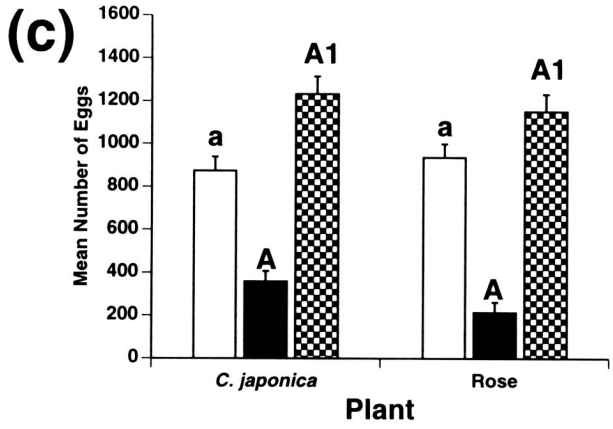
C. japonica *versus Model*

Females showed a strong preference toward *C. japonica* over the model leaves with a wax ridge, with nearly three times more eggs being laid on the plant than on the model (Figure 2c).

Observations of the behavior of mated female *E. postvittana* in a small cage with a choice between either *C. japonica* leaves or laminated card leaves with a wax ridge revealed no difference between the mean number of landings on the plant and model leaves (Table 1). However, there was a strong tendency ($P < 0.06$) for females to spend more (3.5 times) time per visit on the *C. japonica* than on the card leaves. This was matched by almost twice the mean number of eggs laid on the *C. japonica* leaves than on the laminated card leaves (significant at $P < 0.07$). Although females spent more time on the *C. japonica* leaves, they showed a tendency to be less active than when on the laminated card.


(a)
Upperside
Underside
Total
Mean Number of Eggs
1800
1600
1400
1200
1000
800
600
400
200
0
a
A
A1
a
B
B1
C. japonica
Apple
Plant


(b)
Mean Number of Eggs
1000
800
600
400
200
0
a
A
A1
a
B
A1
C. japonica
Kiwifruit
Plant


(c)
Mean Number of Eggs
1600
1400
1200
1000
800
600
400
200
0
a
A
A1
a
A
A1
C. japonica
Rose
Plant

Extracts of Surface Waxes

In separate tests with two different extracts made from different sets of leaves, female *E. postvittana* laid significantly more eggs on the upper side of models treated with the pentane extract of *C. japonica* leaves than on the upper side of models treated with pentane only (Figure 3a). In contrast, there were no significant differences between models treated with either dichloromethane or methanol extracts of *C. japonica* leaves and their respective solvent only-treated controls (Figure 3b and c).

Tactile Stimuli

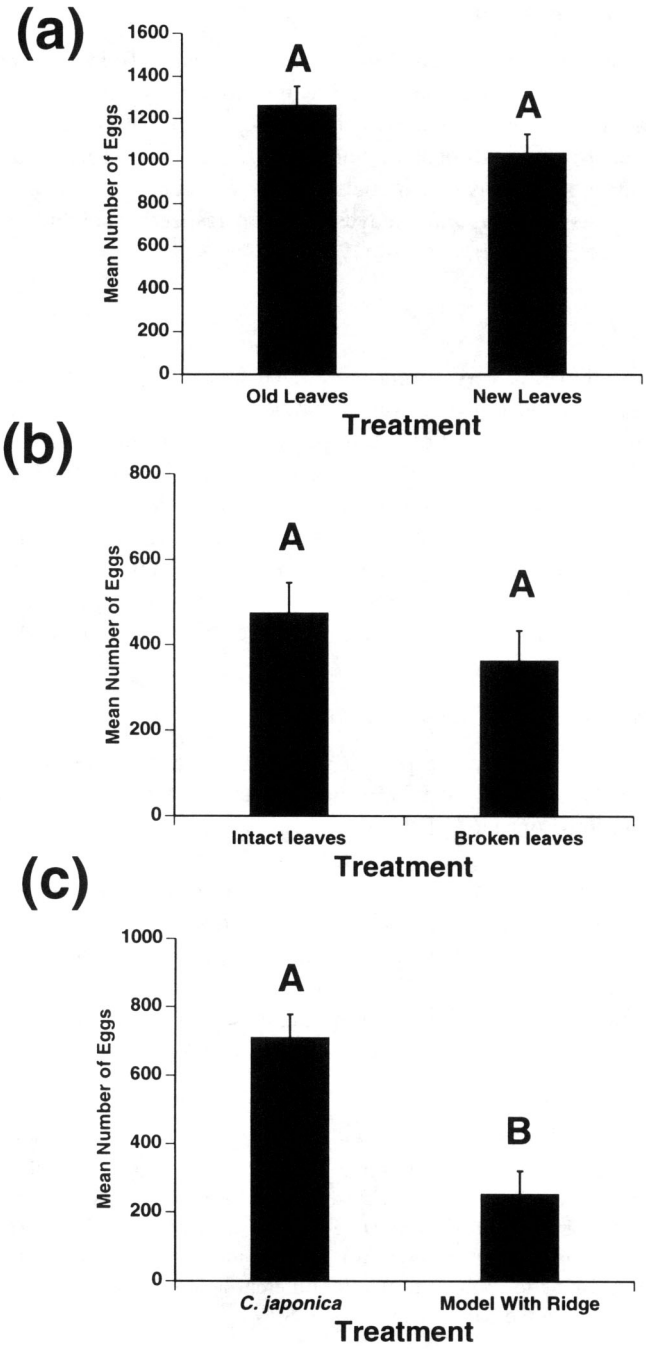
Females showed a very strong preference for laying eggs on the wax leaf-cast models compared to laying on the smooth wax models, with nearly four times the number of eggs laid on the upper side of the former than on the upper side of the latter models (Figure 4a). In another experiment, females showed a significant preference for laying eggs on the upper side of real *C. japonica* leaves as opposed to the upper side of the wax leaf-cast models (Figure 4b). There was no significant difference between the mean number of eggs laid on the upper side of wax leaf-cast models with 10 LE of a pentane extract of *C. japonica* leaves and wax leaf-cast models treated with pentane (Figure 4c).

Orientational Effects of Volatile Chemicals

Of the 95 mated females placed in the wind tunnel with a *C. japonica* plant, 41 (43.2%) took flight within the test period. Most of the females flew rapidly to the floor or sides of the tunnel, although five females (5/95, 5.3%) exhibited some upwind flight, and two (2.1%) of these landed on the plant. A similar pattern was observed when rose was placed at the upwind end of the tunnel. Nineteen of the 40 females (47.7%) introduced into the tunnel took flight, four (10.0%) exhibited some upwind flight, but none landed on the plant.

In the cage with a plant at either end, there was no significant difference in number of eggs laid on the downwind plant and on the upwind plant (Figure 5a). The distribution of eggs laid on the upwind and downwind plants was not

FIG. 1. Mean numbers of eggs laid on the upperside of the leaf and on the under side of the leaf by female *Epiphyas postvittana* in binary choice tests between *Camellia japonica* and (a) apple (*Malus domestica* var. Royal Gala), (b) kiwifruit (*Actinidia deliciosa*), and (c) (*Rosa* X var. Noaschnee). Significant differences between treatments (Wilcoxon signed-rank test) are indicated by different letters of the same case (upper or lower) or number atop the bars. Error bars are SEM.


(a)
1600
1400
1200
1000
800
600
400
200
0
Mean Number of Eggs
A
A
Old Leaves
New Leaves
Treatment
(b)
800
600
400
200
0
Mean Number of Eggs
A
A
Intact leaves
Broken leaves
Treatment
(c)
1000
800
600
400
200
0
Mean Number of Eggs
A
B
C. japonica
Model With Ridge
Treatment

significantly different from that of a random distribution on the two plants. At the conclusion of the experiment, equal numbers of females were found in the upwind and downwind halves of the cages, and equal numbers of females were found on the plants as on the rest of the cage (Figure 5b). The distribution of females on the two plants was not different from that of a random distribution on the plants.

Nonorientational Effects of Volatile Chemicals

Female *E. postvittana* in an airstream containing volatiles from *C. japonica* leaves laid significantly more eggs on the laminated card model than did females in the blank airstream (Figure 6a). There were no significant differences in the number of eggs laid on the laminated card model between females in either a moist or a control airstream (Figure 6b).

In the choice test of *C. japonica* versus laminated card model leaves, by far the greater proportion of eggs was laid on the leaf than on the model in both the airstream containing *C. japonica* volatile chemicals and in the control airstream. Analysis of the data showed that what was in the airstream did not have a significant effect ($P < 0.58$), but the ovipositional substrate did ($P < 0.0001$). There was no significant interaction between what was in the airstream and the ovipositional substrate ($P < 0.26$). In fact, the proportions of eggs laid on the leaf out of the total eggs laid on leaf and model were very similar in both airstreams (Figure 6c).

Effect of Antennectomy

There was a very strong whole-plot effect, with antennectomized insects laying fewer eggs in their arena than the control insects (Figure 7). Similarly, there was a significant split-plot effect, with *C. japonica* being the preferred substrate for oviposition. There was also a significant interaction between the effect of antennectomy and the effect of ovipositional substrate. This was apparent in the data, with control females showing a strong preference toward *C. japonica*, while antennectomized females laid roughly equal numbers of eggs on *C. japonica* and the card.

FIG. 2. Mean numbers of eggs laid by female *E. postvittana* in binary choice tests between (a) old and new leaves of *C. japonica*, (b) intact and broken *C. japonica* leaves, and (c) models with *C. japonica* leaves or models with laminated card model leaves with a paraffin wax ridge on the upper surface (data only shown for eggs laid on upper surface of leaves and models). Significant differences (Wilcoxon signed-rank test) between means are indicated by different letters atop the bars. Error bars are SEM.

TABLE 1. DATA FROM OBSERVATIONS OF MATED FEMALE *Epiphyas postvittana* IN CHOICE SITUATION BETWEEN MODELS WITH *Camellia japonica* LEAVES OR LAMINATED CARD LEAVES

Behavioral observation	*C. japonica*	Card	Significance (*P*)
Number of landings on (Mean ± SD)	4.7 ± 0.69	3.9 ± 0.69	<0.45[a]
Time/visit (min) on (Mean ± SD)	74.2 ± 21.2	21.3 ± 24.0	<0.06[b]
Number of active bouts/visit (Mean ± SD)	2.2 ± 0.51	3.3 ± 0.51	<0.16[a]
Number of eggs laid on (Mean ± SD)	325.8 ± 61.5	171.9 ± 61.5	<0.07[c]

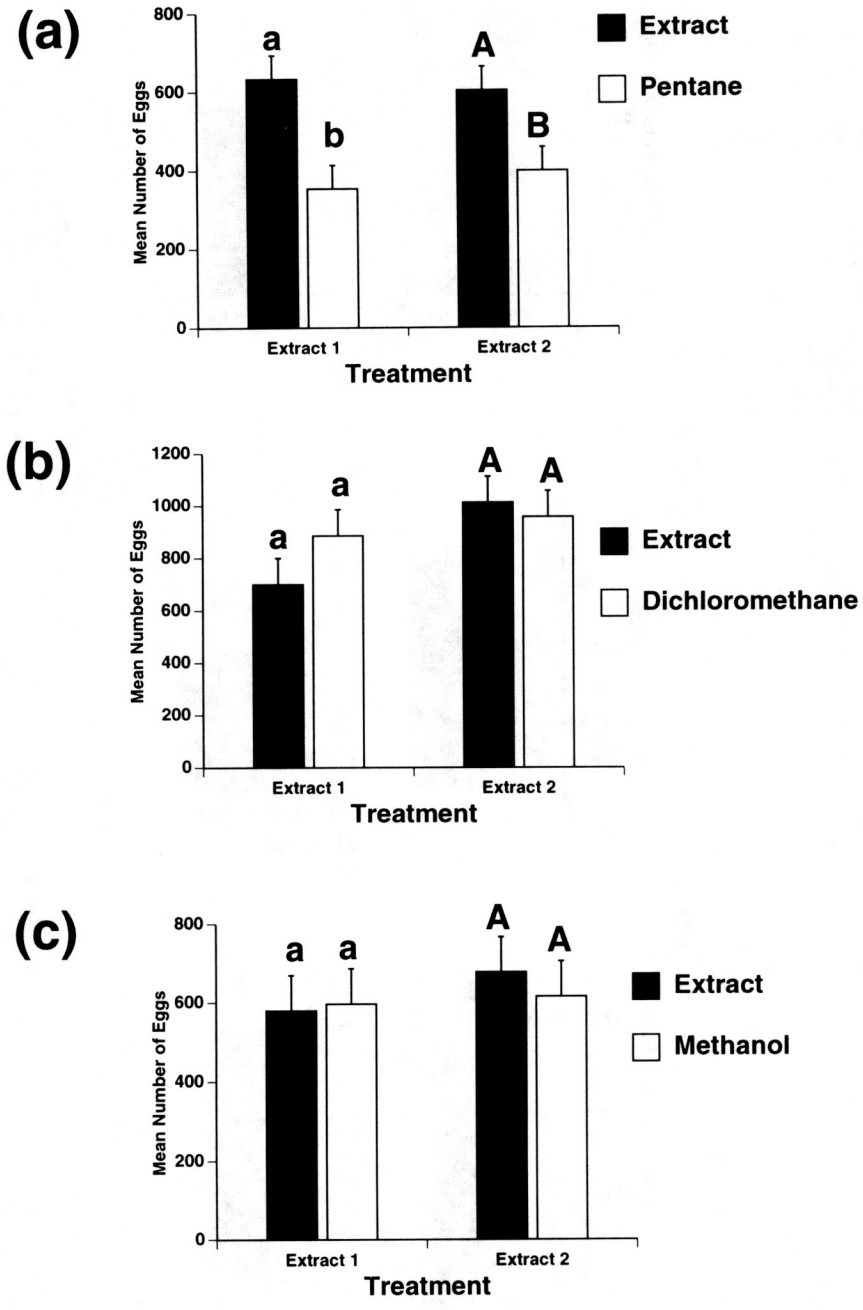
[a]Data analyzed by ANOVA.
[b]Data analyzed by Wilcoxon 2-group test.
[c]Data analyzed by ANOVA after transformation of data to $\sqrt{(x + 0.5)}$ values.

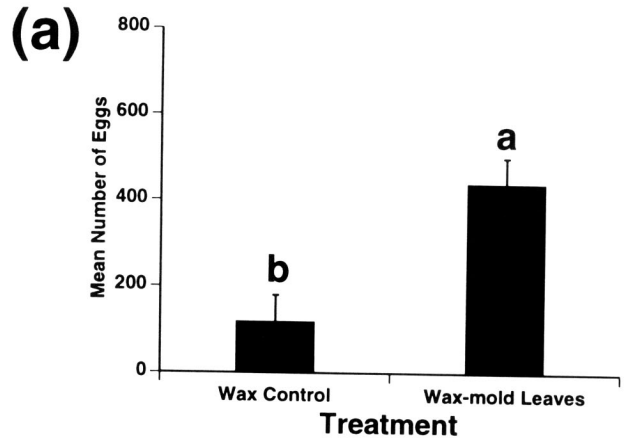
DISCUSSION

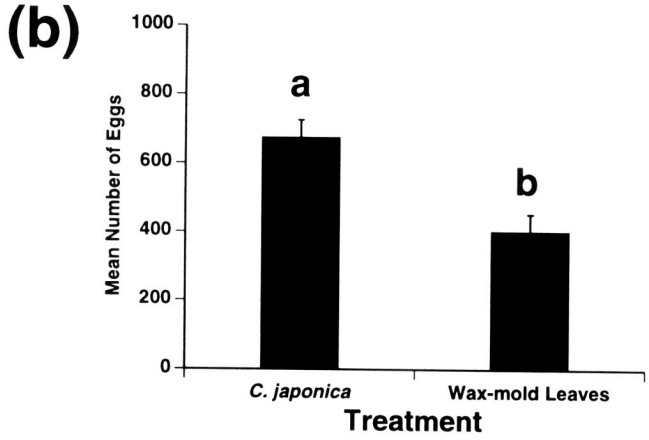
Females of the generalist herbivore, *E. postvittana*, will oviposit on a large number of plant species (Danthanarayana, 1975; Tomkins et al., 1991; this study, and Foster and Howard, unpublished). Although female *E. postvittana* showed little discrimination between the plant species in this study (and many other plant species, Foster and Howard, unpublished), they did show a high degree of discrimination between a plant, *C. japonica*, and a synthetic model, with females laying many more eggs on the plant than on the model. Using this discrimination, we were able to add plant factors to the model in order to isolate the factors from *C. japonica* leaves that stimulated oviposition.

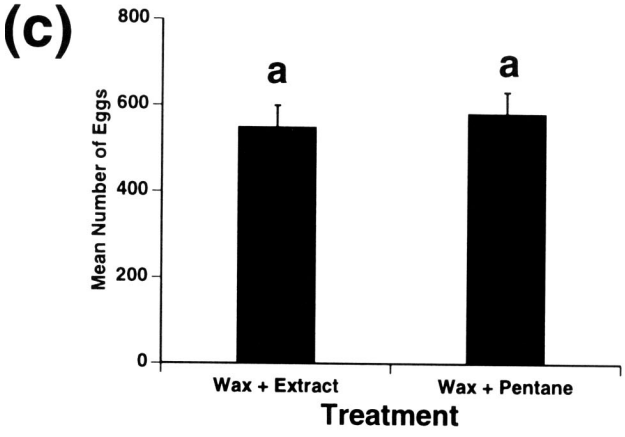
In our laboratory bioassays, it appears as though the selection of a plant for oviposition is mediated principally by plant stimuli perceived while on the plant. In contrast, plant-specific stimuli that can be perceived from a distance appeared to have relatively little effect on host selection behavior, at least within the confines of our bioassays. In support of this statement are the behavioral

FIG. 3. Mean numbers of eggs laid by female *E. postvittana* on the upper side of models with laminated card leaves treated on the upper side with (a) a pentane extract of *C. japonica* leaves versus a pentane control, (b) a dichloromethane extract of *C. japonica* leaves versus a dichloromethane control, (c) a methanol extract of *C. japonica* leaves versus a methanol control. Significant differences (Wilcoxon signed-rank test) between means are indicated by different letters. Error bars are SEM. Two different extracts with the appropriate solvent were tested.


(a)
800
600
400
200
0
Mean Number of Eggs
a
b
A
B
Extract
Pentane
Extract 1
Extract 2
Treatment
(b)
1200
1000
800
600
400
200
0
Mean Number of Eggs
a
a
A
A
Extract
Dichloromethane
Extract 1
Extract 2
Treatment
(c)
800
600
400
200
0
Mean Number of Eggs
a
a
A
A
Extract
Methanol
Extract 1
Extract 2
Treatment


(a)
800
600
400
200
0
Mean Number of Eggs
a
b
Wax Control
Wax-mold Leaves
Treatment


(b)
1000
800
600
400
200
0
Mean Number of Eggs
a
b
C. japonica
Wax-mold Leaves
Treatment


(c)
800
600
400
200
0
Mean Number of Eggs
a
a
Wax + Extract
Wax + Pentane
Treatment

observations in a cage in which females landed similar numbers of times on the plant and model, but stayed on the plant for a greater time and laid more eggs on the plant than on the model. If stimuli perceived over a distance were important for host selection in this situation and thus largely responsible for the greater number of eggs on the plant, then one would have expected to have seen a higher frequency of landing on the plant than on the model. This is not to say that there is no role for plant stimuli perceived at greater distances in host selection by female *E. postvittana*, but that they probably do not account for the preference of plant over model leaves within our bioassay. Visual stimuli, such as the spatial arrangement or size of leaves, are probably important factors affecting host selection in *E. postvittana* (Foster et al, 1997). Yet, in this bioassay it is likely that any visual differences between the plant and model had little appreciable effect on the behavior of females.

It is clear from the results presented here that tactile features of the leaf surface of *C. japonica* are important stimuli influencing oviposition by females, as indeed they are for other insects (e.g., Harris and Miller, 1988; Harris and Rose, 1990; Roessingh and Städler, 1990; Renwick, 1989; Renwick and Chew, 1994; Degen and Städler, 1997). Previous studies have shown that leaf texture, particularly smoothness, influences oviposition by female *E. postvittana* (Danthanarayana, 1975; Tomkins et al, 1991; Foster et al., 1997). Using wax leaf casts, we have shown that, in addition to smoothness, the textural features of the leaf surface of *C. japonica* are also important. Most probably these textural features are the large mid-vein and the smaller leaf veins that branch out in a dendritic fashion from the mid-vein and that were prominent on our wax casts. As such surface characteristics are common to leaves of many plant species, it seems reasonable to assume that this response by females explains, at least in part, the generalist nature of female *E. postvittana*. The converse of this effect, that females are not stimulated to lay on rough, hairy leaf surfaces, has been noted previously (Danthanarayana, 1975; Tomkins et al, 1991).

In addition to leaf texture, extracts of surface chemicals stimulated oviposition by females. Of the three extracts tried, only the non-polar pentane extract elicited significant oviposition by females. The role of plant epicuticular waxes in stimulating oviposition by insect herbivores is being increasingly rec-

FIG. 4. Mean numbers of eggs laid by female *E. postvittana* on the upper side of models in binary choice tests between (a) models with paraffin wax casts of molds of the adaxial surface of a *C. japonica* leaf (wax-mold leaves) versus models with smooth wax model leaves, (b) models with wax-mold leaves versus models with *C. japonica* leaves, and (c) models with wax-mold leaves treated with a pentane extract of *C. japonica* leaves versus models with wax-mold leaves treated with pentane. Significant differences (Wilcoxon signed-rank test) between means are indicated by different letters. Error bars are SEM.

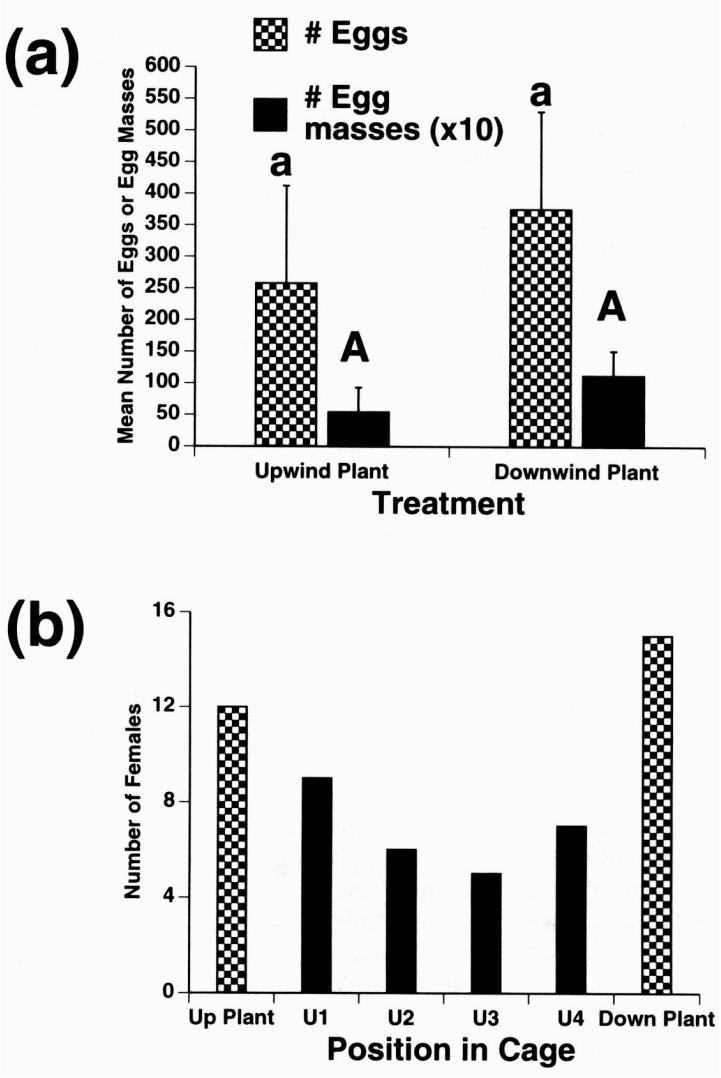
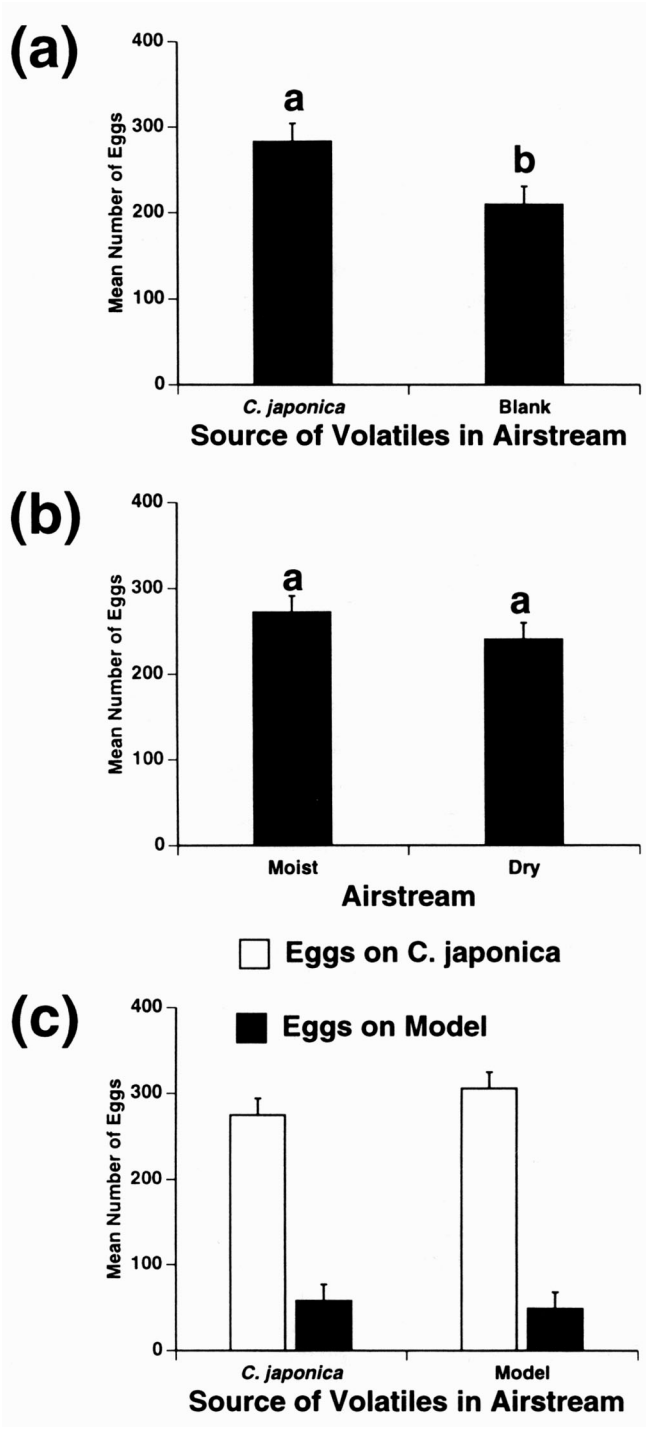


FIG. 5. (a) Number of eggs and masses of eggs (×10) laid by female *E. postvittana* on an upwind and a downwind plant in a cage in a wind tunnel, and (b) distribution of females in the cage at the end of the experiment on the upwind plant (Up Plant), most upwind quarter of the tunnel (U1), quarter just upwind of the center of the tunnel (U2), quarter just downwind of the center of the tunnel (U3), the most downwind quarter of the tunnel (U4), and on the downwind plant (Down Plant). In (a), there were no significant differences (Wilcoxon 2-group test) between the upwind or downwind plants for numbers of eggs or numbers of egg masses. Error bars are SEM.

ognized (Foster and Harris, 1992; Eigenbrode and Espelie, 1995). Recent work has shown that alkanes can stimulate oviposition in some insects. Various branched and unbranched alkanes, as well as a number of carboxylic acids and γ-elemene found in host plants stimulate oviposition by the generalist herbivore *Helicoverpa zea* (Breeden et al., 1996). Plant waxes by themselves do not stimulate oviposition by the diamondback moth, *Plutella xylostella.* However, when paraffin wax or a mixture of 10 *n*-alkanes was added to the known oviposition stimulant, the glucosinolate sinigrin, there was an increase in oviposition on treated substrates relative to that on substrates treated with sinigrin alone (Spencer, 1996). Pentane extracts of corn, pepper, and potato leaves stimulate oviposition by the European corn borer, *Ostrinia nubilalis* (Udayagiri and Mason, 1995). A number of *n*-alkanes from C-26 to C-30 present in the epicuticle of corn leaves stimulate oviposition in *O. nubilalis* (Udayagiri and Mason, 1997). We have recently found that a number of *n*-alkanes in the epicuticular waxes of *C. japonica* stimulate oviposition by female *E. postvittana* (Foster and Howard, unpublished). That these are the principal stimulatory compounds in the pentane extract of *C. japonica* is supported by the experiment in which pentane extract of *C. japonica* was added to the paraffin wax leaf casts. In this case, there was no significant increase in the number of eggs laid on these casts over that on a pentane control, suggesting that the *n*-alkanes of the paraffin wax (Spencer, 1996) alone were as stimulatory as the paraffin wax plus pentane extract of the *C. japonica*.

Neither the dichloromethane nor the methanol leaf extracts of *C. japonica* stimulated oviposition by female *E. postvittana*. The dichloromethane extract would probably contain polar lipids, alkanes, and other nonpolar compounds present in the pentane extract. That this extract did not stimulate oviposition suggests that the stimulatory compounds present in the pentane extract either were not removed from leaves or their effect was balanced by something that inhibited oviposition. Within a given context, the expression of a behavior of an insect results from the appropriate balance of stimulatory and inhibitory stimuli (Miller and Strickler, 1984; Harris and Foster, 1995). If indeed the dichloromethane extract of *C. japonica* contains substances that inhibit oviposition, then this suggests that these substances are not accessible to the insect on the plant surface, as the intact plant surface is highly stimulatory.

As discussed previously, the similar frequencies of landing on the plant and model suggest that volatile plant chemicals have limited, if any, involvement in mediating oriented host finding for female *E. postvittana*, at least over the distances from the host plant that were tested. Indeed, in the wind-tunnel experiments only a very small proportion of females showed any flight upwind, and only about 2% of the females actually landed on the plant during the 1 hr of the assay. Furthermore, in the cage in the wind tunnel, the distributions of


(a)
400
300
200
100
0
Mean Number of Eggs
a
b
C. japonica
Blank
Source of Volatiles in Airstream
(b)
400
300
200
100
0
Mean Number of Eggs
a
a
Moist
Dry
Airstream
Eggs on C. japonica
(c)
Eggs on Model
400
300
200
100
0
Mean Number of Eggs
C. japonica
Model
Source of Volatiles in Airstream

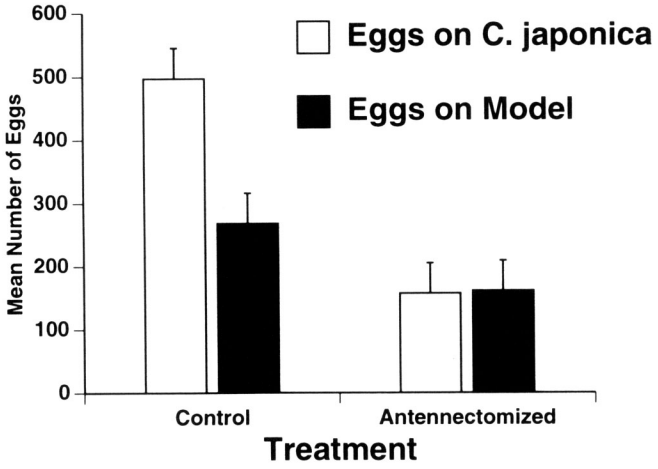


FIG. 7. Mean numbers of eggs laid by female *E. postvittana* in arenas containing a binary choice between *C. japonica* and a laminated card model for antennectomized females and sham-operated control females. The experiment was run as a split-plot design. Significant effects were observed for the whole plot effect (effect of antennectomy, $P < 0.0001$), the split-plot effect (effect of ovipositional substrate, $P < 0.033$), and the crossed effect of antennectomy and ovipositional substrate ($P < 0.028$). Error bars are SEM.

females and of eggs laid on the plants were similar with respect to plants upwind and downwind in the cage, suggesting that these distributions were random with respect to wind direction. In spite of these negative results, more rigorous behavioral studies with females in a wider variety of physiological states under more natural conditions are needed to determine whether volatile chemicals elicit any orientation in females or not.

FIG. 6. (a) Mean numbers of eggs laid on a laminated card by female *E. postvittana* in arenas with an airstream passing over *C. japonica* leaves versus in arenas with a control airstream, (b) mean numbers of eggs laid on a laminated card by female *E. postvittana* in arenas with an airstream passing over moist cotton wool balls versus in arenas with an airstream passing over dry cotton wool balls, and (c) numbers of eggs laid in arenas containing a binary choice between *C. japonica* and a laminated card model that had either an airstream passing over *C. japonica* leaves or a control airstream. In (a) and (b), significant differences (ANOVA) between means are indicated by different letters atop the bars. Error bars are SEM.

Although plant volatile chemicals are known to have a role in host finding by female insects (Miller and Strickler, 1984; Visser, 1986; Ramaswamy, 1988; Honda, 1995), particularly in specialist herbivores (e.g., Lecomte and Thibout, 1981; Fein et al., 1982; Guerin et al., 1983; Judd and Borden, 1989; Tingle et al., 1989; Haynes and Baker, 1989; Visser, 1986), there are very few examples of generalist herbivores exhibiting such behavioral responses to plant volatile chemicals in spite of the numerous reports of electroantennogram responses to such chemicals (Visser, 1986). Landolt (1989) showed that female cabbage loopers, *Trichoplusia ni*, flew upwind in response to volatile chemicals emitted by cabbage, soybean, tomato, and celery. Tingle and Mitchell (1991) found that mated female *Heliothis virescens* flew upwind in response to volatile chemicals from extracts of cotton and tobacco, although an earlier study reported that females did not respond to volatile compounds from cotton (Ramaswamy, 1988). It remains to be determined whether these are isolated examples or whether plant volatile chemicals have a more widespread role in the host-finding behavior of females of generalist herbivores.

While we found no clear evidence for a role of volatile chemicals in host finding, the increased egg laying in an airstream containing *C. japonica* volatile chemicals suggests that they may be involved during host assessment, when the female is on the plant. However, in the same bioassay setup, when given a choice between a model and a plant, females showed a strong preference toward the plant, regardless of whether plant volatile chemicals were in the airstream or not. Presumably, this was because the stimulatory effect on the plant surface, due to the contact stimuli and possibly localized perception of the volatile chemicals on the leaf, was still much greater than when the insect was on the model. Further supporting such a role for plant volatile chemicals was the greater number of eggs laid on *C. japonica* leaves than on the wax leaf casts containing both tactile and *n*-alkane chemical stimuli, which suggests that the plant leaves contained additional stimuli. The precise mechanism of this increased egg laying stimulated by volatile chemicals remains to be determined through in-depth behavioral studies.

Given the qualification that antennectomy may have direct effects on behavior not related merely to perceptory changes (Städler, 1984), which may not be accounted for by the control (see Ramaswamy et al., 1987), our data nevertheless show that antennectomy resulted in females appearing to be unable to discriminate between a plant and a model and females laying fewer eggs. The lack of discrimination between plant and model by antennectomized females suggests that these substrates are perceived as equally stimulatory, implying that texture, surface waxes, and volatile compounds of *C. japonica*, are either not perceived or that without stimulation of the antennae (by whatever stimuli), the other stimuli have little effect on the female (see Harris and Foster, 1995). If the former is the case, this suggests that in *E. postvittana* the antennae are critical

for differentiating most, if not all, plant stimuli. This is consistent with preliminary behavioral observations in which we have observed females moving their antennae frequently to contact the leaf surface before settling to oviposit (Howard and Foster, unpublished). The fewer eggs laid by antennectomized females is consistent with reduced rates of egg laying on a less stimulatory substrate (compared to on more stimulatory substrates), a phenomenon that has been observed in other herbivorous insects, particularly specialists that have been deprived of suitable hosts (Miller and Strickler, 1984). We have also observed that *E. postvittana* females given less stimulatory oviposition substrates lay fewer eggs over their lifetime than do females given more stimulatory substrates.

Our studies have revealed some of the factors from *C. japonica* that stimulate oviposition by *E. postvittana*. Given the apparent nonspecific nature of some of these factors, including leaf surface texture and nonpolar leaf surface waxes, as well as the similar ovipositional preferences by females for *C. japonica* and other plants (this study, Foster and Howard, unpublished), it seems likely that the generalist nature of *E. postvittana* may be explained, in part, by the responses of adult females to general plant stimuli. Further characterization of these plant-specific stimuli, as well as in-depth behavioral studies, will allow a greater understanding of the behavioral mechanisms by which this insect finds, accepts, and selects between different hosts and will perhaps allow the development of new control strategies, based on manipulating host selection behavior (Foster and Harris, 1997).

Acknowledgments—We thank Anne Barrington for supplying the insects, Dr. Marion Harris for comments on an earlier version of the manuscript, and Melissa Miller for statistical advice. This work was funded through a grant (contract CO6512) from the New Zealand Foundation for Research Science and Technology.

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Journal of Chemical Ecology, Vol. 24, No. 7, 1998

Erratum

In the paper "Semiochemicals Mediating Spacing Behavior of Bird Cherry-Oat Aphid, *Rhopalosiphum padi*, Feeding on Cereals" (*J. Chem. Ecol.* 23:2599–2607) by A. Quiroz, J. Pettersson, J. A. Pickett, L. J. Wadhams, and H. M. Niemeyer, the ratio of MHO, MHOH, and 2-T in Tables 2 and 3 on pps. 2603 and 2604, respectively, should be reported as 4:1:1.

0098-0331/98/0700-1277/$15.00/0

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Letters to the Editors

HOW NOT TO USE SYSTEMATICS TO TEST ADAPTIVE HYPOTHESES

Comments on Wink and von Nickisch-Rosenegk[1]

At the urging of comparative method mavens (e.g., Harvey and Pagel, 1991), and with the advent of user-friendly computer programs that produce phylogenetic trees with the click of a mouse, there is an increasing temptation for nonsystematists to base their interpretations of adaptive features among taxa on a hypothesis of phylogenetic relationships. Although it may now be easy to generate a cladogram or some other branching diagram upon which the character of interest may be traced, this does not necessarily mean that the study has been appropriately designed and executed, and thus that the ensuing interpretation is meaningful. Molecular systematics has been an especially attractive Pandora's box for the uninitiated (Brower and DeSalle, 1994), and abuse of sequence data to draw unwarranted phylogenetic inferences is rampant. With the proliferation and growing accessibility of modern analytical techniques, there is an increasing need to ensure that standards of research are maintained in the literature by careful peer review and editorial discretion. In my view, an instance of the failure of this process is illustrated by a recent paper in *Journal of Chemical Ecology*. In the spirit of Hurlbert's (1984) classic admonition on the abuses of statistical methods by naive ecologists, this critique is intended to point out to the journal's readers, editors, and reviewers the errors that can occur when a phylogenetic study is poorly conceived and executed.

General Comments. Wink and von Nickisch Rosenegk (1997) (hereafter W and VN-R) used a phylogenetic analysis of sequences from the mitochondrial 16S rRNA gene to infer the multiple origins of pyrrolizidine alkaloid (PA) and cardiac glycoside (CG) sequestration in Arctiidae and Nymphalidae (Lepidoptera). The selection of taxa in the study is insufficient to test this claim, because it does not represent a balanced sample of the diversity among the clades being examined. Furthermore, the independent origins of these traits in the various lepidopteran groups considered is not a surprising result, given current knowl-

[1]The comments presented here and those I would have written if I had received Wink and Von Nickisch-Rosenegk's manuscript to review prior to publication in the Journal of Chemical Ecology, 23:1549–1568.

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edge of lepidopteran phylogenetic relations based on morphological and molecular evidence (see below). Although a thoughtful review of sequestration chemistry is presented, the authors conclude that their results are confirmatory of an uncontroversial hypothesis, when their data do not in fact support that hypothesis. To restate the obvious is not very interesting, but to do so based on an erroneous interpretation of the data is just plain wrong. I would not have recommended publication of this paper.

Specific Comments. The hypothesis tested is stated on p. 1552: "if these traits [PA and CG sequestration] evolved only once, we should expect to find, by independent means, that species that share these traits are also phylogenetically related." Frankly, it is difficult to imagine that anyone would seriously entertain the hypothesis that danaine butterflies and ctenuchine moths are more closely related to one another than they are to other nymphalid butterflies and arctiid moths, respectively. Even Linnaeus (1758) placed *Danaus plexippus* among the butterflies (*Papilio*, subgenus *Danaus*), and recognized arctiids such as *Panaxia dominula* as belonging among the noctuoid moths (*Phalaena*, subgenus *Noctua*). More recent hypotheses of lepidopteran relationships based on morphology (e.g., Hampson, 1898–1913; Meyrick, 1912; Janse, 1937–1939; Forbes, 1954; all reviewed in Kitching, 1984; Brock, 1971; Common, 1975; Kristensen, 1976; Nielsen, 1989; Minet, 1991) and on molecules (Weller et al., 1994; Weller and Pashley, 1995) all agree that butterflies and arctiid moths are not phylogenetically intermingled. W and VN-R likewise implicitly acknowledge this by the repeated references to traditional classification (e.g., Tikhomirov, 1979). The hypothesis tested by their study is therefore a rather thinly stuffed straw man.

The description of the methods used to align the sequence data is inadequate. The authors report (p. 1554) that the sequences were aligned "using the program CLUSTAL (implemented in the program package HUSAR) and corrected for missing information and gaps." Alignment of DNA sequences provides the interpretation of character homologies upon which the subsequent tree-building algorithms will operate. Without stating the alignment parameters used to seed the analysis, it is impossible to recover the CLUSTAL alignment that the authors found. Not only are these parameters omitted, but neither program is referenced [a recent reference for CLUSTAL is Higgins et al. (1992); I am not familiar with HUSAR]. Furthermore, "corrections" were done by hand to the computer-generated alignment, but what these might be and how they were performed is not revealed. Such manipulations may introduce bias to the alignment that affects the resultant tree topologies and further reduces the repeatability of the method. Although the raw sequences may be available from EMBL, the alignment is not reproducible based on the description presented, rendering the results untestable. A figure showing the aligned sequences would have been much more informative than the three-page table of pairwise distances the authors chose to present.

The description of the phylogenetic methods employed is also inadequate. Two analyses were performed: Jukes-Cantor neighbor joining (NJ) using MEGA 1.0 (Kumar et al., 1993) and maximum parsimony (MP) using PAUP 3.1.1. (Swofford, 1993). NJ, a phenetic method that forms groups based on minimizing corrected pairwise distances, typically produces a single, fully-resolved topology, and the MP analysis[2] is reported to have produced a single most parsimonious cladogram. However, the topologies shown in Figures 1a and b both contain polytomies, because the results of each analysis are presented as bootstrap consensus trees. Bootstrap values are reported to be "significant" (p. 1554), wrongly implying that bootstrapping is an appropriate statistical measure of the accuracy of phylogenetic reconstruction (Sanderson, 1995; Carpenter, 1996). Finally, the discrepancies between the results of the two data analyses are not addressed, and no rationale is given for using two methods of phylogenetic inference that are philosophically incompatible (Brower et al., 1996).

Another problem is the selective disregard for results deemed spurious: "The placement of the noctuid *Spodoptera* in the clade of Nymphalidae in the NJ reconstructions . . . has no phylogenetic relevance . . . , since moths and butterflies are unrelated and not sister groups (Scoble, 1996)" (p. 1554). Aside from the fact that butterflies and some moths *are* sister groups [which Scoble (1996) clearly discussed], it is not acceptable to ignore inconvenient nodes on the topology, such as the paraphyletic butterflies (both trees imply that danaines and arctiids are more closely related to one another than either is to *Papilio*), yet endow with great significance those nodes that support the favored adaptive hypothesis. If all nodes with low bootstrap values were collapsed, neither of the analyses would imply any unambiguous origin of PA sequestration.

Last and most important, even if the problems discussed above are ignored and the trees are accepted at face value, W and VN-R's choice of taxa is decisively inappropriate to address the question being asked. Because virtually all the species sequenced sequester PA, the claim that the phylogenetic data support the hypothesis that "PA sequestration evolved independently in moths and butterflies" (p. 1561) is, ironically, false. When the character ST (PA storage) is mapped on either the NJ or the MP tree presented in W and VN-R's Figures 1a and b, two alternative, equally likely scenarios are supported. Either PA sequestration is independently derived in two clades (Danainae and Arctiidae), or it is gained once in Danainae + Arctiidae and lost in *Spodoptera*. The second optimization is shown in Figure 1. This is, of course, an artifact of insufficient representation of relevant clades occurring on the branches between

[2]The analysis performed was a single heuristic search, which can terminate without discovering the globally parsimonious solution to the data, if the data contain homoplasy that results in tree "islands" (see Maddison, 1991). Unfortunately, because the alignment was not available, I was not able to test the sufficiency of the parsimony analysis by running additional heuristic searches on the data set. Some systematic journals now encourage submission of the data matrix with a manuscript so this sort of testing may be performed by reviewers.

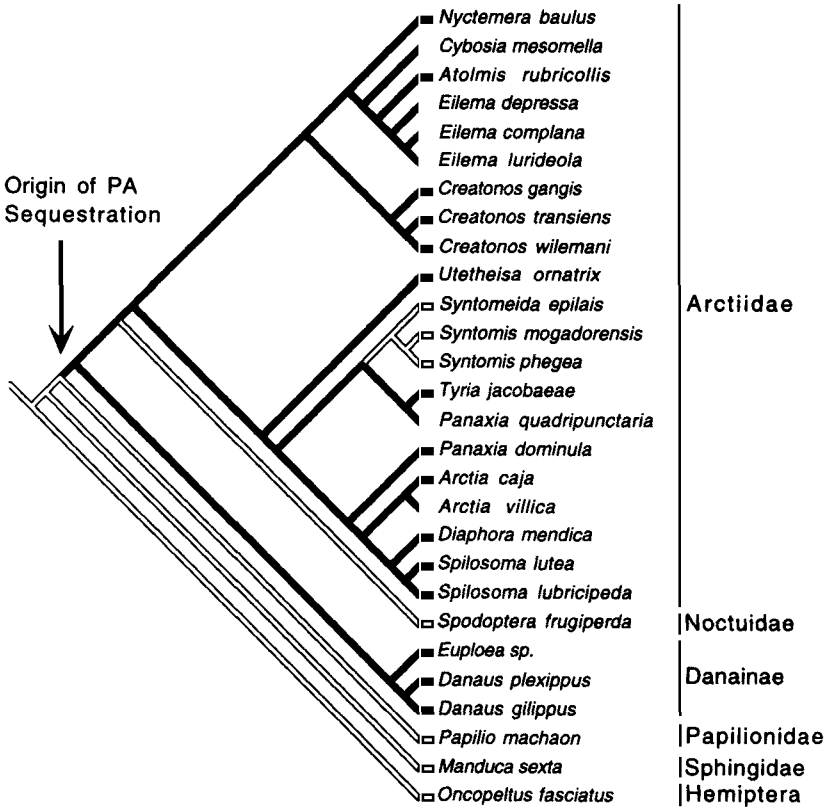

FIG. 1. The single origin of pyrrolizidine alkaloid storage among taxa sampled by W and VN-R, with hypothetical ancestral states as implied by parsimony under accelerated transformation in MacClade (Maddison and Maddison, 1992). Tree redrawn from topology in W and VN-R's Figure 1b. Black = present, white = absent. Boxes next to taxon names indicate the states in terminal taxa, no box indicates missing data for that taxon in W and VN-R's Figure 1a. States are inferred for taxa with missing data by parsimonious interpretation of states in sister groups. Multiple conspecific individuals of *Creatonotos transiens*, *C. wilemani*, and *Eilema depressa* are not shown, and polytomous nodes were arbitrarily resolved, but neither of these differences affects the inferred reconstruction of the character's change on the tree. An equally parsimonious reconstruction under delayed transformation (not shown) implies that PA sequestration is independently derived in danaines and arctiids.

Danainae and Arctiidae. If even one additional nonsequestering butterfly or moth had been included that joined the tree between the danaines and the arctiids, the analyses would have supported W and VN-R's hypothesis. That they do not, in my view, is the fatal flaw of the paper.

In summary, this study is not described in sufficient detail to be repeatable, and its taxon sampling strategy is remarkably poor. If the resulting trees are given the benefit of the doubt, they fail to unequivocally support the hypothesis of independent evolution of PA sequestration. Even if the data did support the hypothesis, they would merely confirm yet again a phylogenetic pattern that has been repeatedly corroborated for more than two centuries.

I reemphasize that my comments are not meant to vilify W and VN-R, who have made many excellent contributions to our knowledge of the chemical ecology of arctiid moths. Instead, they are intended to point out that systematics is a complex theoretical discipline, and not a cookbook procedure that can be adopted as a casual sideline to one's main research area. Appropriate experimental design is just as important in phylogenetic studies as it is in chemical ecology and other biological sciences. I urge all chemical ecologists interested in the comparative approach to collaborate with working systematists who possess an understanding of the complexities of phylogenetic inference and a familiarity with analytical tools relevant to the taxa and characters of interest. Further, I urge the editors of this journal to see that manuscripts with a substantial phylogenetic component are reviewed by peers with sufficient knowledge of systematic methodology to competently evaluate the techniques employed, the quality of resulting topologies, and the soundness of conclusions drawn from them. Only by such attention can the quality of comparative papers in the *Journal of Chemical Ecology* be guaranteed.

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RESPONSE BY MICHAEL WINK, EVA von NICKISCH-ROSENEGK, AND LUC LEGAL

Our study on the use of 16S rDNA sequences to reconstruct phylogenetic relationships in Arctiidae and Nymphalidae and to correlate them with the trait to sequester, transform, and utilize dietary pyrrolizidine alkaloids or cardiac glycosides (Wink and von Nickisch-Rosenegk, 1997) was one of the first in the field of chemical ecology. A similar approach had been published for the sequestration of pyrrolizidine alkaloids and cardiac glycosides and host plant selection in leaf beetles of the genus *Oreina* (Dobbler et al., 1996). Since our study was admittedly preliminary and not final summary, we can understand that it could evoke criticism of both the logic of the approach and the technical performance.

The critique of A. V. Z. Brower concerns two main issues: (1) adequacy of the techniques employed, and (2) validity of the interpretation.

Our paper was the first publication in a continuing study to understand the evolution of the Arctiidae and to draw conclusions on their systematics and chemical ecology. von Nickisch-Rosenegk (VN-R) started the laboratory work, while Luc Legal (LL) presently continues the project on a much wider scale with a more balanced selection of the appropriate taxa. Since several projects in the laboratory of the senior author (MW) are in the field of molecular phylogeny, the corresponding methods are well established and the pitfalls well known (Helbig et al., 1995; Käss and Wink, 1995, 1996, 1997a,b; Kaufmann and Wink, 1994; Leisler et al., 1997; Lenk and Wink, 1997; Seibold et al., 1993; Wink, 1995; Wink and Kaufmann, 1996; Wink et al., 1997; Wittmann et al., 1995). Since most of these studies were in other fields than entomology and published mostly in European journals, this fact might have easily escaped an entomologist in the United States.

It is indeed a problem to align sequences of rDNA, since gaps, insertions, and deletions are often encountered in the loop regions of these RNAs. We regularly start with an alignment program (Higgins et al., 1992), such as CLUSTAL V (using a gap penalty of 10) that we can access in the program package HUSAR. In a second step the alignment is corrected manually, in order to minimize variation (Michel and Costa, 1998). Although even the final alignment of rDNA sequences will always remain somehow subjective, these small variations (according to our experience) hardly affect the topology of the resulting trees. The publication of the alignment would indeed help other scientists in the field to repeat the analysis. Since printing space is valuable and limited, these lengthy alignment tables are increasingly being omitted from publications. We have included the alignment used for our analysis in this paper to illustrate the degree of gaps and insertions (Figure 1), but have limited it to 10 taxa, in order to save space. The interested reader can obtain the complete alignment from the authors.

Many systematicists and especially cladists (Brower et al., 1996) prefer the

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D.gillipus      TTTTTGAAAATAATTTAAAGTCTAATCTGCCCACTGATA---AATATTAAAGGGCTGCAGTATATTGACTGTA
P.machaon       TTTTTGGAAATAATTTAAAGTCTAATCTGCCCACTGATA-AATATATTAAAGGGCTGCAGTATTTGACTGTA
S.frugiperda    TTTTTGAATTTAATATAAAGTCTAATCTGCCCACTGATT-AATATATTAAAGGGCTGCAGTATATTGACTGTA
S.lubricipeda   TTTTTGTAAATAATATATAAAGTCTAATCTGCCCACTGATG--ATTAATTAAAGGGCTGCAGTATATTGACTGTA
C.wilemani      TTTTTGAAAATAATATATAAAGTCTAATCTGCCCACTGATAAAAAAAATTGAAGGGCTGCAGTATATTGACTGTA
E.villica       TTTTTGAAAATAATATATAAAGTCTAATCTGCCCACTGATA-TTTTAATTGAAGGGCTGCAGTATATTGACTGTA
C.quadripunctaria TTTTTGAAAATAATATATAAAGTCTAATCTGCCCACTGATT--TTTAATTGAAGGGCTGCAGTATATTGACTGTA
U.pulchella     TTTTTGGAAATAATATATAAAGTCTAATCTGCCCACTGATT--TTTAATTAAAGGGCTGCAGTATTTGACTGTA
A.phegea        TTTTTGAAAATAATATATAAAGTCTAATCTGCCCACTGATT--TTTAATTAAAGGGCTGCAGTATATTGACTGTA
E.complana      TTTTTGAAAATAATATATAAAGTCTAATCTGCCCACTGATA---ATAATTAAAGGGCTGCAGTATATTGACTGTA

D.gillipus      CAAAGGTAGCATAATCATTAGTCTTTAATTGAAGACTTGTATGAAGGATTTAATGAAATATAAATCTGTCTCT
P.machaon       CAAAGGTAGCATAATCATTAGTCTCTAATTGGTGACTTGTATGAAAGATTGGATGAAATATAAACTGTCTC-
S.frugiperda    CAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAAAGATTGGATGAAATATAAAGCTGTCTCT
S.lubricipeda   CAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAAAGATTGGATGAAGTATAAACTGTCTCT
C.wilemani      CAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAAAGATTGGATGAAATATAAACTGTCTCT
E.villica       CAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAAGGATTGGATGAAGTATAAACTGTCTCT
C.quadripunctaria CAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAAAGATTGGATGAAGTATAGACTGTCTCT
U.pulchella     CAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAAAGATTGGATGAAATATAAACTGTCTCT
A.phegea        CAAAGGTAGCATAATCAATAGTCATTTAATTGATGACTTGAATGAAAGATTGGATGAAATATAAACTGTCTTT
E.complana      CAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAATGATTGGATGAAGTATTAACTGTCTTG

D.gillipus      TATTTATAAT-TAGAATTTAATTTTTTAGTTTAAAAAGCTAAAATAGAGACGAGAAGACCCTATAGA
P.machaon       TAATTAATTTATAAAATTTAATTTTTTAGTAAAAAAAGCTAAAATAATTTTAAAAGACGAGAAGACCCTATAGA
S.frugiperda    TTATTATTTTATAGAATTTAATTTTTAATAAAAAAGTTAAAATAAATTTAAAAGACGAGAAGACCCTATAGA
S.lubricipeda   AAAATATATAATAAAATTTAATTTTTAAATTAAAAAAGTTTAAATAAAAAAGACGAGAAGACCCTATAGA
C.wilemani      AAAATAAAAA-TATAATTTAAATTTTTAAATTTAAAAAAGTTTAAATAAAAAAAGACGAGAAGACCCTATAGA
E.villica       AAAAATATGAATAGAATTTAATTTTTAAATTTAAAAAAGTTTAAATAAAAAAAGACGAGAAGACCCTATAGA
C.quadripunctaria AAAGTAAATTTATAGAATTTAATTTTTAAATTTAAAAAAGTTTAAATAAAAAAAGACGAGAAGACCCTATAGA
U.pulchella     AAAATAAATTTATAATAATTTAAATTTTTAAATTTAAAAAAGTTTAAATAATTTAAAAAAGACGAGAAGACCCTATAGA
A.phegea        AATATAAATTTATAAAATTTAATTTTTAAATTTAAAAAAGTTTAAATAAAAAAAGACGAGAAGACCCTATAGA
E.complana      TAGATAAATTTATAGAATTTAATTTTTAAATTTAAAAAAGTTTAAATATATTAAAAAAGACGAGAAGACCCTATAGA

D.gillipus      GTTTTATAATTAATTAATTTTTAGATTATATATATAA-TTTT-AAATTAA------AAGTA-AATTAATTAT--
P.machaon       GTTTTATAATTTTATTTGATTTATTATTAATATG-TAAATTTTTAAAATTAA------AATTA-AATAAATTAT--
S.frugiperda    GTTTTATATTTAAAGTAATTGAGATTATTTA--TAAAGTTTTTTAATT---------AATTAT-TTTAATAATAT
S.lubricipeda   GCTTTATTTATTTATTGAATTATAATTATTTG--TAAAT---AAAATTTTTT-----AATTT-AATTATTTATAT
C.wilemani      GTTTTATTTATAATTTAAAATAATTATTTA--TAAATTT-AAAATTTTTT-----AATTT-ATTTATAAAT--
E.villica       GTTTTATTTATAATTTAATTTAATTATTTA--TAA-T---AAAATTTTTAATTT-AATTTAATAAAT--
C.quadripunctaria GTTTAATAAATAATTATTATAATTATTTA--TAA-T--AAAAATTTTTT-----AATTTAATTAATTTATAT
U.pulchella     GTTTTATAATAATTTTTAATTAAGATTATTTA--TAAATTTTAAGAATTTTTT-----AATT-AATT-TTATATAT
A.phegea        ATTTTTATAAATAGTTAAATTTAAAATAAAT-TTTAAAT--AAAAATTTTTT-----AATTTTAATGTT-TAT--
E.complana      GTTTTATAGTTTTATATATTTATAATTATTAA--TAA-T---AAAATATTTTTT---AATTATTTAAATTATAT--
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FIG. 1. Aligned nucleotide sequences of part of the 16S RNA gene of 10 Lepidoptera species. Bold print was used to highlight potential secondary structures (Maidak et al., 1996). Specific parts of the sequence involved in the secondary structure were also compared to the sequence and secondary structure of *Papilio machaon*'s LSU (Aubert et al., 1998). In italic are the nucleotides that were omitted from the phylogenetic analysis considering some possible ambiguities of alignment. Total number of nucleotides: 502; kept for analysis: 462; (−) = gap.

character state method maximum parsimony (MP) for tree-building. It would be out of the scope of this note to discuss the extensive and often acrimonious and contradictory battle that can be found in the literature on the advantages and disadvantages of character state versus distance methods (see publications cited in Felsenstein, 1988; Stewart, 1993; Nei, 1996). The same applies to the employment of bootstrap analyses (Felsenstein, 1985) to obtain a statistical estimate, i.e., whether a furcation is significantly supported by the data or not (see discussion in Nei, 1996; Sanderson, 1995).

However, we prefer a pragmatic way and use several methods, such as MP, distance matrix methods, including the use of neighbor joining (NJ), and increasingly, maximum likelihood (ML) to reconstruct phylogenetic relationships. In our experience, data sets based on a balanced sampling usually produce trees of almost congruent topology. Also Nei (1996) concluded "... it is now clear that any method [of tree building] is not almighty, and there are situations in which one method is more efficient than others in obtaining the true tree and that, unless the evolutionary rate varies drastically with evolutionary lineages, all three methods [MP, distance methods, ML] considered here generally give the same or similar topologies." If a clade is supported by these independent methods, which employ different mathematical algorithms, we are confident in the results; ambiguous furcations rather indicate that the topology cannot be resolved with a present data set. In a systematic publication, it would certainly be important to discuss the implications of incongruencies in detail, but since a systematic analysis was not intended, but rather an evolutionary and ecological correlation (in which these small ambiguities did not influence the interpretation), we have deliberately omitted this sort of discussion in our original paper.

Brower implies that a totally different conclusion could be drawn if our MP and distance trees are combined together. (Figure 1 in Brower that was constructed from our published trees appears to be wrong, since it places *Creatonotos* as a sister group to the Lithosiinae, which they are never, in either MP or distance trees. Furthermore, the Old World *Utetheisa pulchella* have now become *U. ornatrix*, an American taxon): Brower argues that PA sequestration could have been acquired much earlier from ancestors to both Nymphalidae and Arctiidae. To test this assumption we have reanalyzed our data but have included sequences of more Nymphalidae, Papilionidae, and Sphingidae (which do not sequester PAs) in our present analysis. Our new analysis unequivocally confirms our published result that PA sequestration apparently evolved in the monophyletic Arctiidae and in some subfamilies of Nymphalidae independently (Figure 2), and clearly shows that this assumption was not based "on a fatal flaw" as Brower wrongly suggests.

During the reanalysis in which we sequenced several more individuals from the same and other arctiid taxa used in the original publication, we discovered

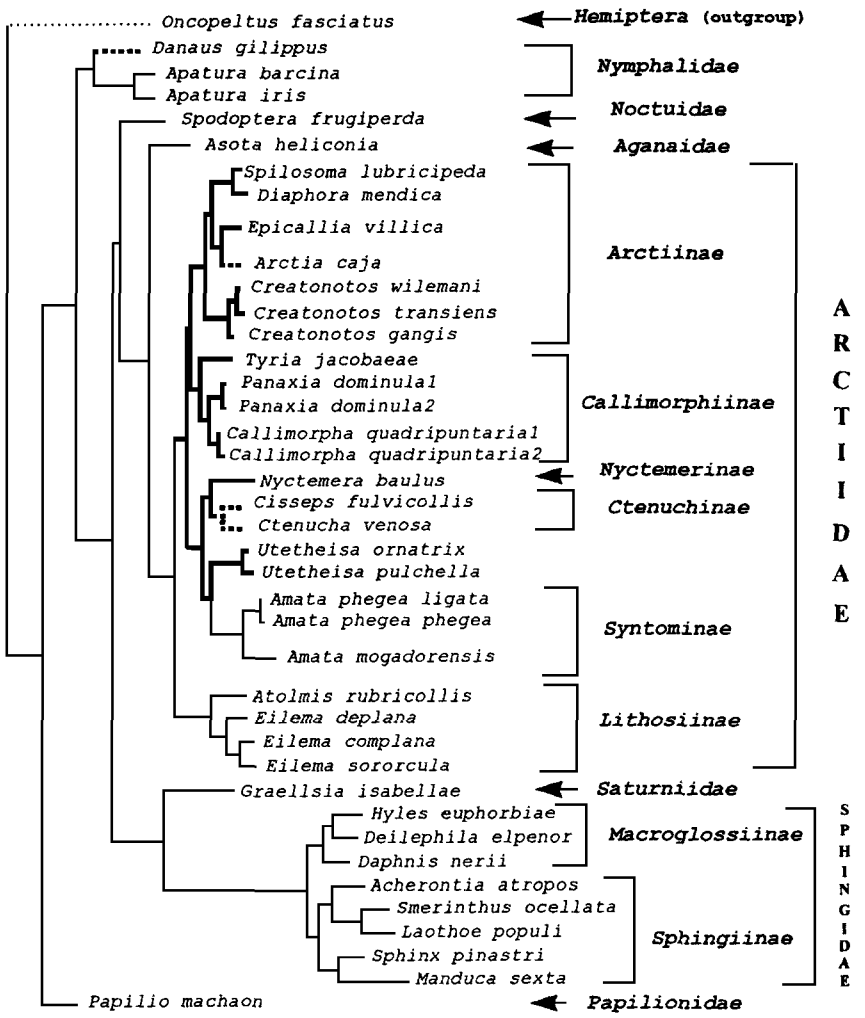

FIG. 2. Phylogenetic tree generated from gap-free 16S RNA sites. The total data set of 40 taxa was run on a Pentium PC with the PAUP 4d60 to obtain a maximum likelihood tree, with an estimated 0.16 fraction of variable sites, whose rates of evolution were assumed to follow a gamma distribution with shape parameter 0.37 (four rate categories represented by mean; rates of substitution were assumed to obey a six-parameter, general time-reversible model with $rAC = 0.40$; $rAG = 6.94$; $rAT = 4.48$; $rCG = 3 \times 10^{-10}$; $rCT = 1.51$; $rGT = 1$). Branches of taxa printed in bold are species that were found to sequester pyrrolizidine alkaloids; a dotted line represents species that were found to sequester cardiac glycosides; taxa that were found to sequester both types of compounds are printed with bold and dotted lines.

a mistake in the original data set, which had shown that *Panaxia dominula* and *Callimorpha* (*Panaxia*) *quadripuncatria* did not cluster together but in different clades. *P. dominula* and *C. quadripunctaria* are indeed related taxa, as suggested by their numerous shared morphological characters (Figure 2). In Figure 2, we have changed a few names of genera and species according to the latest systematics of Arctiidae, particularly the synonymy of *E. depressa* = *E. deplana* and a probable split between the two subfamilies Ctenuchinae and Synthomiinae, which includes the genus *Amata* (no longer *Syntomis*) (Minet, personal communication). A more complete and extended phylogenetic analysis of the Arctiidae will be published in due course (L. Legal and M. Wink, in preparation).

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ATTRACTION OF MATURE *Ceratitis capitata* FEMALES TO 2-HEPTANONE, A COMPONENT OF COFFEE FRUIT ODOR

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Abstract—In indoor laboratory-cage and outdoor field-cage assays, we evaluated the attraction of released, protein-fed, mature Mediterranean fruit fly females to six volatile compounds emitted by attractive crushed ripe coffee fruit: 3-methyl-1-butanal, decanal, 3-methyl-1-butanol, 2-(*Z*)-pentenol, 2-(*E*)-hexenol, and 2-heptanone. Previous tests in a wind tunnel indicated that each of these six compounds was more attractive than clean air to females. In laboratory cage assays, none of the compounds elicited a response significantly greater than that to water. In field-cage assays, 2 or 4 μl of 2-heptanone in 5 ml of water (but none of the other compounds at 2 or 4 μl in 5 ml of water) consistently attracted protein-fed medflies at significant levels that averaged about five times greater than attraction to water alone, although about 40% less than attraction to odor of coffee fruit extract. All types of mature protein-fed females tested (laboratory-cultured virgin, laboratory-cultured mated, wild mated) in field-cage assays responded similarly to 2-heptanone, whereas same-age (9- to 11-day-old) protein-deprived females did not respond significantly to 2-heptanone. Response of protein-fed females to 2-heptanone increased progressively with increasing dose (1, 2, 4, or 8 μl in 5 ml of water). Addition of 2 μl of 2-heptanone or 2 μl of any of the other compounds tested to 5 ml of water extract of coffee fruit did not enhance attractiveness of the extract. Relative to response to water, protein-fed females consistently exhibited a significant positive response to odor of coffee fruit extract but no significant response to odor of Nulure (a proteinaceous food attractant). Together, our

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findings suggest that mature protein-fed females were responding to 2-heptanone as though it were an oviposition-site stimulus rather than a feeding-site stimulus.

Key Words—*Ceratitis capitata*, 2-heptanone, coffee odor, attractants.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is an economically important tephritid fly whose host range encompasses more than 300 species of fruits and vegetables (Liquido et al., 1991). The presumed ancestral origin of medflies is Africa, and the presumed ancestral host of medflies in Africa is the fruit of coffee, *Coffea arabica* (Vargas et al., 1995). Recently, the odor of ripe (red) *C. arabica* fruit was found to be more attractive than the odor of ripe fruit of several other species of *Coffea* as well as that of fruit of several lower-ranking hosts (Prokopy and Vargas, 1996; Prokopy et al., 1997). The odor of ripe *C. arabica* fruit was more attractive than the odor of less mature fruit or the odor of foliage or twigs of this species (Prokopy et al., 1997). In addition, Prokopy et al. (1997) showed previously that the odor of a 24-hr-old water extract of ripe *C. arabica* fruit was more attractive than the odor of 24-hr-old extracts of such fruit with methanol, methylene chloride, or hexane, and that the odor of ripe *C. arabica* fruit that had been frozen, thawed, and crushed was just as attractive as the odor of crushed unfrozen fruit. Finally, in trials in commercial coffee fields without ripe fruit present, traps baited with juice from ripe coffee fruit captured significantly more female medflies than traps baited with trimedlure or water (Vargas et al., 1997).

Based on the above information, Warthen et al. (1997) employed headspace analysis techniques to identify 28 volatile compounds emitted by crushed ripe *C. arabica* fruit that had been frozen after picking and thawed just prior to volatile collection. They used a wind tunnel to assess the attractiveness of each compound to mature, protein-fed laboratory-cultured female medflies under dual-choice conditions, wherein response to odor of each compound was compared to response to clean air. In these assays, medflies responded positively to nine of the compounds: 3-methyl-1-butanal, decanal, 3-methyl-1-butanol, 2-(*Z*)-pentenol, 2-(*E*)-hexenol, 2-heptanone, 2-(*Z*)-hexanol, 2-heptanol, and 3-octanol. Follow-up assays in which an indoor olfactometer was used showed that among these nine compounds, the first six were the most attractive (Jang et al., unpublished data).

In indoor laboratory cages as well as in patches of nonfruiting host trees in outdoor field cages, we evaluated the response of medflies to each of the first six compounds listed above. In addition, we asked the following questions: What effect does fly physiological state (protein-fed versus protein-deprived, virgin versus mated) and fly origin (lab-cultured versus wild) have on fly response

to each compound in relation to response to the odor of a water extract of coffee fruit and the odor of a proteinaceous attractant (Nulure)? What effect does addition of each compound to a water extract of coffee fruit, as opposed to addition to water alone, have on fly response?

METHODS AND MATERIALS

Source of Flies. Medflies originated either from a laboratory colony in culture for about 30 generations at the USDA Tropical Fruit, Vegetable and Ornamental Crops Research Laboratory in Honolulu, Hawaii, or from natural-population larvae that infested field-collected coffee fruit. Unless otherwise indicated, from eclosion until tested 9–11 days afterward (for lab-cultured flies) or 14–19 days afterward (for wild-origin flies), both sexes were held together in screened cages (30 × 30 × 30 cm) and supplied with enzymatic yeast hydrolysate, sucrose, and water (these were termed protein-fed flies) or simply sucrose and water (these were called protein-deprived flies). No fruit was supplied, and flies were held under conditions of about 24°C, 50% relative humidity, and 13 hr natural day length. Flies held in this manner were presumed to be mated. Lab-cultured flies became mature at 5–6 days of age, whereas wild-origin flies became mature at 10–11 days of age.

Bioasssays. In laboratory-cage trials, we employed an approach similar to that used by Robacker and Warfield (1993) for studying attraction of *Anastrepha ludens* Loew flies to sources of odor. Each cage was 30 × 30 × 30 cm, covered with screen on top and two sides (front and back were solid surfaces), and contained 40 females of a single type introduced 24 hr earlier. A filter paper disc (3 cm diam.) containing test substance was placed at the screened top near each of the four corners. Each paper was raised 1 cm above the cage top by using a 2.5-cm-diam. glass ring to ensure that olfaction and not contact chemoreception was the factor eliciting fly response. A trial lasted 5 min, during which a count was taken every 30 sec of the number of flies on the cage top partly or fully beneath each filter paper. There were four trials (replicates) per cage with 1 hr or more between trials. Treatment positions were rotated clockwise until each treatment occupied each corner once.

In field-cage trials, we used an approach similar to that of Prokopy et al. (1997). All trials were carried out from 09:00 to 12:00 hr and from 13:00 to 16:00 hr in four cylindrical, 3-m-tall × 3-m-diam. clear nylon-screen enclosures placed beneath the protection of a partly opaque plastic roof on the grounds of the University of Hawaii field station at Kainaliu (Kona), Hawaii. Flow of wind through the cages was not regulated but rarely exceeded 2 km/hr. Each cage contained five potted nonfruiting guava trees whose bases were grouped near the cage center and that together formed a foliar canopy of 2.6 m diam., extending to within 20 cm of the cage wall and reaching the cage ceiling. Before use,

foliage and branches of each tree were rinsed thoroughly with water. At each of six positions within each cage, we hung a single container of test substance. The positions were about 1 m apart along the circumference of an imaginary circle about 25 cm inward from the periphery of the canopy and about 45 cm below the cage ceiling. Containers of test subtances were clear glass Petri dishes (9 cm diam. × 1.5 cm tall). All but the outer 1 cm of the clear plastic lid of each container consisted of nylon netting that permitted air flow.

About 10 min before the start of a trial, we released as a group about 100 lab-cultured or 60 wild flies of each sex at the base of the canopy. Separate field cages were maintained for each origin or physiological state of fly studied. Flies that arrived on the screened area of the lid of a container within the 5-min period allotted to each replicate were considered as responding to the odor of the contents. They were sexed and removed within 30–60 sec of arrival via an aspirator held by one of us, who continually circled the perimeter of the foliage canopy watching for flies arriving on treatment containers. Even though we released approximately as many males as females for each experiment, fewer males responded to test odors, and among males that did respond, response patterns to the same treatment were more variable across experiments. Hence, we chose to exclude from consideration here all data on male responders. In previous tests in field cages, medfly males from the same laboratory culture likewise proved inconsistent in response pattern to coffee odor (Prokopy and Vargas, 1996).

For the first replicate, treatment positions were randomized. Treatments were rotated clockwise within the canopy after each replicate until each treatment occupied each position once, requiring a total of about 30 min to complete a set of six replicates. For the next set of six replicates, we deployed treatments in a newly arranged random order. From 09:00 to 12:00 hr, we were able to complete three sets of replicates per field cage, provided weather conditions were favorable (i.e., no rainfall and temperatures between 24 and 32°C). The same was true for trials conducted between 13:00 and 16:00 hr. By the end of 3 hr, the number of flies in each field cage that responded to treatments had reached a low level, with most nonresponders having flown to the cage wall. We did not collect such nonresponding flies before the start of the next set of replicates but instead allowed them to remain in the cage. At the beginning of each set of replicates, we sprinkled canopy foliage with water to minimize fly response to treatments on the basis of thirst.

Sources of Test Odor. Water extract of coffee fruit was prepared by soaking fresh-picked whole red berries of *C. arabica* cv. arabica in water (1 g fruit/per ml water) for 24 hr at 25°C, after which the water was poured through a strainer and stored at 4°C until use one to three days later. Nulure solution was formulated according to Wakabayashi and Cunningham (1991) as follows: 9% Nulure (Miller Chemical Co., Hanover, Pennsylvania), 5% sodium borate, and

86% water. Each of the test compounds was obtained from a commercial supplier. Distilled water served as a control treatment.

In laboratory-cage assays, we soaked each filter paper disc in distilled water and then applied 1 μl of test substance to the paper. In field-cage assays, we used 5 ml of either water extract of coffee fruit, Nulure solution, or distilled water per Petri dish or introduced 5 ml of distilled water (or 5 ml of water extract of coffee fruit) into a Petri dish and added either 2 μl (for experiments in Table 2, below) or 4 μl (for experiments in Table 3, below) of the named compound (neat, or undiluted form). Compounds were added to Petri dishes about 5 min before the start of a trial and remained detectable (by the human nose) throughout a set of six replicates (~30 min), after which a fresh batch was prepared for the next set of replicates.

Statistical Analysis. Data were analyzed by two-way analysis of variance (ANOVA). Treatment means were compared by using the least significant difference test criterion (0.05 level). For analysis of data from laboratory cage tests, the 16 replicates per treatment per female type per experiment were grouped into four sets of four replicates each. For each treatment, the value of a single set of four replicates consisted of the total number of females counted beneath the filter paper of that treatment across all 10 counts per 5-min trial and across all four positions of that treatment on a cage top. For analysis of data from field cage tests, the 36 replicates per treatment per female type per experiment were grouped into six sets of six replicates each. For each treatment, the value of a single set of six replicates consisted of the summed numbers of females arriving on the screen covering a Petri dish for all six positions of that treatment. We reasoned that grouping the data into sets of replicates for ANOVA was the most valid approach to minimizing the varying effects of treatment position (on a laboratory cage top or within a tree patch canopy) on fly response to replicates within a set.

RESULTS

Laboratory-Cage Tests. Among protein-fed laboratory-cultured females (Table 1), significantly more responded positively to the odor of Nulure but not to the odor of coffee fruit extract compared to the response to water (experiment 1). Females did not respond to any of the six compounds tested more often than to water (experiments 2 and 3).

Among protein-deprived laboratory-cultured females (Table 1), more responded positively to the odor of Nulure as well as coffee fruit extract compared to response to water (experiment 1). Females were more responsive to the odor of decanal and 2-heptanone (but not to the odor of any of the other four compounds tested) than to water (experiments 2 and 3).

Field-Cage Tests. In the first set of experiments, we assessed responses of

TABLE 1. LABORATORY-CAGE ASSAYS: RESPONSE OF PROTEIN-FED OR -DEPRIVED LABORATORY-CULTURED *C. capitata* FEMALES (9–11 DAYS OLD) TO ODOR OF WATER EXTRACT OR VOLATILE COMPOUNDS FROM RIPE COFFEE FRUIT OR NULURE EMITTED FROM FILTER PAPER

Odor source	Females (mean ± SE) at odor source per replicate[a]	
	Protein-fed	Protein-deprived
Experiment 1		
Coffee fruit extract	7.8 ± 3.7ab	25.0 ± 2.7b
Nulure	13.5 ± 3.7a	88.8 ± 15.2a
Water	3.8 ± 0.8b	8.3 ± 2.8c
Water	4.5 ± 1.8b	8.0 ± 1.8c
Experiment 2		
3-Methyl-1-butanal	4.0 ± 1.1a	15.3 ± 5.0ab
Decanal	4.5 ± 1.3a	24.3 ± 3.3a
3-Methyl-1-butanol	2.3 ± 1.3a	17.8 ± 4.3ab
Water	2.3 ± 0.9a	13.0 ± 2.4b
Experiment 3		
2-(*Z*)-Pentenol	2.8 ± 0.6a	16.8 ± 4.9ab
2-(*E*)-Hexenol	4.3 ± 0.9a	14.8 ± 3.6ab
2-Heptanone	2.3 ± 0.5a	25.3 ± 4.2a
Water	1.5 ± 0.6a	12.5 ± 4.8b

[a]Sixteen replicates per treatment per female type. Values in each column in each experiment followed by the same letter are not significantly different at the 0.05 level according to least significant difference tests.

protein-fed and protein-deprived laboratory-caged females to each compound introduced into water or into a water extract of coffee fruit.

Among protein-fed females, more were attracted to the odor of coffee fruit extract than to the odor of Nulure in each experiment, with no greater attraction to the odor of Nulure than to water in any experiment (Table 2). None of the six compounds in water were as attractive as coffee fruit extract and none differed significantly from Nulure in attractiveness, but 2-(*Z*)-pentenol and 2-heptanone were significantly more attractive than water (experiments 1 and 2). None of the six compounds in coffee fruit extract significantly enhanced attractiveness of such an extract above that of coffee fruit extract alone (experiments 3 and 4).

Among protein-deprived females, significantly more in each experiment were attracted to the odor of Nulure than to the odor of coffee fruit, which was more attractive than water in each experiment (Table 2). None of the six compounds in water were as attractive as Nulure (experiments 1 and 2). Indeed, none were significantly more attractive than water, and none differed signifi-

TABLE 2. FIELD-CAGE ASSAYS: ATTRACTION OF PROTEIN-FED OR -DEPRIVED LABORATORY-CULTURED *C. capitata* FEMALES (9–11 DAYS OLD) TO ODOR OF WATER EXTRACT OF RIPE COFFEE FRUIT, VOLATILE COMPOUNDS FROM RIPE COFFEE FRUIT INTRODUCED INTO WATER OR WATER EXTRACT OF RIPE COFFEE FRUIT, OR NULURE IN PETRI DISHES

Experiment	Volatile compounds introduced into	Odor source	Arriving females (mean ± SE) per replicate[a]	
			Protein-fed	Protein-deprived
1	Water	Coffee fruit extract	1.4 ± 0.3a	0.7 ± 0.2b
		3-Methyl-1-butanal	0.1 ± 0.1b	0.1 ± 0.1bc
		Decanal	0.1 ± 0.1b	0.1 ± 0.1bc
		3-Methyl-1-butanol	0.2 ± 0.1b	0.4 ± 0.1bc
		Nulure	0.4 ± 0.1b	3.4 ± 0.7a
		Water	0.1 ± 0.1b	0.2 ± 0.1c
2	Water	Coffee fruit extract	1.5 ± 0.3a	0.7 ± 0.2b
		2-(*Z*)-Pentenol	0.6 ± 0.1b	0.4 ± 0.1bc
		2-(*E*)-Hexenol	0.4 ± 0.2bc	0.2 ± 0.2bc
		2-Heptanone	0.6 ± 0.2b	0.3 ± 0.2bc
		Nulure	0.5 ± 0.2bc	3.8 ± 0.6a
		Water	0.1 ± 0.1c	0.1 ± 0.1c
3	Coffee fruit extract	Coffee fruit extract	1.1 ± 0.2a	1.0 ± 0.2b
		3-Methyl-1-butanal	1.2 ± 0.2a	0.7 ± 0.2b
		Decanal	0.7 ± 0.3ab	0.7 ± 0.1b
		3-Methyl-1-butanol	1.2 ± 0.3a	1.2 ± 0.4b
		Nulure	0.5 ± 0.2bc	5.5 ± 0.8a
		Water	0.1 ± 0.1c	0.1 ± 0.1c
4	Coffee fruit extract	Coffee fruit extract	1.6 ± 0.3a	0.8 ± 0.2b
		2-(*Z*)-Pentenol	1.9 ± 0.4a	0.6 ± 0.2bc
		2-(*E*)-Hexenol	0.9 ± 0.2b	1.1 ± 0.3b
		2-Heptanone	1.8 ± 0.3a	1.3 ± 0.3b
		Nulure	0.6 ± 0.3bc	3.2 ± 0.8a
		Water	0.1 ± 0.1c	0.1 ± 0.1c

[a]Thirty-six replicates per treatment per female type. Values in each column in each experiment followed by the same letter are not significantly different at the 0.05 level according to least significant difference tests.

cantly in attractiveness from coffee fruit extract (experiments 1 and 2). None of the six compounds in coffee fruit extract enhanced the attractiveness of such an extract above that of extract alone (experiments 3 and 4).

In the second set of experiments, we assessed responses of protein-fed virgin and protein-fed mated laboratory-cultured females as well as protein-fed mated wild females to those four compounds that attracted the most protein-fed females in field cage experiments 1 and 2 of Table 2. As in experiments 1 and

TABLE 3. FIELD-CAGE ASSAYS: ATTRACTION OF MATURE PROTEIN-FED *C. capitata* FEMALES OF DIFFERENT TYPES TO ODOR OF WATER EXTRACT OR VOLATILE COMPOUNDS FROM RIPE COFFEE FRUIT INTRODUCED INTO WATER IN PETRI DISHES

Type of fly	Odor source	Arriving females (mean ± SE) per replicate[a]
Experiment 1, laboratory-cultured, virgin	Coffee fruit extract	1.1 ± 0.3a
	3-Methyl-1-butanol	0.1 ± 0.1c
	2-(*Z*)-Pentenol	0.1 ± 0.1c
	2-(*E*)-Hexenol	0.3 ± 0.1bc
	2-Heptanone	0.5 ± 0.1b
	Water	0.1 ± 0.1c
Experiment 2, laboratory-cultured, mated	Coffee fruit extract	1.1 ± 0.2a
	3-Methyl-1-butanol	0.1 ± 0.1c
	2-(*Z*)-Pentenol	0.2 ± 0.1bc
	2-(*E*)-Hexenol	0.2 ± 0.1bc
	2-Heptanone	0.5 ± 0.1b
	Water	0.1 ± 0.1c
Experiment 3, wild, mated	Coffee fruit extract	0.8 ± 0.2a
	3-Methyl-1-butanol	0.1 ± 0.1c
	2-(*Z*)-Pentenol	0.1 ± 0.1c
	2-(*E*)-Hexenol	0.1 ± 0.1c
	2-Heptanone	0.4 ± 0.1b
	Water	0.0 ± 0.0c

[a] See footnote of Table 2.

2 of Table 2, each of the four compounds was introduced into water. The results were essentially the same for each type of fly tested (Table 3). In each case, response was greater to coffee fruit extract than to any of the four compounds or water. In each case, response was greater to 2-heptanone than to water, but not to 3-methyl-1-butanol, 2-(*Z*)-pentenol, or 2-(*E*)-hexanol in comparison with water.

Finally, we evaluated the response of protein-fed laboratory cultured females to different doses of 2-heptanone in water (Table 4). The degree of response increased with increasing dose (1, 2, 4, or 8 μl) per test container. All doses (except 1 μl) were more attractive than water, but none was as attractive as coffee fruit extract.

DISCUSSION

Our findings revealed that in laboratory-cage bioassays, protein-fed laboratory-cultured medfly females discriminated rather poorly among sources of

TABLE 4. FIELD-CAGE ASSAY: ATTRACTION OF PROTEIN-FED LABORATORY-CULTURED *C. capitata* FEMALES (9–11 DAYS OLD) TO ODOR OF WATER EXTRACT OF RIPE COFFEE FRUIT OR ODOR OF DIFFERENT DOSES OF 2-HEPTANONE INTRODUCED INTO WATER IN PETRI DISHES

Odor source	Dose (μl)	Arriving females (mean ± SE) per replicate[a]
Coffee fruit extract		2.8 ± 0.5a
2-Heptanone	1	0.5 ± 0.2cd
2-Heptanone	2	0.6 ± 0.1c
2-Heptanone	4	0.8 ± 0.2bc
2-Heptanone	8	1.1 ± 0.2b
Water		0.2 ± 0.1d

[a] See footnote of Table 2.

odor tested (except for Nulure, which elicited a significant positive response). In contrast, in field-cage bioassays, discrimination was good to excellent. Among the six coffee fruit volatile compounds tested in water in field cages, 2-heptanone always elicited a greater attraction of protein-fed females (mated or virgin, laboratory-cultured or wild) than did water. This was not true for any of the other compounds tested: 3-methyl-1-butanal, decanal, 3-methyl-1-butanol, 2-(*Z*)-pentenol, or 2-(*E*)-hexenol. The level of attraction of protein-fed females to 2 or 4 μl of 2-heptanone in water in field-cage bioassays averaged about five times greater than attraction to water alone, and about 40% of the level of attraction to coffee fruit extract, except in the final experiment, where 8 μl of 2-heptanone was required to elicit this level of response. Protein-fed females in field-cage bioassays always responded to a greater extent to the odor of coffee fruit extract than to odor of Nulure, whereas the reverse was true for protein-deprived females, which did not exhibit greater attraction to odor of any of the six coffee fruit volatile compounds tested than to water.

In the laboratory-cage bioassay approach used here, protein-fed medfly females were more responsive to Nulure than to coffee fruit extract by a factor of nearly 2:1, whereas in field-cage bioassays of protein-fed females, response to coffee fruit extract was greater than to Nulure by a factor of at least 2:1. Moreover, in laboratory-cage bioassays, the response of protein-fed females to the odor of coffee fruit extract was only about twice that to water, whereas in field-cage bioassays, response to odor of coffee fruit extract was always at least 10 times greater than to water, averaging 13 times greater across all experiments. Part of this disparity in outcome between laboratory- and field-cage bioassays could lie in differences in the manner in which odor sources were deployed: on

filter paper in laboratory-cage assays and in Petri dishes in field-cage assays. We believe, however, that such disparity played a minor role because at the end of the trials in both situations, moisture was present and all sources emitted a distinctive odor (to the human nose). We believe that a principal cause of disparity lay in differences in structure between the bioassay arenas. In laboratory cages, odor sources were separated by less than 30 cm, and flies usually walked toward the odor source, whereas in field cages, odor sources were separated by about 1 m, and flies almost invariably flew to odor sources. In addition, in laboratory cages, the same fly could have been counted up to 10 times as a responder, whereas in field cages, a fly was counted as a responder only once. Finally, in a broader sense, the presence of branches and foliage on trees in the field cages probably afforded a setting more conducive to natural patterns of resource foraging behavior than that afforded by a laboratory cage. That field-cage bioassays involving the use of host tree patches or open field bioassays afford a greater degree of discrimination among sources of stimuli than do laboratory-cage bioassays has also been reported in other studies on tephritid flies (e.g., Prokopy et al., 1994, 1995; Robacker et al., 1996).

Even though all six of the coffee fruit volatile compounds tested showed evidence of being at least moderately attractive to mature protein-fed medfly females in earlier dual-choice wind-tunnel assays (Warthen et al., 1997), only one (2-heptanone) proved consistently attractive to similar-type flies in field-cage bioassays here. A possible reason for this could be that attraction to each compound relative to clean air is pronounced under dual-choice conditions but substantially disappears (except in the case of 2-heptanone) under multiple-choice conditions involving competing sources of odor. Alternatively, the amount of each compound used per trial in wind-tunnel assays (5 μl pipetted onto filter paper) may have been ideal for eliciting attraction to each compound in that setting, whereas except for 2-heptanone, the amount used per trial here (2 or 4 μl in 5 ml of water) may have been less than ideal for eliciting attraction in our field-cage setting. In additional field cage tests (subsequent to those reported here and data not shown) involving evaluation of the six compounds at 5 μl of compound in 5 ml of water, 2-heptanone again was significantly (about 5 times) more attractive than water to mature, protein-fed, laboratory-cultured females. None of the other five compounds was significantly more attractive than water. More than twice as many females were attracted to 2-heptanone than to 2-(*E*-hexenol (the only other compound showing numerically greater attractiveness than water). Conceivably, some or all of the five compounds that were not significantly more attractive than water in field cage assays might become so if amounts other than 2-5 μl of compound per 5 ml of water were to be used.

The compounds tested here are not peculiar to coffee fruit but have been identified as components of volatiles from blueberry fruit (2-heptanone) (Lugemwa et al., 1989), stone fruit [2-(*E*)-hexenol] (Light and Jang, 1987), citrus fruit [2-(*E*)-hexenol, decanal] (Light and Jang, 1987), and papaya fruit

[2-(*E*)-hexenol] (Light and Jang, 1987), all of which are listed as hosts of medfly (Liquido et al., 1991). Interestingly, 2-heptanone was shown to elicit positive elctroantennogram responses from blueberry maggot flies, *Rhagoletis mendax* Curran (Lugemwa et al., 1989), while a closely related compound (2-butanone) has been found to elicit a positive antennogram response (Eisemann and Rice, 1992) as well as a positive attractive response (Drew, 1987) from the Queensland fruit fly, *Bactrocera tyroni* (Froggat).

Previously, in wind-tunnel tests, Light and Jang (1996) found that a blend of three esters (none of which were attractive on their own to gravid medfly females) enhanced the attractiveness of the odor of ripe nectarines when added to nectarine odor in a small amount in neat form (3.3 μl of each ester). The reasoning underlying the approach of Light and Jang (1996) was that individual components or mini-blended components of attractive fruit might not elicit a response in their own right but could do so when "synergized" by other untested components of fruit odor. Here, we found no significant enhancement of the odor of coffee fruit extract by addition of 2 μl (neat form) of any of the compounds tested (including 2-heptanone) to such extract. Perhaps the amount of compound added was too little to produce a synergistic effect.

Our finding that relative to the response to water, protein-fed females in field-cage assays consistently exhibited a positive response to 2-heptanone and coffee fruit extract but not to the odor of Nulure, coupled with opposite responses by protein-deprived females, strongly suggests that protein-fed females were responding to 2-heptanone as though it were an oviposition site stimulus rather than a feeding site stimulus. Protein-fed females of all types tested (wild, laboratory-cultured, virgin, mated) exhibited a similar response pattern to 2-heptanone and coffee fruit extract. We did not test responses of immature (younger) protein-fed females because earlier it was found that such females are comparatively unresponsive to coffee fruit odor (Prokopy and Vargas, 1996). Physiological-state-dependent response patterns similar to those seen here have been shown for other tephritid flies. For example, both *B. tryoni* and *Rhagoletis pomonella* (Walsh) flies denied access to protein and lacking fully developed eggs are less attracted to egg-laying sites than to proteinaceous food, whereas the opposite is true for females provided with protein and carrying fully developed eggs (Prokopy et al., 1991, 1995).

In conclusion, we suggest that future studies on attraction of medflies to components of coffee fruit volatiles focus on a comparison between the response to 2-heptanone and blends of compounds that include 2-heptanone and some or all of the other five compounds tested here. We also suggest that the scope of bioassays be expanded to include field assays in patches of ripe host fruit (where competition exists from natural volatiles) as well as evaluation of attractive components of coffee fruit odor (such as 2-heptanone) in combination with attractive food odor (such as Nulure, or its synthetic equivalent) and attractive male-produced pheromone (Jang and Light, 1996).

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COMPARISON OF CERALURE AND TRIMEDLURE CONTROLLED-RELEASE FORMULATIONS FOR MALE MEDITERRANEAN FRUIT FLIES IN C&C TRAPS

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Abstract—Ceralure or trimedlure polymeric TNO panels in C&C traps were compared for attractancy by the capture of male Mediterranean fruit flies, *Ceratitis capitata* (Wiedemann). This study contrasts the effectiveness of ceralure with trimedlure, the former of which is an iodo-analog of trimedlure. Field tests in Hawaii with released flies showed that the active ceralure B1 isomer in a commercial mixture of ceralure isomers consistently caught as many male flies as active trimedlure C isomer in a commercial mixture of trimedlure isomers at one-half the molar quantity of trimedlure C. These panels caught 6.4 and 5.2 times, for ceralure and trimedlure, respectively, more than the standard 2 g trimedlure AgriSense aged plug over a 26-week period. The persistence of ceralure is demonstrated by residual analyses of aged panels that revealed the presence of 2.75 times more ceralure than trimedlure in panels after 130 days of field exposure.

Key Words—Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), medfly, trimedlure, ceralure, attractant, panel, C&C traps, TNO panels.

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INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is a worldwide agricultural pest of fruits, nuts, and vegetables (Liquido et al., 1991). The presence of the pest in the continental United States is prevented through vigorous detection, quarantine, and eradication in subtropical areas where hosts exist (Anonymous, 1996a). A network of traps is used to monitor any accidental entries of the medfly. The parapheromone that is used in these traps for attracting male medflies is trimedlure (TML), *tert*-butyl 4- and 5-chloro-2-methyl cyclohexane-1-carboxylate (Beroza et al., 1961). The controlled-release of TML from polymeric materials in reference to the release of TML from cotton wicks has been studied extensively in an effort to develop an optimal formulation (Leonhardt et al., 1984, 1986, 1987, 1989, 1994; Rice et al., 1984). A 2-g TML polymeric plug dispenser is the present standard for controlled-release of TML. Commercial TML is a mixture of 90–95% *trans* isomers (McGovern et al., 1986), which are designated A, B1, B2, and C (McGovern and Beroza, 1966) because of the GC elution sequence; the other 5–10% of the mixture consists of *cis* isomers. The *trans* designation refers to the diequatorial relationship between the vicinal 1-carboxylic acid ester group and the 2-methyl group. There are four *trans* isomers, since the halogen can be in the 4 or 5 position in either an axial or equatorial position. The most attractive isomer with an axial 4-chloro group is TML-C (McGovern et al., 1966, 1987), or more specifically one of its enantiomers, (1*S*,2*S*,4*R*)-TML-C (Sonnet et al., 1984; Doolittle et al., 1991). USDA specifications for commercial TML indicate a 38–45% TML-C content.

Another related parapheromone that is used to monitor the male medfly is ceralure (CER) (McGovern and Cunningham, 1987), ethyl 4- and 5-iodo-2-methylcyclohexane-1-carboxylate. Commercial CER is also a mixture of 90–95% *trans* isomers (McGovern et al., 1986), which are designated A, B1, B2, and C (McGovern and Beroza, 1966) as with TML. The most attractive isomer with an equatorial 5-iodo group is CER-B1 (Leonhardt et al., 1994, 1996; Avery et al., 1994; Warthen et al., 1994). USDA specifications for commercial CER require a 26% CER-B1 content.

Avery et al. (1994) investigated formulations in Hold Fast Stickem of 0.04–4.8 g TML or CER on yellow laminated foam boards (six-week study in Hawaii) and of 0.2–2 g TML or CER on yellow polyethylene panels (seven-week study in Guatemala). These investigations did not show the difference in persistence of the two parapheromones, but both delivery systems were effective for two to four weeks and attractive for six to seven weeks, and thus attracted better than the 2-g TML standard polymeric plug. A new controlled-release formulation of CER (15% by weight) with a commercial polymer (PDI Inc., Blain, Minnesota) was cast into thin rectangular molds. Each of these molds (2.5 × 1.87–10 × 5 cm) was placed on one side of plastic yellow panels

previously coated with Hold Fast Stickem. CER content was 0.18–0.25 g, but medfly captures were not recorded because of low populations of medflies.

Leonhardt et al. (1994) continued the investigation of TML and CER controlled release by developing a polyethylene matrix panel, but these panels became brittle on field exposure and gave low release rates of parapheromone. Another formulation with a polymer coating on cardboard panels proved to be stable in the field and gave high fly captures up to six weeks. They (Leonhardt et al., 1994) also tested Farma Tech International polymeric matrix panels, which contained 12.3 or 23.4 g of TML and were coated with Hold Fast Stickem. Fly captures from Hawaii showed that the panels were highly effective for 134 days. These panels were an alternative to the short-lived panel traps, which consisted of TML and Hold Fast Stickem spread on the surface of a plastic panel, used in California to delineate limits of known infestations. AgriSense polymeric matrix panels with 10 g TML or 10 g CER and coated with Hold Fast Stickem were also tested, but there was less medfly attraction with a slower rate of release than the Farma Tech panels.

Warthen et al. (1997) continued the study of various polymeric panels for the controlled release of TML, but with the introduction of the new three-panel C&C trap, which separated the center parapheromone panel from the two Hold Fast Stickem panels. The use of the 20-g TML polymeric TNO panel C was highly effective in delivering attractant doses for at least 18 weeks, resulting in fly capture to 6.3 times that of the standard 2-g TML AgriSense aged plug. Warthen et al. (1997) also showed the superiority of CER over TML as an attractant during a 13-week study with PVC panels, enveloped by Seabright bifold yellow cardboard coated with Hold Fast Stickem, containing 10 g CER.

The present study continues the development of these controlled-release polymeric panels by comparing larger doses of parapheromone using a 25-g TML–polymeric TNO panel C and a new formulation of 25-g CER–polymeric TNO panel C in the new C&C traps (Warthen et al., 1997). The mass trapping of released sterile male medflies under field conditions was carried out during a 26-week period, 48 days longer than the Farma Tech International 10-g TML and 10-g CER panel study by Leonhardt et al. (1994) and 57 days longer than the 20-g TML–polymeric TNO panel C study by Warthen et al. (1997). The catches were also compared to those for standard 2-g TML polymeric plugs in Jackson traps. Residuals of TML and CER in the panels were determined at various points of aging.

METHODS AND MATERIALS

Chemicals, Plugs, Panels, Traps. The TML (Lot 505010249), used in AgriSense plugs, was supplied by AgriSense (Fresno, California). TML (Lot

10292) used in the polymeric TNO panels (developed under license from TNO, The Netherlands), was supplied by Farma Tech International (Fresno, California). Both lots of TML met the USDA specifications of 38–45% w/w TML-C. The CER, supplied by AgriSense, in the polymeric TNO panels met USDA specifications of 26% w/w CER-B1. The composition of polymeric TNO panels, B and C, is proprietary. Standard Jackson traps (Harris et al., 1971) were used for the 2-g fresh and aged TML plugs. The polymeric TNO panels (15 × 15 cm) were used in C&C traps. This new trap is the invention of R. T. Cunningham and J. M. Cook (Farma Tech International). The device consists of a metal hook or hanger and a plastic holder, about the size of a chalkboard eraser (12.7 × 5 cm), that has three panels hanging down from the holder. The center polymeric TNO panel contains 25 g parapheromone. Two creamy white paperboard panels coated on both sides with Hold Fast Stickem (Seabright Laboratory, Emeryville, California) each hang down parallel to and 2.5 cm from either side of the polymeric TNO panel. All three panels slide neatly into the holder, making them easy to remove, inspect, and replace (Anonymous, 1996b). The paperboard panels were replaced weekly if the fly catch was high or cleaned by removal of flies if the fly catch was low.

Field Bioassay. A 26-week study was conducted in a mature *Macadamia integrifolia* Maiden & Betche orchard near Hilo, Hawaii, to compare CER in a TNO C panel to TML in a TNO C panel from September 1993 to March 1994. A TML panel B was also utilized for comparison. The amount of TML used in TNO panels B and C was 25 g, and the amount of CER used in panel C was 25 g. One parapheromone panel was used in each C&C trap. A reference treatment of 2 g TML (AgriSense plug) in a Jackson trap was included; the plug was replaced monthly. Another similar treatment was included for comparison, but was allowed to age and not replaced monthly. Treatments were hung from consecutive trees at 1.5 m above the ground and 20 m apart in a randomized complete-block design (Cochran and Cox, 1957). Blocks were arranged in two parallel rows and approximately 36,000 sterile adult male and female laboratory-reared flies (3–5 days old) were released for each bioassay period from holding box cages that were carried between the two rows of blocks.

Five replicates of each panel were carried out over 26 weeks and trap catches were counted weekly and recorded by the procedure of Rice et al. (1984). The untransformed data were subjected to ANOVA and Fisher's PSLD test (Fisher, 1966) (Table 1). Because of the climatic variations in field environment during the bioassay, the fly capture data were normalized by dividing the means of captures from aged parapheromone by the means of captures from the fresh polymeric plug (2 g TML) for each counting period and multiplying by 100 (Figure 1 below).

Residual Content Analysis. Small samples (2.5 cm^2) of the TML and CER panels C were taken on varying days during the aging process for GC analyses

TABLE 1. MEAN CATCH OF RELEASED MALE MEDITERRANEAN FRUIT FLIES WITH TRIMEDLURE[a] AND CERALURE[b] PANELS IN C&C TRAPS VERSUS STANDARD FRESH TRIMEDLURE IN JACKSON TRAPS (HILO, HAWAII)

	Mean[c] catch (±standard error) per trap for each parapheromone after indicated weeks aged in the field								
Lure	0–2	3–5	6–8	9–11	12–14	15–17	18–20	21–23	24–26
TML									
2.0 g, fresh[d]	81.4 ± 17.9c	95.0 ± 13.8b	97.2 ± 11.9b	87.1 ± 13.4b	101.9 ± 18.9c	107.1 ± 15.2b	152.6 ± 14.9b	107.6 ± 17.5b	131.3 ± 16.3c
2.0 g, aged[e]	66.3 ± 16.6c	95.3 ± 16.3b	85.3 ± 10.5b	42.6 ± 7.4b	32.3 ± 5.8c	12.8 ± 4.8c	24.7 ± 4.8c	20.3 ± 7.4c	17.7 ± 6.3d
25 g, panel B[f]	161.8 ± 19.7ab	188.7 ± 22.0a	363.8 ± 35.9a	410.3 ± 54.1a	343.2 ± 58.4ab	234.1 ± 45.4a	278.1 ± 25.0a	176.7 ± 23.6a	217.7 ± 19.2b
25 g, panel C[f]	138.8 ± 14.4ab	248.2 ± 36.9a	305.4 ± 38.0a	315.7 ± 37.9a	287.7 ± 51.6b	186.1 ± 25.1a	234.1 ± 26.1a	174.4 ± 29.5a	194.2 ± 12.9b
CER									
25 g, panel C[f]	122.4 ± 23.2b	191.9 ± 34.2a	383.4 ± 42.4a	412.9 ± 41.2a	431.1 ± 65.5ab	196.1 ± 41.6a	305.5 ± 51.5a	211.9 ± 28.1a	305.1 ± 28.7a
F	8.71	9.70	22.10	26.35	26.20	12.23	16.80	19.40	36.90
EMS	2718.86	6942.76	14298.41	17930.92	15983.43	9547.03	11469.19	4459.91	4650.23
P	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

[a]Trimedlure contained 40% (w/w) trimedlure-C isomer.
[b]Ceralure contained 26% (w/w) ceralure-B1 isomer.
[c]Means followed by the same letter within a column are not significantly different ($P > 0.05$; Fisher PLSD test), randomized complete block field plot design with five replicates. Statistics are on untransforemd data for all weeks.
[d]AgriSense plug in standard Jackson trap (replaced once/month).
[e]AgriSense plug in standard Jackson trap.
[f]Polymeric TNO panel (developed under license from TNO, Delft, The Netherlands, and supplied by Farma Tech) in C&C trap.

of residual parapheromone content. These samples were soaked in 600 ml of acetone and hexane (1:1 v/v) for one to three days before GC analyses. The residual parapheromone contents (Leonhardt et al., 1994, 1996) of the panels were then graphed (Figure 2 below).

RESULTS AND DISCUSSION

Table 1 shows the comparison of mean catches of male medflies where there were equal weights of TML and CER in panels C in C&C traps in relation to standard fresh and aged TML plugs in Jackson traps over a period of 26 weeks. These two panels, along with the TML panel B all showed significantly more fly catches than the fresh or aged TML plugs for weeks 0–26. Comparison of TML panel B with TML panel C showed no statistically significant differences. Comparison of TML panel C with CER panel C showed significantly more catches for CER only at 24–26 weeks.

Due to climatic variations in the field environment during the bioassay, normalized data are shown in the bar graph (Figure 1). The normalized mean male medfly catch was greater for CER than that for TML for the majority of the weeks aged.

Residual data plots (Figure 2) reveal that there is 2.75 times more CER than TML present in the C panel after 130 days. This larger amount of residual CER explains the greater catches than those with TML for normalized data (Figure 1). Exposure of a TML panel C for only 30 days (Figure 2) results in 10 g residual TML remaining, whereas exposure of a CER panel C for the same time period results in 16.8 g residual CER remaining. Residual CER amounts of 10 g occur in CER panels C only after 170 days of exposure. The added weight of the iodine atom contributing to the molecular weight of CER decreases its rate of release for an added benefit of persistence.

Although this research was conducted with equal weights of each parapheromone in the panels, this study's comparison is really one made between TML-C and CER-B1, the attractive isomers of TML and CER, respectively (Warthen et al., 1994; McGovern et al., 1990). Therefore, 40% of TML (25 g $\times$ 40% = 10 g TML-C) and 26% of CER (25 g $\times$ 26% = 6.5 g CER-B1) are compared here. Since there is a 21.6% less molar amount of CER in equal weights of the two isomers due to the larger molecular weight of CER, 10 g TML-C versus 6.5 g CER-B1 is a comparison of 0.043 mol versus 0.022 mol of attractive isomers, respectively. Therefore, there is approximately twice as much attractive isomer in the TML panel C (TML-C) as there is in the CER panel C (CER-B1) on a molar basis, yet the CER panel C catches at least as many flies as does the TML panel C. This demonstrates the potency and persistence of CER. If TML-C were used alone, less chemical would be needed.

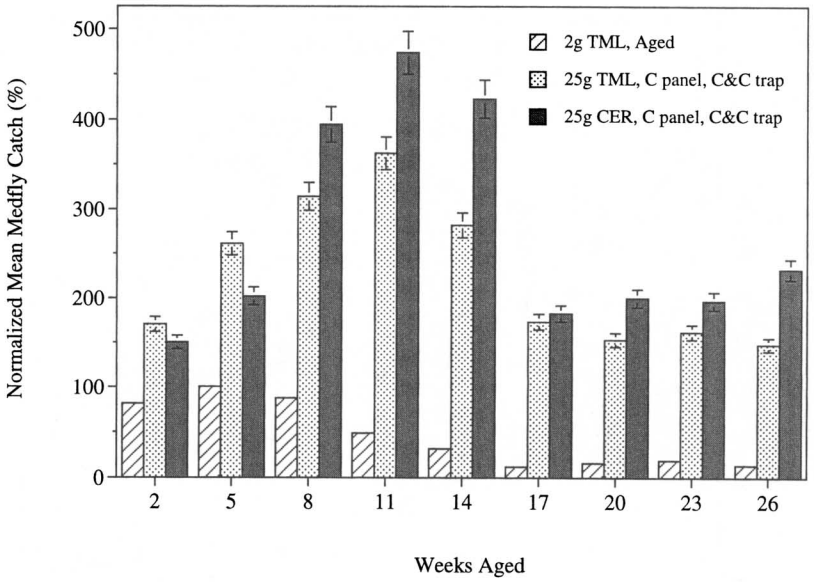

FIG. 1. Comparison of male Mediterranean fruit fly catch for 25-g TML panel C in C&C trap, 25-g CER panel C in C&C trap, and 2-g TML, aged AgriSense plug for 26 weeks. Data from Table 1: fly capture data were normalized by dividing the means of captures from aged parapheromone by the means of captures from the fresh polymeric plug (2 g TML) for each counting period and multiplying by 100; the 2-g TML fresh standard was replaced on the days of medfly catch determination.

If CER-B1 were used alone, the amount of chemical needed could be reduced by another 50% for the same attractancy level, assuming a linear dose–response curve. Use of the specific enantiomers that are attractive would reduce the amount of chemical needed by another 50%.

CONCLUSIONS

We conclude that a 25-g CER polymeric TNO panel C in a C&C trap is more effective than a similar TML panel in a C&C trap for mass capture and monitoring of male Mediterranean fruit flies for at least 26 weeks. The effectiveness is even more impressive considering that the presence of the attractive CER-B1 isomer in CER is one half the molar quantity of the attractive TML-C isomer in TML on an equal weight basis. The persistence of CER as an attractant is supported by the residual studies, which showed that 2.75 times

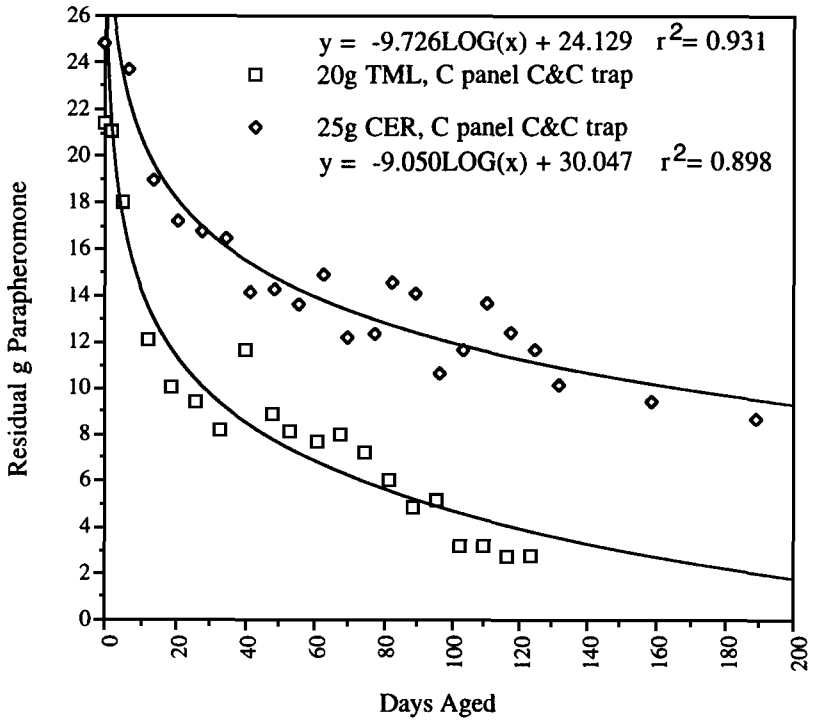

FIG. 2. Residual amount of TML in 20-g TML panel C and residual amount of CER in 20-g CER panel C (from Table 1) during 130 and 190 days of field exposure, respectively, as a logarithmic regression with correlation coefficient.

more CER is present in the C panel than TML at 130 days of exposure. We also conclude that the design of the C&C trap, which avoids placement of Hold Fast Stickem directly on the parapheromone panel and hence inference with controlled release, is critical since it allows two adjacent cardboard panels to carry the Hold Fast Stickem. The combination of this trap design and polymeric panel dispenser increases fly capture 6.4 and 5.2 times, for CER and TML, respectively, above that of the standard 2-g TML AgriSense aged plug over the 26-week period. The design of this trap also allows panels to slide neatly into the holder, making them easy to remove, inspect, and replace, thus permitting less labor-intensive surveillance. The comparison of CER to TML in polymeric TNO panels C in C&C traps provides new knowledge upon which future studies comparing equal molar quantities of racemic CER-B1 and TML-C, as well as their attractive enantiomers, must be carried out.

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VARIATION IN GROWTH, CHEMICAL DEFENSE, AND HERBIVORE RESISTANCE IN SCOTS PINE PROVENANCES

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Abstract—Variation in the growth, chemical defense, and susceptibility to monophagous and polyphagous sucking herbivores was assessed for four Scots pine (*Pinus sylvestris*) provenances in Petri dish and pot experiments. Seed provenances, Muonio (MU), Suomussalmi (SU), Korpilahti (KO), and Saaremaa (SA) represented a 1200-km N–S gradient. Scots pines from SU were least preferred as a host plant by pine-feeding aphids *Schizolachnus pineti* Fabr. and *Eulachnus agilis*, and in pot experiments, pines from SA were most preferred. *S. pineti* also produced the fewest nymphs on pines from SU. Polyphagous *Lygus rugulipennis* laid significantly fewer eggs on pines from MU provenance, which were smaller than seedlings from other provenances. The total and some individual terpene concentrations increased, while the total resin acid concentration decreased, towards the north. The absolute concentrations of total or individual terpenes and resin acids in the needles of unfavored SU plants did not differ significantly from the other provenances. Only the proportional quantity of dehydroabietic acid was higher in SU plants than in KO plants, and it correlated, together with sandaracopimaric acid, negatively with the number of *S. pineti* nymphs in the reproduction experiment. Conversely, absolute concentrations of some terpenes correlated negatively and some resin acids positively with the number of *S. pineti* nymphs. The length and weight of shoots decreased towards the north, adequately explaining the number of *Lygus* eggs and the final numbers of both aphids. The results suggest that seedling size is a better estimator of susceptibility of pine

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seedlings to both specialist and generalist sucking insects than relative or absolute concentrations of monoterpenes and resin acids.

Key Words—*Pinus sylvestris*, seed provenances, herbivore resistance, terpenes, resin acids, seedling growth, aphid, *Lygus* bug.

INTRODUCTION

Compared to the abundant literature on arthropod resistance of plants in managed systems, information on genetically based resistance in natural systems is limited (Kennedy and Barbour, 1992). In forest nurseries tree seedlings are grown in managed systems, but the seed used is usually from natural stands (Nerg et al., 1994) or seed orchards with open pollination. This has led to observations that certain conifer provenances are more attractive to insect herbivores than others (Schowalter and Stein, 1987).

Suitability of preferred host plants for oviposition is usually an indicator of better quality for the offspring (Leather, 1985). According to the plant vigor hypothesis, preferred host plants are larger and grow faster than average plants (Price, 1991). In addition, the growth–differentiation balance hypothesis predicts that actively growing tissues are less able to produce secondary metabolites than differentiated tissues, because growth processes precede differentiation processes (Herms and Mattson, 1992; Cronin and Hay, 1996). It also has been suggested that fast-growing plants and lower defense levels are favored under conditions of high resource availability (Coley et al., 1985). Reduced attractiveness of slow-growing conifers to insect herbivores could be due to their higher concentration of monoterpenes (Hrutfiord and Gara, 1989) or resin acids (Holopainen et al., 1995).

The chemical properties of conifer needles may affect host plant selection, oviposition, and reproduction of insect herbivores. Generalist herbivores are deterred by high concentrations of defense substances, but specialists use them to identify their food plants (Meijden, 1996). Thus, terpenoids are considered toxic or repellent compounds for herbivores, but they also can act as attractants, feeding stimulants, or oviposition cues (Leather, 1987; Gershenzon and Croteau, 1991). The site of terpenoid accumulation can have an important influence on their effectiveness against insect herbivores (Gershenzon and Croteau, 1991). It is also noteworthy that different parts of plants may differ in constitutive levels of chemical defense and also in the degree to which these defenses are inducible (Zangerl and Rutledge, 1996). Terpenoid composition shows large variability among plant organs (Gershenzon and Croteau, 1991) and between trees and is under strong genetic control (Hiltunen et al., 1975; Gref, 1987), but the relative proportions of different terpenoid constituents may be modified by environmental factors, light, water, and nutrients (e.g., Gleizes et al., 1980; Gref and Tenow,

1987; McCullough and Kulman, 1991). Nutrient deficiencies alter the carbon–nutrient balance (Bryant et al., 1983) in plants by reducing the rate of growth more than the rate of photosynthesis (Chapin, 1980). This carbon, in excess of that required for growth, is thought to provide an additional substrate for terpenoid biosynthesis. Geographical variation in terpene concentrations is remarkable (Juvonen and Hiltunen, 1972; Muona et al., 1986; Nerg et al., 1994), but resin acids do not show a strong geographical trend (Nerg et al., 1994).

The ratio of individual monoterpenes has been shown to be crucial for attractivity of pine needles to ovipositing insects. Leather (1987) proposed that pine beauty moths (*Panolis flammea* D. & S.) use the β-pinene–α-pinene ratio as a rough guide for selecting the correct host plant and then fine tune using other monoterpenes as indicators of suitability for egg-laying. Jactel et al. (1996) also concluded that *Dioryctria sylvestrella* Ratz. responded to mixtures or ratios of common pine volatiles, rather than to specific individual compounds.

The purpose of this study was to determine whether variation in growth and chemical defense exists in different Scots pine provenances, and if such variation is related to changes in insect herbivore performance. We compared growth and defense chemistry of seedlings and their acceptability to monophagous and polyphagous sucking insects using four provenances from a 1200-km N–S gradient (Nerg et al., 1994). Polyphagous *Lygus* bugs (Holopainen, 1986; Schowalter and Stein, 1987) and pine-feeding lachnid aphids (Carter and Maslen, 1982) that are able to damage young conifers by their feeding were used as model insects.

METHODS AND MATERIALS

Petri Dish Experiments. Scots pine (*Pinus sylvestris* L.) seeds were collected from three different geographical sites in Finland and one in Estonia along a 1200-km N–S gradient representing open pollinated natural stands. The four seed provenances were Muonio (MU) (latitude 67°56′), Suomussalmi (SU) (65°10′), Korpilahti (KO) (62°0′), and Saaremaa (SA) (58°22′). The weight of a thousand grains was 4.5 g, 4.0 g, 4.4 g, and 5.4 g, respectively. For host plant selection experiments with the grey pine aphid (*Schizolachnus pineti* Fabr.) and spotted pine aphid (*Eulachnus agilis* Kalt.), Scots pine seeds from the four provenances were placed on moist filter paper and kept in a growth chamber at 19°C/12°C (day/night) temperature, and under a 22L:2D photoperiod with maximum daytime irradiance about 360 μmol photons/m^2/sec, simulating the weather conditions typical of June in central Finland (Holopainen et al., 1995). After 12 days, four seedlings representing four different provenances were placed in 15 replicate plastic Petri dishes (diameter 14 cm) on moist filter paper with 20 apterous aphids that were released in the center of each dish. Needle sucking

aphids, which prior to experiments were feeding on 3-year-old Scots pines in the growth chamber, were obtained from a > 1-year-old laboratory colony. Two experiments with *S. pineti* and one experiment with *E. agilis* were conducted at room temperature (21°C) in the laboratory. Numbers of aphids feeding on different seedlings were observed five to eight times during a 22-hr observation period.

Experiments with Potted Seedlings. Scots pine seeds from the four provenances were sown in February and grown in the greenhouse at 23°C/18°C (day/night) temperature and with maximum daytime irradiance during sunny days of about 900 μmol/m^2/sec, and during cloudy days about 140 μmol photons/m^2/sec. After two months, seedlings were transplanted, in random order, in the corners of plastic 0.8-liter pots (filled with a 1 : 1 v/v mixture of peat and sand) so that only one seedling from each seed provenance was in the same pot. After a growing period of about 10 weeks in the greenhouse, host plant selection experiments with the spotted pine aphid (*E. agilis*) and the grey pine aphid (*S. pineti*) (same laboratory colony as described above) were conducted consecutively. There were twenty replicate pots and 12 aphids were placed in the center of each pot. Experiments were conducted at room temperature in the laboratory. Numbers of aphids feeding on different seedlings were observed five times during a 45-hr observation period.

For determination of reproductive rate of *S. pineti*, 27 replicate pots with 4 seedlings, one seedling from each provenance, were used, and two aphids (from the same laboratory colony as described above) were transferred to each seedling at the beginning. To prevent aphids moving from one seedling to another, seedlings were separated from each other with crossing paper barriers. One of the two aphids was removed after they had settled down within 24 hr. This experiment was conducted in the growth chamber. The numbers of nymphs produced were counted three times a week and a final aphid count was done seven weeks after the start of the experiment.

The oviposition preference of the tarnished plant bug (*Lygus rugulipennis* Popp) was determined after 14 weeks of growth. Eight replicate plastic containers (36 × 26 × 22 cm) with six replicate pots per container, each pot having four seedlings representing the studied provenances, were used. Into each container, three *L. rugulipennis* females and three males (collected from Scots pine stock in a forest nursery in central Finland) were released. Before the experiment, insects were kept on *Epilobium angustifolium* at 10°C. Containers were covered with nylon gauze and transferred to the growth chamber. The number of eggs laid on each seedling was counted after 12 days by using a dissecting microscope.

Chemical Analyses. Pine shoots from the reproduction experiment were collected in liquid nitrogen and stored at −80°C until analysis. Terpenes were

extracted from fresh pine needles with *n*-hexane (Kainulainen et al., 1992) for 1 hr. 1-Chlorooctane was used as an internal standard. Resin acids were extracted from freeze-dried needles following the procedures of Gref and Ericsson (1985). Heptadecanoic acid was used as an internal standard. Samples were analyzed by gas chromatography–mass spectrometry (Hewlett Packard GC-type 5890, MSD type 5970) by using a 30-m-long HP-5 capillary column (0.25 mm ID, 0.25-μm film thickness, Hewlett Packard) (Nerg et al., 1994).

Seedling Growth. The length of shoots and fresh and dry weights of shoots were determined after the host plant selection and oviposition pot experiments. The fresh and dry weight of roots was also measured after the host plant selection experiment.

Statistical Analyses. Log(n + 1)-transformed aphid numbers, log(n + 1) transformed egg numbers, concentrations of terpenes and resin acids, and seedling growth measurements were analyzed by one-way analysis of variance and the provenance means were compared with the Tukey HSD procedure. Correlation analyses were done by using Pearson correlation.

RESULTS

Petri Dish Experiments. In the first host plant selection experiment in Petri dishes, the final numbers of aphids feeding on seedlings were lowest on the SU provenance. However, no significant differences between provenances were found (Figure 1A and B). In the second experiment, plants from SU and SA were preferred significantly less by *S. pineti* than plants from KO (Figure 1C).

Experiment with Potted Seedlings. In the host plant selection experiment with potted seedlings, the final numbers of *E. agilis* were significantly higher in plants from SA than in plants from elsewhere (Figure 2A). *S. pineti* preferred plants from SU and MU significantly less than plants from SA and plants from SU less than plants from KO (Figure 2B). The final numbers of both aphids correlated positively with seedling biomass parameters (Table 1). In the reproduction experiment, the cumulative numbers of *S. pineti* nymphs were significantly lower in plants of SU provenance than in those of SA and KO provenances, after 24 days from the start of the experiment (Figure 3).

Seedling provenance had an effect ($F_{3,7} = 14.35$, $P < 0.001$, 1g(n + 1) transformed numbers) on number of eggs laid by *L. rugulipennis*. Almost equal amounts were laid on SA, KO, and SU provenance, but MU provenance was significantly less preferred as an oviposition site (Figure 4). The number of eggs per seedling was best explained by the shoot length of the seedlings [$r^2 = 0.5816$, $P < 0.001$, 1g(n + 1)-transformed numbers] (Figure 5), but there was also a significant positive correlation between eggs laid and dry weight of shoot ($r^2 = 0.1443$, $P < 0.001$).

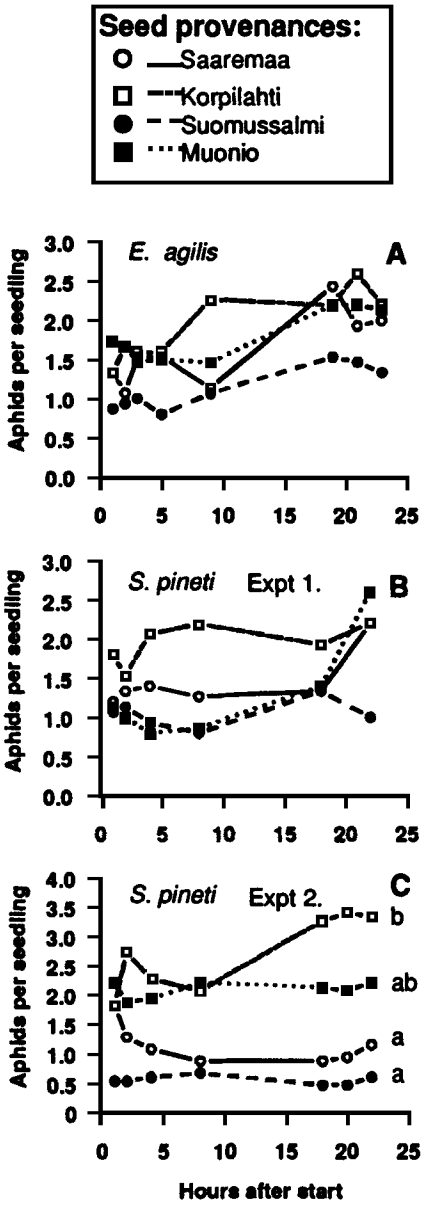

FIG. 1. Mean number of *E. agilis* (A) and *S. pineti* (B and C) settled to feed on Scots pine seedlings of different seed provenances in two different Petri dish experiments. Final observations followed by different letters differ significantly ($P < 0.05$) according to Tukey's test.

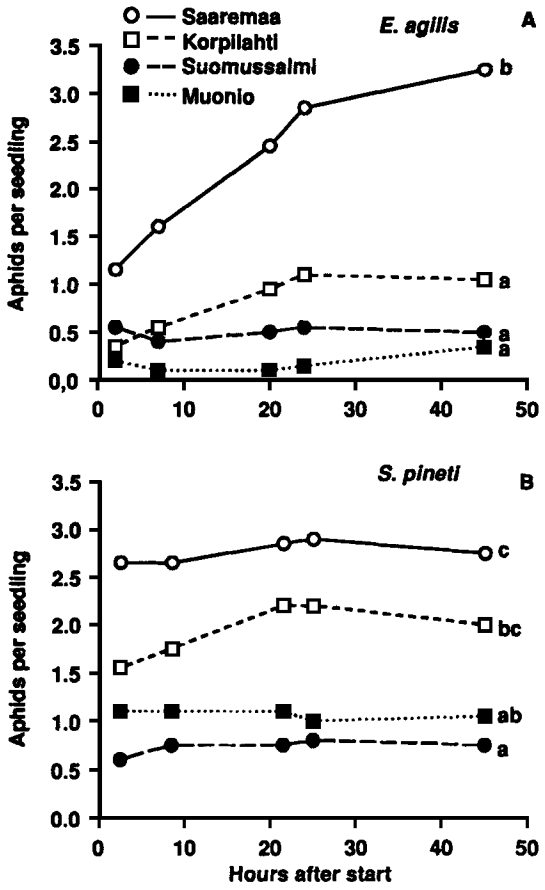

FIG. 2. Mean number of *E. agilis* (A) and *S. pineti* (B) settled to feed on Scots pine seedlings of different seed provenances in experiment with potted seedlings. Final observations followed by different letters differ significantly ($P < 0.05$; $N = 20$) according to Tukey's test.

The most common monoterpenes in pine needles were α-pinene and 3-carene. The total terpene concentration increased towards the north and was significantly higher in plants from MU than from SA (Figure 6). The concentrations of some individual monoterpenes, tricyclene, α-pinene, camphene, β-pinene, limonene, and bornylacetate increased towards the north. In contrast, the absolute quantity of 3-carene was not affected by seed provenance, but the proportional quantity of 3-carene decreased towards northern provenances. Simultaneously, the proportional quantity of α-pinene increased. Concentrations

TABLE 1. CORRELATION COEFFICIENTS AND THEIR SIGNIFICANCE BETWEEN BIOMASS PARAMETERS AND NUMBER OF *Schizolachnus pineti* AND *Eulachnus agilis* APHIDS IN POTTED SCOTS PINE SEEDLINGS IN HOST PLANT SELECTION EXPERIMENT (N = 52)

	S. pineti		*E. agilis*	
	r	P	r	P
Shoot length	0.400	<0.01	0.618	<0.001
Shoot dry weight	0.605	<0.001	0.633	<0.001
Seedling dry weight	0.576	<0.001	0.646	<0.001

of all individual terpenes and total terpenes correlated negatively with the number of *S. pineti* nymphs in the reproduction experiment (Table 2).

The most common resin acids in pine needles were abietic and dehydroabietic acids (Table 3). Total resin acid concentration decreased towards the north and was significantly lower in plants from MU than in plants from elsewhere (Figure 6). Of individual resin acids, the concentrations of palustric + levopimaric and neoabietic acids were significantly higher in plants from KO

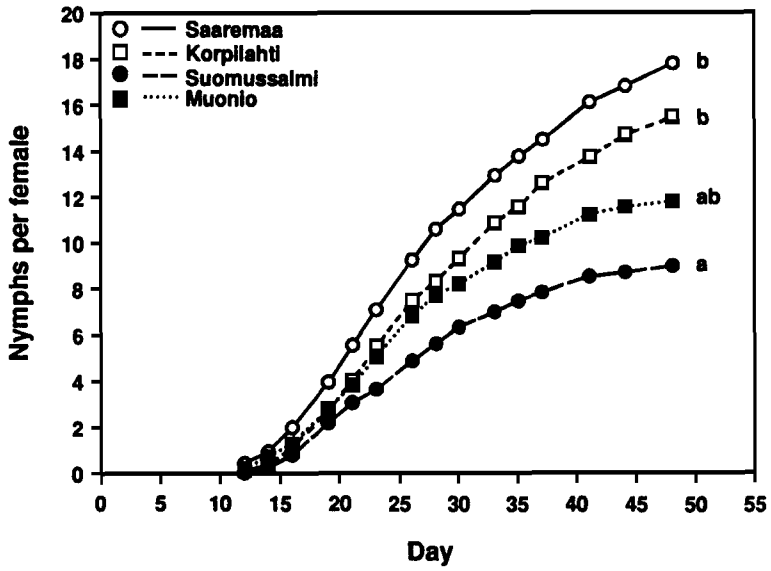

FIG. 3. Reproduction of *S. pineti* on Scots pine seedlings of different seed provenances. Cumulative observations followed by different letters differ significantly ($P < 0.05$; N = 27) according to Tukey's test.

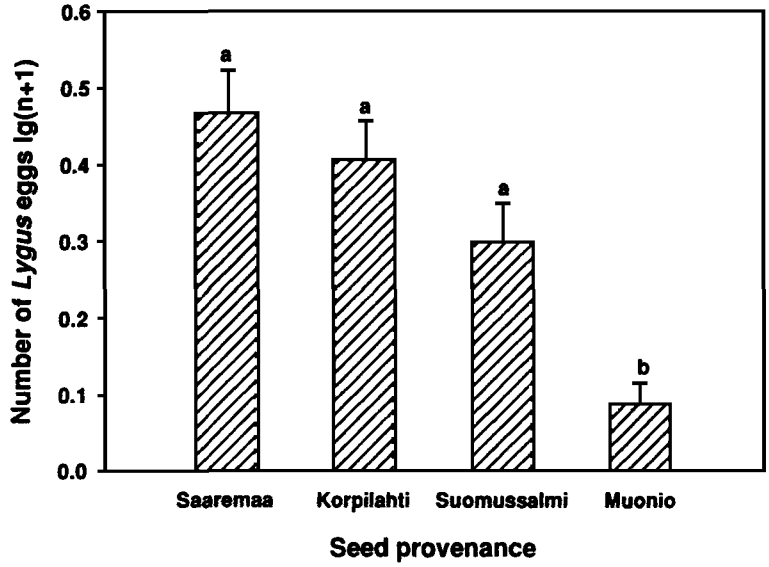

FIG. 4. Mean number (+SE) of eggs laid per Scots pine seedling of different seed provenances by three *L. rugulipennis* females. Bars indicated by different letters differ significantly ($P < 0.05$; $N = 48$) according to Tukey's test.

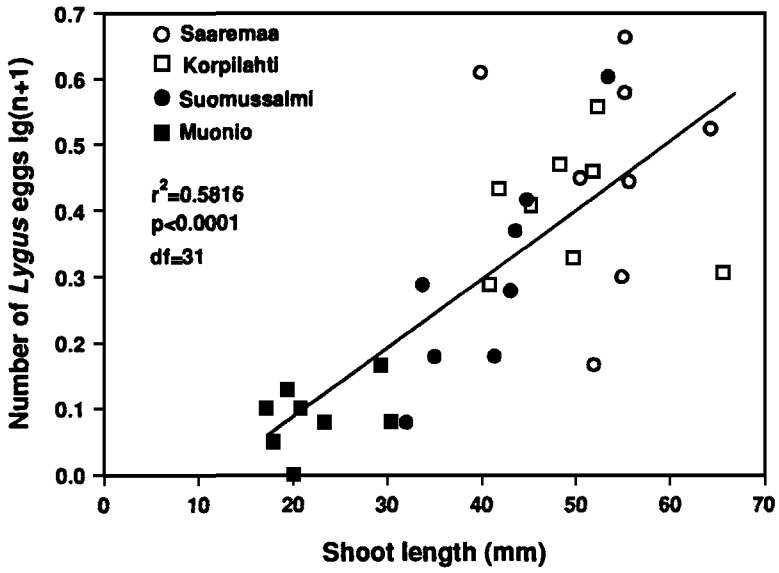

FIG. 5. The relationship between pine seedling shoot length and number of eggs laid by *L. rugulipennis*. Seed provenances are illustrated with different symbols.

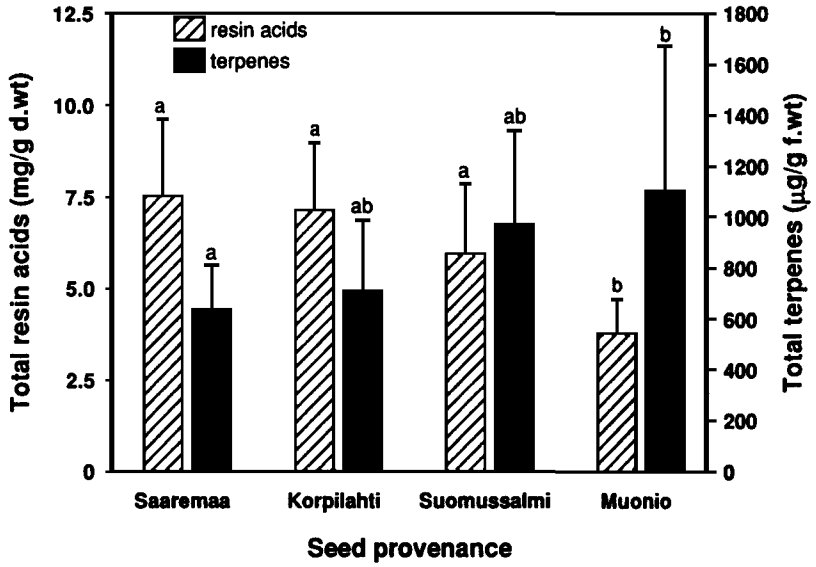

FIG. 6. Mean concentration (+SD) of total resin acids and terpenes in Scots pine needles of different seed provenances. Bars tapped by different letters differ significantly ($P < 0.05$; $N = 10$ for terpenes and $N = 8$ for resin acids) according to Tukey's test.

TABLE 2. CORRELATIONS OF ABSOLUTE CONCENTRATIONS OF INDIVIDUAL AND TOTAL TERPENES WITH TOTAL NUMBER OF NYMPHS PRODUCED BY *Schizolachnus pineti* [log(n + 1)-TRANSFORMED NUMBERS] AND THEIR SIGNIFICANCE ($N = 40$)

Compound	r	P
Tricyclene	−0.265	0.098
α-Pinene	−0.248	0.122
Camphene	−0.309	0.053
Sabinene	−0.036	0.824
β-Pinene	−0.321	0.043
Myrcene	−0.261	0.104
3-Carene	−0.031	0.852
Limonene	−0.388	0.013
Bornylacetate	−0.306	0.055
α-Copaene	−0.175	0.281
Longifolene	−0.266	0.098
β-Caryophyllene	−0.236	0.143
α-Humulene	−0.224	0.164
Total terpenes	−0.344	0.030

TABLE 3. MEANS OF PROPORTIONAL QUANTITY (%) OF DIFFERENT RESIN ACIDS IN PINE NEEDLES OF DIFFERENT SEED PROVENANCES[a]

Resin acid	Seed provenance			
	Saaremaa	Korpilahti	Suomussalmi	Muonio
Pimaric	0.50(0.42)	0.46(0.17)	0.35(0.24)	0.30(0.46)
Sandaracopimaric	3.20(0.51)a	3.13(0.64)a	3.72(0.47)a	4.72(0.34)b
Isopimaric	4.06(2.88)	5.91(5.31)	3.78(2.45)	3.01(1.41)
Palustric + levopimaric	9.35(2.57)	12.25(1.51)	11.74(7.21)	9.02(2.09)
Dehydroabietic	21.27(4.20)ab	18.78(5.43)a	26.47(7.98)b	37.09(3.91)c
Abietic	51.90(4.57)a	48.34(7.58)ac	40.88(12.97)bc	35.10(2.95)b
Neoabietic	9.72(2.71)	11.13(2.92)	13.06(8.01)	10.76(2.28)

[a]Standard deviations are in parentheses. Different letters in the same line indicate significant difference between provenances according to Tukey's test ($P < 0.05$).

than from MU, and abietic acid was significantly higher in plants from SA and KO than from MU. Conversely, the proportional quantities of sandaracopimaric and dehydroabietic acids were significantly higher in plants from MU than in plants from elsewhere (Table 3). Concentrations of primaric ($r = 0.346$, $P = 0.048$) and palustric + levopimaric ($r = 0.407$, $P = 0.019$) acids correlated significantly positively with the number of *S. pineti* nymphs in the reproduction experiment, but the proportional quantities of sandaracopimaric and dehydroabietic acids correlated significantly negatively with the number of *S. pineti* nymphs (Table 4).

The length of pine shoots was dependent on seed provenance ($F = 42.41$,

TABLE 4. CORRELATIONS OF PROPORTIONAL QUANTITIES OF RESIN ACIDS WITH TOTAL NUMBER OF NYMPHS PRODUCED BY *Schizolachnus pineti* [$\log(n + 1)$-TRANSFORMED NUMBERS] AND THEIR SIGNIFICANCE ($N = 33$)

Compound	r	P
Pimaric	0.296	0.095
Sandaracopimaric	−0.369	0.034
Isopimaric	0.074	0.684
Palustric + levopimaric	0.236	0.187
Dehydroabietic	−0.412	0.017
Abietic	0.246	0.167
Neoabietic	0.023	0.898

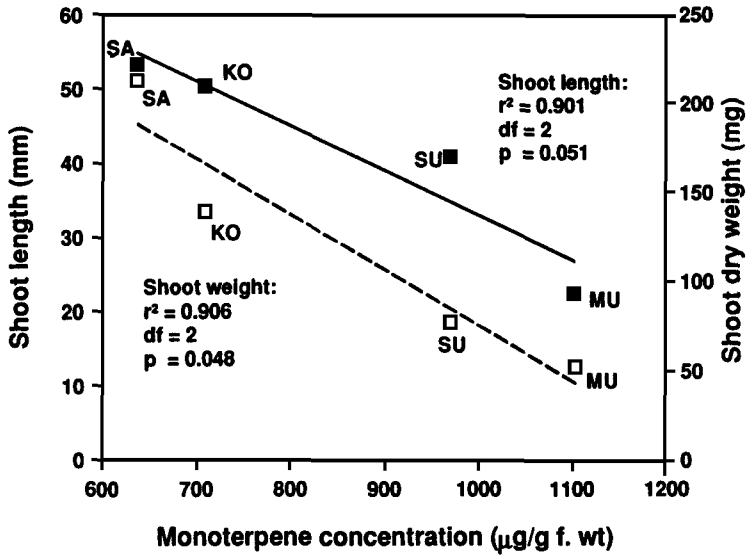

FIG. 7. The relationships between total terpene concentration and shoot length and dry weight. Provenances: SA = Saaremaa; KO = Korpilahti, SU = Suomussalmi, MU = Muonio. Filled squares = shoot length, open squares = shoot dry weight.

$P < 0.001$), and it decreased towards the north (Figure 7). Shoots were significantly longer in SA and KO plants than in SU and MU plants (Tukey, $P < 0.05$). In addition, shoots were significantly longer in plants from SU than in plants from MU. The dry weight of shoots also was dependent on seed provenance ($F = 81.98$, $P < 0.001$) and decreased towards the north (Figure 7). Dry weight of shoots was significantly higher in SA plants than in plants from elsewhere and also higher in KO plants than in SU and MU plants (Tukey, $P < 0.05$). The shoot length and shoot dry weight correlated negatively with the monoterpene concentration in pine needles (Figure 7) and positively with the resin acid concentration (shoot length: $r^2 = 0.994$, $P = 0.003$, $df = 2$; shoot dry weight: $r^2 = 0.779$, $P = 0.117$, $df = 2$). Root biomass was significantly higher in plants from SA than in plants from SU and MU (Tukey, $P < 0.05$).

DISCUSSION

Monophagous and polyphagous insect herbivores responded differently when they were offered a choice of Scots pine seedlings from different provenances over a 1200-km N–S gradient. Pine-feeding aphids selectively seemed

to avoid plants from SU, which originate from harsher conditions than would be expected from the geographical location. In the reproduction experiment, the monophagous aphid *S. pineti* produced fewer nymphs on SU plants, whereas the polyphagous *L. rugulipennis* did not discriminate among the three southern provenances as to oviposition site, with the most northern MU provenance being the least preferred.

Our results indicate that Scots pine needles have marked differences in chemical composition (terpenes and resin acids) among provenances, and herbivore performance is likely affected by this. Jactel et al. (1996) found that more volatile compounds may be more stimulating to herbivore performance; however, the effects of volatile monoterpenes on insect herbivore performance are quite variable. Some monoterpenes stimulate the oviposition of pine beauty moth *Panolis flammea* (Leather, 1987), but the concentrations of 3-carene in Scots pine needles did not affect sawfly success (Lyytikäinen, 1993). Monoterpenes had no effect on jack pine budworm larvae (McCullough et al., 1993). At the other extreme, *Pissodes strobi* prefers fast-growing spruce terminals with low myrcene content as an oviposition site (Hrutfiord and Gara, 1989). In the present study, total terpenes increased towards the north, and this may explain the lesser preference of polyphagous *L. rugulipennis* in the oviposition experiment. The results conform to the hypothesis of Meijden (1996) and support the study of Leigh et al. (1985), who found that *Lygus* bugs avoid puncturing plant structures that contain monoterpenes and sesquiterpenoids.

Tomlin and Borden (1996) found that the criteria for acceptance of a host for oviposition appear to be more stringent than for feeding. In our feeding experiments, high total or individual terpene concentrations in SU provenance plants did not explain the rejection of plants from this provenance by monophagous aphids. Plants from SA were most preferred by aphids in almost every experiment, and they had the lowest total terpene concentration. It appeared that all individual and total terpenes negatively affected the reproduction of *S. pineti*. Of the individual compounds, limonene and β-pinene had the strongest negative correlation with reproduction. Lindgren et al. (1996) found limonene to be toxic to pine weevils. In contrast, limonene and also longipinene and copaene were present in higher quantities and camphene in lower quantities in *Dioryctria sylvestrella*-attacked maritime pines than in unattacked trees (Jactel et al., 1996). In this study, high variation among trees in terpene concentration caused high deviation within seed provenances. Feeding of specialist aphids did not clearly respond to terpenes, and better reproduction of aphids on plants with low terpenes does not support the hypothesis of Meijden (1996).

Resin acids are deterrents for insect herbivore performance in many experiments. Sitka spruce trees resistant to *Pissodes strobi* had greater amounts of individual resin acids than susceptible trees (Tomlin et al., 1996). Tomlin et al. (1996) also concluded that different suites of resistance traits impart this resis-

tance among clones. Björkman et al. (1991) found a positive relationship between sawfly development time and resin acid concentrations, and Kainulainen et al. (1996) concluded that total resin acids can deter aphid performance. In this study, total resin acids were highest in SA provenance plants and decreased towards the north. Absolute concentrations of some resin acids had a positive correlation with aphid nymphs produced and could thus act like stimulants for aphid performance. The proportional quantities of sandaracopimaric and dehydroabietic acids revealed that they might act as repellents for *S. pineti* reproduction. In the study of Jactel et al. (1996), mixtures of secondary compounds were more important in determining herbivore performance than were individual compounds. Compared to terpenes, resin acids seemed to be more important in determining herbivore performance in this study.

Insect performance may be linked to several nutrients and/or secondary metabolites, which may also have combined effects (Clancy et al., 1988; Berenbaum, 1995). For example *L. rugulipennis* preferred pine seedlings with higher nitrogen content as an oviposition site (Holopainen et al., 1995), and sawfly success had a positive correlation with needle nitrogen content (Lyytikäinen, 1993). Kainulainen et al. (1996) found a positive correlation between the ratio of nitrogen to total resin acids and aphid growth rate. In this study, the nutritional quality of pine seedlings was not determined; however, the better nutritional quality of larger pine seedlings could explain the oviposition preference of *L. rugulipennis*. It is also possible that larger seedlings are easier targets for oviposition.

Seed size is usually a good, and often the most important, predictor of early growth of seedlings (Houssard and Escarré, 1991; Jurado and Westoby, 1992; Ouborg and Van Treuren, 1995). Light-seeded seedlings are likely to have lower final biomass than heavier-seeded seedlings, even though light-seeded seedlings can germinate sooner (Jurado and Westoby, 1992; Garnier and Freijsen, 1994). Horner and Abrahamson (1992) found that females of the gall-making herbivore *Eurosta* preferentially oviposited on plants with higher bud diameter, leaf area, and growth rate. In this study, plants of southern SA provenance had the highest seed weight and, as predicted, the highest seedling weight. On the other hand, the seeds were almost equal in size at KO and northern MU provenances, but in MU, pine seedlings grew slower and were smaller. It is known that plant species occupying slow-growth habitats tend to have naturally slow relative growth rate, even when grown in favorable conditions with higher nutrient availability (Chapin, 1980). The oviposition of polyphagous *L. rugulipennis* was not dependent on seed size, but in almost every aphid experiment, monophagous aphids performed most poorly on seedlings from SU provenance with small seed size. In addition, all measured seedling biomass parameters explained adequately the final numbers of both aphids in pot experiment. Because of short and regular distances between seedlings in pots in the host plant selection

experiment, aphids should have been able to settle on any seedling with equal probability, and this was not observed.

L. rugulipennis favored southern provenance plants, which had larger shoots, lower total terpene, and higher total resin acid concentration. However, the proportional quantities of some individual resin acids were lower in southern than in northern provenance plants, and this might make seedlings more suitable for oviposition. The potentially better nutritional quality of bigger seedlings could make them more suitable for oviposition. Results from our aphid experiments were not clear. Biomass measurements explained aphid performance, but it appeared that seed size explained the suitability of different provenances for aphids. Deviating terpene or resin acid concentrations do not explain the rejection of SU provenance plants. The reason for this rejection remains unclear. It is important to note that the proportional quantities of sandaracopimaric and dehydroabietic acids were repellents to *S. pineti* reproduction. Total and individual terpenes negatively affected aphid reproduction as well.

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ESSENTIAL AMINO ACID COMPOSITION OF FLESHY FRUITS VERSUS MAINTENANCE REQUIREMENTS OF PASSERINE BIRDS

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Abstract—Nutritional analyses were performed on 27 fruit species that are eaten by frugivorous birds in east Mediterranean habitats in Israel. The essential amino acid (EAA) profile [compared by principal component analysis (PCA)] of these fruits indicated two distinct groups of fruits. The main group consisted of 23 species that were similar in their relatively low total EAA quantities and imbalanced EAA profiles. On average, the EAAs phenylalanine and tyrosine were most concentrated and histidine least (tryptophan was not measured). Comparing the relative amounts of EAAs in fruits with required amounts for maintenance of granivorous passerine birds revealed that these fruits are deficient in all or most EAAs. The sulfur-containing amino acids (methionine and cystine) were most limiting relative to the required amount (41–61% below requirement), while four others (arginine, lysine, isoleucine, and leucine) were severely deficient (30–37% below requirement). These results complement reports suggesting that frugivorous birds have lower total protein demands than granivores. The second group of four fruit species had relatively high total EAA contents. Each of these fruits appeared to contain especially large quantities of some EAAs, but it was uncertain whether some high concentrations could have resulted from interactions in the pulp during preparation and chemical analysis.

Key Words—Frugivory, essential amino acids, protein, fleshy fruits, nutrition, secondary compounds, plant–animal interactions, passerines.

INTRODUCTION

There is evidence that frugivorous birds are unable to subsist on a diet consisting of only one or several species of fleshy fruits (Berthold, 1976; Izhaki and Safriel, 1989; Izhaki, 1992). Yet, other studies have indicated that the consumption of

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several fruit species together may be adequate for maintaining body mass (Simons and Bairlein, 1990; Herrera et al., 1994; Bairlein, 1996). Protein deficiency has often been suggested to explain body mass loss and negative nitrogen balance of frugivores in cage experiments (Moss and Parkinson, 1975; Berthold, 1976; Bairlein, 1987; Levey and Karasov, 1989; Sedinger, 1990). The ability of frugivorous birds to maintain a positive nitrogen balance, however, is not solely a function of the absolute quantity of pulp protein (Izhaki, 1992).

Crude protein contents are known for hundreds of wild fleshy fruits (see review in Jordano, 1992). However, positive nitrogen balance is not solely a function of crude protein content. Most crude protein values are derived by Kjeldahl analysis. Since there is a large nonprotein nitrogen component in many forages (Holt and Sosulski, 1981; Sedinger, 1984), including ripe fleshy fruits (Izhaki, 1993), the true protein contents of many fruits as calculated by the Kjeldahl method are probably overestimated (Milton and Dintzis, 1981; Izhaki, 1993). Moreover, many of the nonprotein nitrogen components of fruits are secondary compounds that may interfere with protein metabolism (Izhaki and Safriel, 1989). Finally, the quality of dietary protein is a function of amino acid profile, a character not measured by Kjeldahl analysis (Milton and Dintzis, 1981; Izhaki, 1993).

Ten amino acids are essential for birds because they cannot be synthesized in sufficient quantities to meet cellular requirements and must be acquired from the diet (Robbins, 1993; Murphy, 1996). The quality of protein in the diet is largely dependent on the concentrations of these essential amino acids (EAAs) relative to an animal's requirement. Fruit pulp may lack one or more amino acids, as demonstrated by Pannell and Koziol (1987). Moreover, protein utilization by an animal depends upon how closely, after correcting for digestibility, the proportions of the essential amino acids the protein provides correspond with the proportional amino acid requirements of the animal (Baker, 1977). Although the body is able to compensate for slight deficiencies of some amino acids, the amino acid balance is highly critical and makes the organism sensitive to alterations in the amino acid pattern of the diet (Harper, 1964).

Little information is available about the amino acid composition of wild fleshy fruits (Herbst, 1986; Pannell and Koziol, 1987; Izhaki, 1993) or amino acid requirements of frugivores. EAA requirements have been studied extensively in commercially important fowl (National Research Council, 1984), and in passerine birds they have been investigated recently by Murphy (1993, 1996), who measured the minimum EAA requirements of a wild granivorous passerine, the white-crowned sparrow (*Zonotrichia leucophrys gambelii*). Maintenance requirements of specific EAAs have been reported for two other passerine species, *Junco hyemalis* (Parrish and Martin, 1977; Westerhaus, 1983; Merritt, 1986) and *Melopsittacus undulatus* (Earle and Clark, 1991). These results are summarized in Murphy (1996).

The objectives of this paper are to: (1) characterize EAA composition of fleshy fruits and (2) compare the maintenance requirements of EAAs of granivorous passerine birds to EAA availability in fleshy fruits. It should be emphasized that there is evidence that frugivores have lower protein requirements than granivores (Bairlein, 1991; Izhaki 1992; Murphy 1993; Bairlein and Gwinner; 1994). Unfortunately no data are available on EAA requirements of frugivores, so the comparison of the EAA profile of fruits with the requirements of granivores is the best approximation available for passerine birds.

METHODS AND MATERIAL

Fruit Species. Amino acid contents of 27 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, 7 shrubs, 7 climbers, and 2 herbaceous plants. 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.

Amino Acid Analysis. Fresh ripe fruits were collected in the field, the seeds were removed, and the pulp was oven dried at 40°C to constant mass. The dried pulp was ground to powder, and 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. Here I report only the results for the EAAs. No analysis was made for tryptophan because it is destroyed by acid hydrolysis (Gehrke et al., 1985). I included concentrations of phenylalanine + tyrosine and methionine + cystine in analyses because the nutritional requirement of the former within each pair can be partially compensated for by the latter (National Research Council, 1984). Detailed results that specify the full amino acid content for each fruit species (excluding *Solanum nigrum*) were published earlier (Izhaki, 1993).

The average EAA composition was compared with EAA requirements for maintenance of the white-crowned Sparrow (*Zonotrichia leucophrys gambelii*) (Murphy, 1993, 1996) to determine percent deficiency. These EAA requirements were based on diets containing 10.75–12.5 kJ apparent metabolizable energy (AME) per gram. In a previous study, it was found that a diet of three fruit species (*Rubia tenuifolia*, *Rhamnus alaternus*, and *Ephedra aphylla*) contained 12.3 kJ AME (Izhaki, 1992) and the comparison seemed valid.

To detect the total value of each fruit species in a balanced EAA diet, I calculated a compatibility index (C, Table 3 below) by averaging the differences between EAA requirements (*AAR*) and their content (*AAC*) as follows:

$$C = 1/n \sum_{N}^{i=1} \frac{AAR - AAC}{AAR}$$

where N is the total number of analyzed EAAs. This index gives equal weight to each of the EAAs regardless of their different importance and role in bird metabolism. Therefore, I have used this index for ranking fruit species rather than an absolute value of EAA composition.

Statistical Analysis. Principal component analysis (PCA) was performed to examine relationships among EAAs in the fruit pulp with a correlation matrix (SAS, 1988). The objective of PCA is to reduce the number of independent variables and to explain variation observed in the data with fewer dimensions. The raw input data included the EAA composition (milligrams of EAAs in 100 g dry pulp) in each of the 27 analyzed fruit species (see Table 4 below).

RESULTS AND DISCUSSION

Amino Acid Composition Pattern of Wild Fruits. The EAA profile of the 27 fruit species assayed in this study is summarized in Table 1 and Figure 1. The eigenvalues from PCA indicate that the first component accounted for 86.5%

TABLE 1. PRINCIPAL COMPONENT ANALYSIS OF CORRELATION MATRIX OF ESSENTIAL AMINO ACID CONTENT OF 27 WILD FRUIT SPECIES COLLECTED IN NORTHERN ISRAEL[a]

	Component		
	I	II	III
Eigenvalue	7.79	0.84	0.23
% of variation	86.5	9.5	2.5
Cumulative %	86.5	96.0	98.5
Variable			
Arginine	0.30	−0.46	0.61
Histidine	0.34		−0.52
Lysine	0.34		
Isoleucine	0.35		
Leucine	0.35		
Valine	0.33	0.45	
Methionine + cystine	0.35		−0.42
Phenylalanine + tyrosine		0.66	
Threonine	0.35		

[a]Only loading scores > 0.3 are shown.

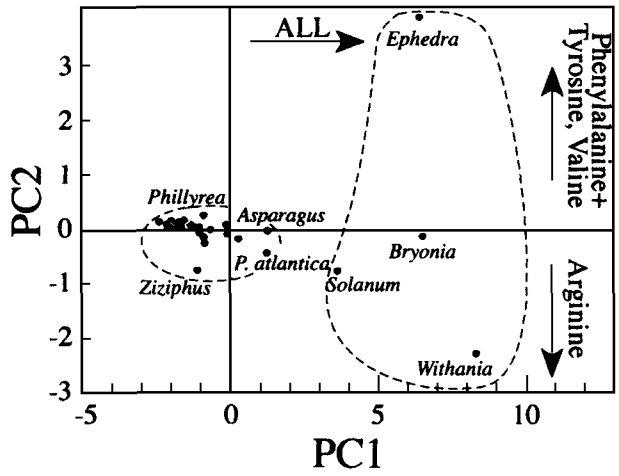

FIG. 1. Scatterplot of the first two components from principal component analysis of essential amino acid content of 27 wild fruit species collected in northern Israel. Detailed list of analyzed species and their EAAs content are given in Table 4.

of the standardized variance, while the second component accounted for only 9.5%. Thus, the first two components explain 96% of the variance (Table 1). The first component is a measure of total EAAs since the first eigenvector had high negative loadings on arginine and high positive loadings on phenylalanine + tyrosine and valine.

The principal component diagram demonstrates that two distinctive groups of fruits may be defined. Group 1 includes 23 fruit species that are relatively similar in their EAA composition (Figure 1). Most of this cluster is located along the negative scale of PC1, indicating relatively low total EAAs (Table 1, Figure 1). *Arbutus andrachne* has the lowest value on this axis but several other species had low values also. *Pistacia atlantica* and *Asparagus aphyllus* fruits are relatively rich in total EAAs in this group (Figure 1). Group 2 includes four species (*Ephedra aphylla*, *Bryonia* spp., *Withania somnifera*, and *Solanum nigrum*), and these have high values for PC1, indicating a relatively high total EAA content. *E. aphylla* appears on the upper scale in PC2, indicating high amounts of phenylalanine + tyrosine and valine. *W. somnifera* has negative values on PC2, indicating a high content of arginine (Figure 1). The average coefficients of variation of all EAAs in group 1 and in group 2 are much lower than for all fruits combined (54%, 47%, and 102%, respectively), indicating that fruit species within each of the two distinctive groups are more similar in their EAA composition pattern than those in all fruits combined.

On average, the most common EAA in ripe fruit pulps was phenylalanine + tyrosine, and histidine was the rarest (Table 2). Tryptophan was not mea-

TABLE 2. AVERAGE AMINO ACID CONTENT (% OF DRY MASS ± SD) OF ALL STUDIED FRUIT SPECIES IN ISRAEL, 23 FLESHY FRUIT SPECIES (GROUP 1), AND GROUP 2[a] FRUITS[b]

Amino acid	Requirements for passerine maintenance	All-fruits ($N = 27$)		Group 1 ($N = 23$)		Group 2 ($N = 4$)	
		Content	% difference	Content	% difference	Content	% difference
Arginine	0.28	0.25 ± 0.26	−11	0.18 ± 0.12	−36	0.69 ± 0.43	146
Histidine	0.08	0.09 ± 0.09	13	0.06 ± 0.03	−25	0.28 ± 0.08	250
Lysine	0.21	0.20 ± 0.20	−5	0.14 ± 0.09	−36	0.60 ± 0.22	186
Isoleucine	0.19	0.18 ± 0.16	−6	0.12 ± 0.06	−37	0.52 ± 0.13	174
Leucine	0.27	0.28 ± 0.23	4	0.19 ± 0.09	−30	0.78 ± 0.16	189
Valine	0.17	0.25 ± 0.27	47	0.16 ± 0.08	−6	0.80 ± 0.34	371
Methionine + cystine	0.27	0.16 ± 0.15	−41	0.10 ± 0.06	−63	0.48 ± 0.13	78
Phenylalanine + tyrosine	0.28	0.45 ± 0.67	61	0.25 ± 0.13	−11	1.64 ± 1.25	485
Threonine	0.13	0.17 ± 0.15	31	0.11 ± 0.05	−15	0.49 ± 0.14	277
Tryptophan	0 − 0.05						

[a] *Ephedra aphylla*, *Bryonia* spp., *Withania somnifera*, *Solanum nigrum*.
[b] The dietary amino acid requirements (% diet) for maintenance of *Zonotrichia leucophrys* adults (after Murphy, 1996) are compared with these contents and appear as % difference between content and requirement.

sured. Since histidine was not detected in any of 10 tropical fruit species assayed by Pannell and Koziol (1987) and was relatively rare in five neotropical fruit species assayed by Herbst (1986), it seems that a shortage of histidine is a general feature of fleshy fruits.

EAA Requirements for Maintenance vs. Content in Ripe Fruits. Comparing the relative amounts of EAAs with required amounts for granivorous passerine birds revealed that the fruits of group 1, on average, are deficient in EAAs (Table 2). Thirteen plant species were deficient in all EAAs and six in five to eight EAAs. Of the 10 EAAs, the sulfur-containing amino acids (methionine + cystine) were the most limiting, relative to the required amount (41–61% below requirement). Four other EAAs (arginine, lysine, isoleucine, leucine) were severely deficient (30–37% below requirement, Table 2). Methionine and lysine also were found to be the most limiting amino acids in fruit consumed by frugivorous bats (Herbst, 1986).

In addition to the shortage in the absolute amount of certain EAAs, fruits may provide an imbalanced profile of EAAs. The index of compatibility revealed that three fruit species (*Arum dioscoridis*, *Pistacia palaestina*, and *Larus nobilis*) provide the most balanced EAA diet ($C < 3\%$), indicating a relatively small average difference between requirement and availability of all EAAs (Table 3). Although no data exist on EAA demands of frugivores, it seems from the present study that granivorous passerines probably could not subsist solely on any one of these fruits since they are all deficient in several EAAs. The deficiency of a single EAA may impede the use of other EAAs that are in adequate supply. Thus, in avian species, deficiency of any single EAA may lead to a reduced rate of protein synthesis and possibly net protein degradation in the whole body (Muramatsu, 1990).

Furthermore, it has been suggested that moderate amino acid imbalances can be overcome by increasing the dietary protein content. The organism is particularly sensitive to small changes in amino acid balance when the total protein content of the diet is low (Harper, 1964). Indeed, this seems to be the case in the fruits of group 1 because their average protein content (Table 3) is only $2.6\% \pm 1.3\%$.

Bairlein (1987, 1996) reported that frugivores fed on very low protein fruits lost weight immediately at the beginning of trials, but in some cases later maintained a constant mass. It is still unclear if the fruit species that were used in these cases supplied a more balanced EAA diet than in cases where the birds could not maintain their body mass.

How Do Frugivorous Birds Satisfy Their EAA Demands in the Wild? It is expected that totally frugivorous species would face great difficulty subsisting on a single fruit species, since most of fruits appear deficient in one or more EAA and/or supply an imbalanced EAA diet. Birds probably cannot eat more of the same fruit species to compensate for its inadequacies. First, the con-

TABLE 3. ANALYSIS OF PROTEIN, EAAs, NONPROTEIN NITROGEN COMPOUNDS, AND COMPATIBILITY INDEX OF PULPS OF 27 FRUIT SPECIES FROM NORTHERN ISRAEL

Plant species	Protein (% dry weight)[a]	EAA amount (% dry weight)[b]	Nonprotein N[c]	Compatibility index (C)[d]
Ephedra aphylla	14.5	7.9	0.30	3.04
Withania somnifera	14.2	7.0	0.20	2.88
Bryonia spp.	12.0	6.3	0.23	2.41
Solanum nigrum	25.4	3.9		1.24
Pistacia atlantica	4.9	2.7	0.41	0.49
Asparagus aphyllus	6.0	2.7	0.18	0.49
Pistacia lentiscus	4.0	2.0	0.23	0.14
Arum dioscoridis	4.2	1.9	0.42	0.027
Pistacia palaestina	3.5	1.8	0.45	0.013
Larus nobilis	3.4	1.8	0.26	−0.008
Smilax aspera	3.0	1.5	0.33	−0.17
Phillyrea latifolia	2.8	1.4	0.25	−0.26
Ruscus aculeatus	2.9	1.4	0.47	−0.26
Rosa canina	2.9	1.3	0.42	−0.27
Osyris alba	2.4	1.3	0.63	−0.31
Viburnum tinus	2.3	1.2	0.18	−0.34
Zizphus spina-christi	2.8	1.3	0.41	−0.35
Tamus orientalis	2.4	1.2	0.50	−0.37
Rhus coriaria	2.0	1.1	0.33	−0.42
Lonicera etrusca	1.9	0.9	0.45	−0.5
Rubia tenuifolia	1.8	0.8	0.31	−0.56
Myrtus communis	1.4	0.7	0.33	−0.59
Crataegus monogyna	1.3	0.7	0.42	−0.62
Rhamnus alaternus	1.4	0.7	0.39	−0.63
Rhamnus lycioides	1.5	0.7	0.52	−0.63
Styrax officinalis	1.2	0.5	0.48	−0.7
Arbutus andrachne	0.7	0.4	0.26	−0.8

[a] Total amino acids (from Izhaki, 1993).
[b] Total EAAs (for detailed EAA content, see Table 4).
[c] (% total Kjeldahl N − % amino acid N)/% total Kjeldahl N (from Izhaki, 1993).
[d] Average differences between EAAs requirements (*AAR*) and their content (*AAC*) calculated as follows:

$$C = 1/n \sum_{N}^{i=1} \frac{AAR - AAC}{AAR}$$

where N is the total number of analyzed EAAs.

sumption rate of fruit-eating birds is constrained by gut-emptying rate (Levey, 1987). Second, amino acid deficiency may cause a prompt reduction in food intake to protect against a high rate of urea production (Rogers and Leung, 1973). Thus, a bird likely would not eat a lot of an amino acid-deficient fruit to compensate for a missing amino acid. However, there is evidence that frugivores differ from nonfrugivores in the way they deal with the particular nutritional quality of fruits and that different species may have different protein requirements (Bairlein, 1991; Bairlein and Gwinner, 1994).

The problems of shortage and imbalance in EAAs apparently is overcome in nature through diet selection. The consumption of relatively small amounts of several different fruit types with dissimilar amino acid patterns can provide an adequate overall nutritional balance for maintenance. Foraging behavior of this kind may help the bird avoid toxicity that could be caused by accumulating large amounts of specific secondary compounds (Mack, 1990). However, severe time constraints can operate on such a foraging strategy for EAA complementation. Therefore, it is more probable that small animals complement food sources of varying balanced protein content (Murphy and Pearcy, 1993; Murphy 1994, 1996). Furthermore, frugivores may be able to select for the most rewarding fruit species in terms of demands for particular nutrients (Bairlein, 1991; Bairlein and Gwinner, 1994).

Nutritional requirements of wild birds fluctuate throughout their annual cycles (Murphy, 1994). The EAA composition of fleshy fruits would be even less sufficient for laying birds, when protein and EAA demands are higher (Murphy, 1996). This is the season when birds are susceptible to malnutrition and potential amino acid deficiencies. However, most passerine birds in the east Mediterranean ecosystems breed during spring when fruits are rare (Izhaki, 1986) and consume insects as their main protein source. An alternative explanation is that they do not consume fruits during the breeding season simply because there are no fruits available (Izhaki and Safriel, 1985). Therefore, a strong line of evidence comes from the tropics, where both fruits and insects are available year-round. If EAAs are not limiting in tropical fruits, one would expect highly frugivorous birds to breed during the peak season of fruit abundances. This is not the case, however, as these birds actually breed during the period of fruit scarcity (Levey 1988, Loiselle and Blake, 1991). Interestingly, this period apparently corresponds with the time of peak insect abundance (Janzen, 1983).

Thus, dietary preferences change during the various seasons, and invertebrates become increasingly important as a nutrient source during the spring breeding season in most passerine birds (Parrish and Martin, 1977; Izhaki and Safriel, 1985). By including insects in their diet, birds can compensate for the EAA deficiency of fruits. For example, birds may obtain sufficient lysine from

TABLE 4. TOTAL PULP EAA COMPOSITION OF 27 FRUIT SPECIES FROM EAST MEDITERRANEAN HABITATS (% DRY WEIGHT)

Plant species	Arginine	Histidine	Lysine	Isoleucine	Leucine	Valine	Methionine + cystine	Phenylalanine + tyrosine	Threonine
Ephedra aphylla (Ephedraceae)	0.302	0.226	0.430	0.478	0.850	1.250	0.410	3.459	0.461
Osyris alba (Santalaceae)	0.193	0.064	0.116	0.110	0.194	0.147	0.095	0.222	0.111
Larus nobilis (Lauraceae)	0.181	0.083	0.238	0.163	0.279	0.216	0.163	0.305	0.163
Rosa canina (Rosaceae)	0.209	0.081	0.128	0.107	0.166	0.145	0.100	0.233	0.126
Crataegus monogyna (Rosaceae)	0.070	0.033	0.085	0.057	0.103	0.088	0.048	0.120	0.068
Pistacia lentiscus (Anacardiaceae)	0.246	0.116	0.275	0.176	0.301	0.222	0.155	0.375	0.174
Pistacia atlantica (Anacardiaceae)	0.374	0.147	0.378	0.220	0.356	0.298	0.210	0.534	0.199
Pistacia palaestina (Anacardiaceae)	0.195	0.094	0.238	0.151	0.247	0.234	0.159	0.356	0.151
Rhus coriaria (Anacardiaceae)	0.104	0.045	0.136	0.101	0.173	0.121	0.077	0.214	0.095
Rhamnus alaternus (Rhamnaceae)	0.064	0.034	0.056	0.069	0.119	0.083	0.055	0.123	0.062
Rhamnus lycioides (Rhamnaceae)	0.066	0.032	0.068	0.050	0.117	0.093	0.048	0.122	0.068
Zizphus spina-christi (Rhamnaceae)	0.541	0.046	0.109	0.072	0.143	0.097	0.058	0.152	0.078
Myrtus communis (Myrtaceae)	0.079	0.039	0.086	0.063	0.117	0.083	0.063	0.140	0.066
Arbutus andrachne (Ericaceae)	0.041	0.016	0.037	0.037	0.057	0.050	0.027	0.058	0.036
Styrax officinalis (Styracaceae)	0.056	0.032	0.051	0.034	0.080	0.069	0.062	0.087	0.051
Phillyrea latifolia (Oleaceae)	0.166	0.052	0.032	0.155	0.247	0.178	0.104	0.291	0.140
Solanum nigrum (Solanaceae)	0.366	0.289	0.486	0.357	0.547	0.447	0.473	0.643	0.309
Withania somnifera (Solanaceae)	1.213	0.389	0.911	0.584	0.866	0.663	0.668	1.132	0.612
Rubia tenuifolia (Rubiaceae)	0.089	0.037	0.059	0.084	0.122	0.108	0.062	0.161	0.075
Viburnum tinus (Caprifoliaceae)	0.131	0.050	0.141	0.110	0.197	0.140	0.106	0.221	0.114
Lonicera etrusca (Caprifoliaceae)	0.108	0.033	0.052	0.107	0.159	0.110	0.066	0.190	0.095
Bryonia spp. (Cucurbitaceae)	0.892	0.208	0.576	0.663	0.868	0.841	0.384	1.315	0.573
Asparagus aphyllus (Liliaceae)	0.299	0.115	0.265	0.268	0.395	0.327	0.248	0.570	0.250
Ruscus aculeatus (Liliaceae)	0.263	0.069	0.155	0.128	0.150	0.157	0.095	0.220	0.113
Smilax aspera (Liliaceae)	0.191	0.071	0.161	0.129	0.214	0.168	0.125	0.346	0.131
Tamus orientalis (Dioscoreacea)	0.179	0.044	0.084	0.110	0.178	0.143	0.080	0.230	0.111
Arum dioscoridis (Araceae)	0.279	0.073	0.114	0.169	0.285	0.276	0.156	0.366	0.174

lysine-rich invertebrates that they consume during the breeding season (Parrish and Martin, 1977).

Relationship Between Amino Acids and Secondary Compounds. The findings that the four toxic fruits had relatively high total protein (Table 3), high concentrations of specific EAAs (Figure 1), and large surpluses relative to requirements for all EAAs (Table 2) raise doubts about whether these results are artifacts. Amino acids are precursors of some secondary compounds (Evans, 1996). Each of the four plant species in group 2 might be rich in certain secondary compounds (or in compounds derived by hydrolysis) that have chemical structures similar to specific EAAs. The chemical analysis method I used might have been insensitive to these differences. For example, *Ephedra* is well known for its relatively high content of alkaloids such as ephedrine. Phenylalanine is the precursor of ephedrine (Evans, 1996). Ephedrine is formed by a union of a C_6–C_1 unit and a C_2 unit. Phenylalanine is the originator of the C_6–C_1 moiety, being converted first to benzaldehyde or benzoic acid. Benzoic acid combines with the intact acetyl group of pyruvic acid to form ephedrine (Grue-Sørensen and Spenser, 1993). Thus, the location of *Ephedra* in Figure 1 may result from its high content of ephedrine rather than phenylalanine and therefore the concentration of total EAAs of *Ephedra* may be lower than reported here (Table 3, Figure 1).

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RESPONSES OF TEA TUSSOCK MOTH, *Euproctis pseudoconspersa*, TO ITS PHEROMONE, (*R*)-10,14-DIMETHYLPENTADECYL ISOBUTYRATE, AND TO THE *S*-ENANTIOMER OF ITS PHEROMONE

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Abstract—Field trials were conducted with each synthetic enantiomer (>98% ee) and blends of the two synthetic enantiomers of the female-produced sex pheromone (10,14-dimethylpentadecyl isobutyrate) of the tea tussock moth, *Euproctis pseudoconspersa*. Male moths were attracted to each enantiomer alone and to various blends of them. Short syntheses of both enantiomers of the pheromone from commercially available (*R*)- and (*S*)-citronellyl bromide and a method of checking the enantiomeric purity of the citronellyl bromide enantiomers are described.

Key Words—*Euproctis pseudoconspersa*, 10,14-dimethylpentadecyl isobutyrate, sex pheromone.

INTRODUCTION

The tea tussock moth, *Euproctis pseudoconspersa* (Strand) (Lepidoptera: Lymantridae), is a serious defoliating pest of tea, *Camellia sinensis*, in China and Japan, resulting in losses in both quality and quantity of tea produced. Furthermore, tea tussock moth larvae have urticating spines that cause severe

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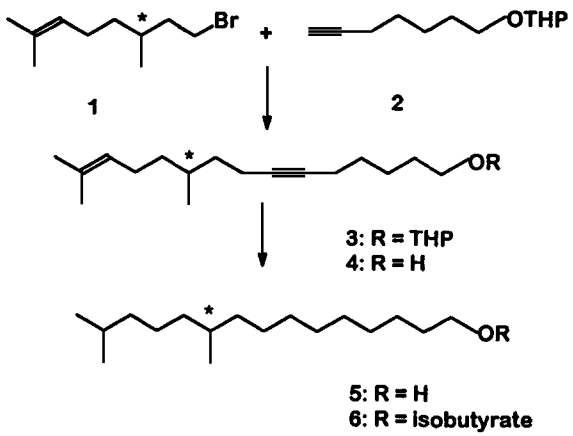
skin irritation upon contact (Ogata, 1958). The major component of the female-produced sex attractant pheromone of the moth was recently identified by two research groups independently as 10,14-dimethylpentadecyl isobutyrate (Wakamura et al., 1994; Zhao et al., 1996). Both groups found that the racemic pheromone was attractive to male moths. The naturally produced pheromone was tentatively established as the *R* configuration about the C_{10} methyl group by comparison of electroantennogram (EAG) responses of male moth antennae to the synthetic enantiomers (Ichikawa et al., 1995). A more recent study reports that the *R*-enantiomer elicits stronger EAG responses from male moth antennae than the *S*-enantiomer or the racemic mixture (Wakamura et al., 1996a). In field trials, however, each enantiomer singly and in blends was equally attractive (Wakamura et al. 1996a). We report the syntheses of each of the enantiomers of the pheromone, a method for checking the enantiomeric purity of the chiral starting materials, and results of field trials in commercial tea plantations in Jiangxi province, China.

METHODS AND MATERIALS

Proton NMR spectra were prepared with a General Electric QE 300 instrument (300 MHz) in $CDCl_3$ solution. EI mass spectra (70 eV) were taken with a Hewlett-Packard (HP) 5970B mass selective detector interfaced to an HP 5890 GC (Avondale, Pennsylvania) fitted with a DB5-MS column (20 m × 0.2 mm ID, J&W Scientific, Folsom, California). Air- and/or water-sensitive reactions were carried out in oven-dried glassware under N_2 atmosphere. THF was purified by distillation from sodium-benzophenone ketyl under argon. Flash chromatography was carried out with 230–400 mesh silica gel (Aldrich Chemical, Milwaukee, Wisconsin).

Synthesis of 10,14-Dimethylpentadecyl Isobutyrate Enantiomers (Scheme 1). Butyllithium (8.8 ml of a 2.5 M solution in hexanes; 22 mmol) was added dropwise to a cooled (0°C) THF solution (25 ml) of the tetrahydropyranyl ether of 6-heptyn-1-ol, **2** (3.92 g, 20 mmol) (Zhao et al., 1996). The solution was stirred for 20 min, followed by the sequential addition of dry *N,N'*-dimethylpropyleneurea (DMPU) (10 ml) and (*R*)-(−)-citronellyl bromide, **1** [4.34 g, 20 mmol; $[\alpha]_D^{20} = -6.8°$ (neat), Aldrich Chemical]. The mixture was warmed to 20°C and stirred overnight, then poured into water, and the mixture was extracted three times with hexane. The combined hexane extracts were washed with water and brine, dried, and concentrated. The crude THP ether, **3**, was taken up in MeOH (25 ml), and *p*-toluenesulfonic acid (~1 g) was added in portions until the solution was acidic to moist litmus paper. The solution was stirred overnight, then 2.5 g of $NaHCO_3$ were added, and most of the MeOH was removed by rotary evaporation. The residue was partitioned between water and hexane, and



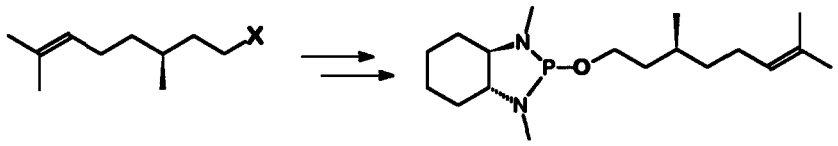
SCHEME 1.

the hexane layer was washed with brine, dried, concentrated, and flash chromatographed (20% EtOAc in hexane), yielding alcohol **4** (4.13 g, 80%). (*S*)-**4** was prepared in analogous fashion from (*S*)-(+)-citronellyl bromide [Aldrich Chemical; $[\alpha]_D^{20} = +6.8°$ (neat)] in 67% yield.

A slurry of 5% Pd on carbon (2 g) in hexane (50 ml) was flushed with Ar, then saturated with H_2. (*R*)-Alcohol **4** (2.5 g, 10 mmol), was added by syringe, and the mixture was stirred until the reaction was complete (8 hr), monitoring the reaction by GC. The mixture was then flushed thoroughly with Ar, filtered through a short plug of Cellite, and flash chromatographed (25% EtOAc in hexane), giving a quantitative yield of saturated (*S*)-alcohol **5**. NMR and mass spectra and GC retention time matched those of the previously synthesized racemic material (Zhao et al., 1996). (*R*)-**5** was similarly prepared from (*S*)-**4** in 83% yield.

Isobutyryl chloride (1 ml, 10 mmol) was added dropwise to a solution of (*S*)-alcohol **5** (1.7 g, 6.6 mmol) and pyridine (1.2 g, 15 mmol) in ether (100 ml) at 0°C. The mixture was stirred overnight, then filtered. The filtrate was washed thoroughly with 1 M $NaHCO_3$, 1 M HCl, and brine, then dried, concentrated, and Kugelrohr distilled (oven temperature 175°C, 0.7 mm Hg), yielding (*S*)-ester **6** (2.1 g, 98%). The mass, NMR, and IR spectra matched those of the racemic material (Zhao et al., 1996). The *R*-enantiomer of **6** was similarly prepared in quantitative yield.

Analysis of Enantiomeric Purity of Starting Material (Scheme 2). (*S*)-(+)-Citronellyl bromide **1** (90 mg, 0.4 mmol) was added to a solution of cesium acetate (800 mg, 4.1 mmol) in DMPU (2 ml), and the mixture was stirred at 60°C for 45 min. The mixture was cooled, poured into water, and extracted



1: X = Br
7: X = OAc
8: X = OH

9

SCHEME 2.

with hexane. The hexane extract was concentrated, giving crude acetate (*S*)-**7**. Ethanol (5 ml) and 2 M aq. NaOH (0.5 ml) were added, and the solution was stirred at room temperature for 4 hr. The solution was then diluted with water and extracted with hexane. The hexane extract was dried over anhydrous Na_2SO_4, concentrated, and Kugelrohr distilled (oven temperature 75°C, 0.5 mm Hg), yielding (*S*)-(−)-β-citronellol **8**. Retention time and mass spectrum matched those of an authentic sample of racemic β-citronellol (Aldrich Chemical).

An aliquot of distilled (*S*)-**8** (9 μl, 0.05 mmol) was added to an oven-dried 5-mm NMR tube, followed by 0.25 ml of dry deuterated benzene, and 0.5 ml of a 0.1 M solution of (3a*R*,7a*R*)-2-dimethylamino-1,3-dimethyl-octahydro-1H-1,3,2-benzodiazaphosphole (Fluka Chemical, Milwaukee, Wisconsin). The tube was sealed and allowed to stand at room temperature for two days, before being analyzed by phosphorus NMR at 202.47 MHz, using a General Electric GN-500 instrument. An analogous sample was prepared from racemic β-citronellol.

Field Trials. Field trials were carried out in commercial tea plantations in Jiangxi Province, People's Republic of China. In the first trial (June 6–12, 1995), pheromone solutions in CH_2Cl_2 were loaded onto rubber septum lures (11 mm, The West Co., Lititz, Pennsylvania; 1 mg dose/septum, five replicates) that were placed in Pherocon 1C sticky traps (Trece, Salinas, California). Traps were hung within the tea fields, suspended from wooden stakes at a height of ~1.5 m, at a spacing of at least 20 m between traps. Male moth captures were tabulated daily and traps were rerandomized every second day. In the second trial (June 6–20, 1996), 1-mg doses of pheromone on rubber septa were used as baits in water pan traps, as previously described (Zhao et al., 1996). Trap catch data were checked for normality and analyzed by analysis of variance, followed by Bonferroni's all pairwise multiple comparison procedure (SigmaStat, 1992).

RESULTS AND DISCUSSION

In the first field trial, each of the synthetic enantiomers alone or in various mixtures attracted equal numbers of moths (Table 1), corroborating the report

TABLE 1. MALE *E. pseudoconspersa* CAUGHT IN TRAPS BAITED WITH DIFFERENT RATIOS OF SYNTHETIC ENANTIOMERS OF 10,14-DIMETHYLPENTADECYL ISOBUTYRATE[a]

Ratio, R:S	Moths caught (Mean ± SE)
100:0	117.6 ± 19.0a
95:5	104.0 ± 11.0a
80:20	105.2 ± 14.4a
50:50	107.4 ± 9.5
20:80	80.6 ± 36.5a
5:95	68.2 ± 19.6a
0:100	69.8 ± 5.7a
Blank	6.2 ± 1.6b

[a]Mean catch per trap of the combined counts from five replicates counted seven times (i.e., treated statistically as five replicates). Total dose/lure: 1 mg. Numbers followed by the same letter are not significantly different (Bonferroni's multiple pairwise comparison procedure, $P = 0.05$).

of Wakamura et al. (1996a). Because it is unusual for pheromone enantiomers to be of similar attractiveness (Mori, 1996), a second test compared attractiveness of each enantiomer alone and that of the racemic mixture. The attractiveness of all three lures was again statistically indistinguishable, with the *R*, *S*, and racemic formulations attracting averages of 11.0 ± 2.85, 9.33 ± 2.32, and 6.67 ± 2.5 moths/trap respectively ($N = 6$, Student-Newman-Kuels test, $P > 0.05$).

Because the results of Wakamura et al. (1996a) were not known when these studies were executed, we were concerned that the unusual field-trapping results might be due to enantiomerically impure pheromone because starting materials [(*R*)- and (*S*)-citronellyl bromides **1**] of unknown chiral purity had been used in the synthesis (the supplier was not able to provide the enantiomeric purity). It was not possible to check the chiral purity of the synthetic pheromone enantiomers directly because the pheromone enantiomers have negligible optical rotations and are inseparable by gas chromatography (GC) on a chiral stationary phase (Ichikawa et al., 1995). Our synthetic sample of racemic **6** also gave a single peak on a chiral Cyclodex-B GC column, operated isothermally, so that the chiral purity of the synthetic pheromone enantiomers could not be checked directly.

However, the conditions used to generate the enantiomers of **6** from each citronellyl bromide enantiomer result in negligible loss of stereochemical integrity at the single chiral center in the molecule. Consequently, as a reasonable alternative to direct determination of the enantiomeric purity of the synthetic pheromone enantiomers, the enantiomeric purity of the starting material was checked instead. Because the citronellyl bromide enantiomers were also not

resolved on a Cyclodex-B GC column, this necessitated conversion of a sample of (*S*)-citronellyl bromide to a derivative whose enantiomeric purity could be readily determined. Thus, (*S*)-citronellyl bromide was converted to (*S*)-citronellol **8**, via the acetate **7**, then to the derivative **9** (Alexakis et al., 1992). A racemic sample of citronellol, derivatized under the same conditions, showed two ^{31}P NMR signals of equal size (137.33 and 136.70 ppm). Only a single peak was discernible with the compound derived from (*S*)-**8**, allowing us to conservatively estimate that the (*S*)-citronellyl bromide starting material had an enantiomeric excess of >98%. Because the commercial citronellyl bromide enantiomers had equal and opposite optical rotations, we conclude that the pheromones produced from them were of high enantiomeric purity, and that *E. pseudoconspera* male moths do not discriminate between the two enantiomers (Table 1).

Our field-trapping results corroborate the analogous report from Wakamura et al. (1996a). This research group had demonstrated in an earlier study that male antennae respond most strongly to stimulation by (*R*)-**6**, with a lesser response to the racemic mixture, and the least response to (*S*)-**6** (Ichikawa et al., 1995). Furthermore, EAG responses of male antennae exposed to either the insect-produced pheromone or (*R*)-**6** were indistinguishable (Wakamura et al. 1996a). Thus, the available evidence suggests that females produce (*R*)-**6**, or a nonracemic mixture containing a large preponderance of (*R*)-**6**, but for unknown reasons, males show no distinct preference for either enantiomer in field-trapping studies. Several examples of this unusual category of pheromone-mediated behavior have been documented in the Insecta (reviewed in Mori, 1996), but to our knowledge, this is the first instance of such a phenomenon in the Lepidoptera. In general, unnatural enantiomers, when presented to moths known to utilize chiral sex attractant pheromone components, either have no discernible biological activity or inhibit attraction of males (Mori, 1996).

For practical purposes, being able to use the cheaper and more readily available racemic material should expedite the commercialization of this pheromone for monitoring purposes. Furthermore, because the reproductive lifetime of female moths is short and highly synchronized in generations, it has been suggested that pheromone-based mating disruption may represent a feasible control strategy for this pest species (Wakamura et al., 1996b).

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IDENTIFICATION OF SEMIOCHEMICALS RELEASED DURING APHID FEEDING THAT ATTRACT PARASITOID *Aphidius ervi*

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Abstract—Herbivore induced release of plant volatiles mediating the foraging behavior of the aphid parasitoid *Aphidius ervi* was investigated using the pea aphid, *Acyrthosiphon pisum*, feeding on broad bean, *Vicia faba*. Behavioral responses were studied using an olfactometer and a wind tunnel. Volatiles obtained by air entrainment of aphid infested plants were more attractive to *A. ervi* than those from uninfested plants, in both behavioral bioassays. GC-EAG of both extracts showed a number of peaks associated with responses by *A. ervi*, but with some differences between extracts. Compounds giving these peaks were tentatively identified by GC-MS and confirmed by comparison with authentic samples on GC, using two columns of different polarity. The activity of pure compounds was further investigated by EAG and wind tunnel assays. Results showed that, of the compounds tested, 6-methyl-5-hepten-2-one was the most attractive for *A. ervi* females, with linalool, (*Z*)-3-hexen-1-yl acetate, (*E*)-β-ocimene, (*Z*)-3-hexen-1-ol, and (*E*)-β-farnesene all eliciting significantly more oriented flight behavior than a solvent control. Foraging experience significantly increased parasitoid responses to these compounds, with the exception of (*E*)-β-farnesene. Time-course GC analysis showed that feeding of *A. pisum* on *V. faba* induced or increased the release of several compounds. Release of two of these compounds (6-methyl-5-hepten-2-one and geranic acid) was not induced by the nonhost black bean aphid, *Aphis fabae*. During the analysis period, production of (*E*)-β-ocimene remained constant, but 6-methyl-5-hepten-2-one, linalool, geranic acid, and (*E*)-β-farnesene appeared during the first day after *A. pisum* infestation and increased in concentration with increasing time of aphid feeding.

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Key Words—Homoptera, Aphididae, Hymenoptera, Braconidae, tritrophic interactions, host foraging, plant volatiles, semiochemicals, induction, synomones, wind tunnel, GC-EAG, *Acyrthosiphon pisum*, *Aphidius ervi*, *Vicia faba*, *Aphis fabae*.

INTRODUCTION

Plant volatiles play an important role in host location not only by phytophagous insects (Visser, 1983, 1986), but also by their parasitoids (Price, 1981; Vinson, 1985; Tumlinson et al., 1992). The functions of such semiochemicals in tritrophic interactions are receiving increased attention, particularly the use of plant volatiles as synomones during host location by the natural enemies of herbivorous arthropods (Dicke and Sabelis, 1988; Turlings et al., 1990b; Vet and Dicke, 1992). Work on tritrophic systems has indicated that herbivore feeding damage stimulates plants to release relatively large amounts of volatiles that are attractive to predators or parasitoids, a process mediated by systemic interactions that serves as a plant defense mechanism (Dicke and Sabelis, 1988; Dicke et al., 1990a,b; Turlings et al., 1990a,b; Turlings and Tumlinson, 1992). Plants infested with herbivorous mites produced a water-soluble endogenous elicitor that was shown to be transported out of the infested leaves (Dicke et al., 1993; Mattiacci et al., 1995). Oral secretions of caterpillars of several moth species have been shown to contain elicitors that induce the emission of volatiles in the host plant (Turlings et al., 1993; Alborn et al., 1997). The induced emission of plant volatiles lagged several hours behind onset of feeding and depended on the herbivore density (Dicke et al., 1993; Tumlinson et al., 1993), with certain compounds being synthesized rapidly de novo (Paré and Tumlinson, 1997). In some cases, the release of herbivore induced volatiles from a damaged plant stimulated the release of the same volatiles from clean plants in the vicinity (Bruin et al., 1992, 1995). Although the induction of synomones was considered to be a general phenomenon (Turlings et al., 1991), identification studies have so far been limited to relatively few plant/insect systems.

Behavioral work has demonstrated the role of plant volatiles in foraging by aphid parasitoids (Read et al., 1970; Powell and Zhang, 1983; Sheehan and Shelton, 1989; Pickett et al., 1991; Powell and Wright, 1992; Wickremasinghe and van Emden, 1992; Grasswitz and Paine, 1993; Guerrieri et al., 1993, 1997; Du et al., 1996, 1997; Powell et al., 1998). Wind tunnel studies have shown flight orientation by *Aphidius ervi* Haliday (Hymenoptera: Braconidae: Aphidiinae) to volatiles released by bean plants, *Vicia faba* (Fabaceae), infested with host aphids, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) (Guerrieri et al., 1993, 1997; Du et al., 1996, 1997; Powell et al., 1998). The response of *A. ervi* females to volatiles released from host aphids alone or from uninfested plants was weak, but there was a significantly stronger response to aphid infested

plants and plants recently damaged by aphids, suggesting that aphid feeding influences the release of plant volatiles that act as synomones. Host aphids feeding on a basal leaf of *V. faba*, which was subsequently removed before testing the rest of the plant for attractiveness, also caused a systemic production of the volatiles for up to 24 hr after the aphids had been removed (Guerrieri et al., 1996 and unpublished data).

A. ervi is an oligophagous species attacking a restricted range of hosts including aphids on legumes and cereals, and the possibility that specific semiochemicals play a key role in its host location required investigation. In addition, although the concept of biochemical interactions among plant/aphid/parasitoid has been proposed (Pickett et al., 1991), identification of associated synomones has not previously been reported. However, the previous detailed wind tunnel studies on host location by *A. ervi* (Guerrieri et al., 1993, 1997; Du et al., 1996, 1997) provided an ideal basis for its use as a model system with which to investigate these interactions (Powell et al., 1998). In this study, volatiles produced by uninfested plants and plants infested with host or nonhost aphids were compared, and compounds that were electrophysiologically active for *A. ervi* were assessed for their behavioral activity.

METHODS AND MATERIALS

Plants. Broad bean plants, *V. faba* cv. The Sutton, were grown under greenhouse conditions (photoperiod 18L:6D). Two-week-old seedlings were used for air entrainments.

Insects. The parasitoid *A. ervi* was reared on colonies of the pea aphid, *A. pisum*, feeding on broad bean plants, *V. faba*. Aphid and parasitoid cultures were maintained in two separate controlled environment chambers at 18 ± 1°C, 75 ± 5% relative humidity, and a 18L:6D photoperiod. In preparation for the tests, female parasitoids, which emerged from mummies isolated in individual glass vials, were mated within 24 hr of emergence and kept in cages for one day with access to honey solution (50%) under the same environmental conditions as described above. Individual parasitoids were tested only once in bioassays. Experienced parasitoids were obtained by allowing them to search and oviposit on the aphid/plant complex for 30 min and experiments were carried out 2–4 hr after this experience (see Du et al., 1997).

Entrainment of Plant Volatiles. All glassware, including the air entrainment equipment, was scrupulously cleaned and heated at 200–230°C overnight before use. Solvents were rigorously purified by redistillation. The Porapak Q (50–80 mesh) used to collect volatiles in air entrainment experiments (Blight, 1990) was washed with redistilled diethyl ether and then conditioned by heating overnight in a stream of nitrogen at 180°C.

Pots containing 10 two-week-old *V. faba* plants, either uninfested or infested with 500 *A. pisum* (host aphid) or black bean aphid, *Aphis fabae* Scop. (non-host), were put separately into belljars (20 liters) that were sealed with Teflon tape. Air was purified by drawing through a molecular sieve (5 Å) and activated charcoal traps before entering the belljars. The airflow was adjusted to 1 liter/min. Collected volatiles were adsorbed on Porapak Q and eluted with freshly distilled diethyl ether (600 μl). The resulting extract was concentrated to 50 μl under a stream of nitrogen and stored at −20°C.

Bioassay. The bioassays, which followed protocols established in the previous study (Du et al., 1996), employed a Y-tube olfactometer and a wind tunnel. In the Y-tube olfactometer, air was drawn through activated charcoal and a molecular sieve (5 Å) before entering the Y-tube so that it was purified of any contaminating odors. The airflow through each of the arms was maintained at 800 ml/min. The air entrainment extracts, equivalent to 1 hr collection, were applied to 1 × 2 cm pieces of Whatman No. 1 filter paper that were then placed in the airstream entering one of the olfactometer arms. Thirty naïve *A. ervi* females were tested individually and presented with a direct choice between the extract entrained from an undamaged, i.e., aphid-free, plant and that entrained from a plant infested with *A. pisum*. The olfactometer arm chosen by each parasitoid over a 5-min period was recorded. A χ^2 test was used to determine significant differences between numbers of parasitoids choosing different odor treatments or arms of the olfactometer.

Similarly, 1 × 2 cm pieces of filter paper, treated with air entrainment extracts (1.5 hr equivalent) or synthetic compounds (10 μg) dissolved in redistilled diethyl ether (10 μl), were attached to the top of a plastic support at an angle of 45° from the horizontal (highest point upwind) and used in the wind tunnel bioassays. Light intensity in the wind tunnel was 3600 lux at the insect release site and the wind speed was adjusted to 23 ± 1 cm/sec. The exhaust air was vented outside the room. Female parasitoids were tested individually by releasing them from an open-ended glass tube placed horizontally in the odor plume, 20 cm downwind from the odor source. The releasing tube was held parallel to the wind direction and 10 cm above the floor of the tunnel, allowing parasitoids sufficient time to detect the odors from each source. At least 50 females were tested against each odor source. The bioassay comparing individual synthetic compounds took place over several days, and one replicate was completed on each day. The order in which compounds were tested was varied randomly each day to eliminate any time variation. Filter paper targets treated with the ether solvent alone were used as a control.

The numbers of parasitoids taking off from the release site, performing direct flights oriented towards the target, and landing on the target were recorded. Each female was allowed a maximum of 5 min to respond and given only one opportunity to fly. A χ^2 test was used to determine whether the number of

parasitoids responding to each of the air entrainment extracts, both for oriented flights and target landings, differed significantly.

In the case of the synthetic chemicals, the mean percentage response per day was calculated for statistical analysis. The proportions responding (i.e. showing oriented flight and landing on the target) were subjected to logit transformation to normalize the data and then analyzed using a two-way analysis of variance to test the effect of experience and chemical on the response (Genstat 5 Committee, 1993). Tukey multiple comparison tests were used to compare individual means.

Electrophysiology. Electroantennogram (EAG) recordings from 2-day-old naïve female parasitoids were made using Ag–AgCl glass electrodes filled with saline solution [composition as in Maddrell (1969) but without the glucose]. The insect was anesthetized by chilling, and the head was excised and mounted on the indifferent electrode. The tip of the recording electrode was removed so that its inside diameter was just wide enough to accept the uncut terminal process of the antenna. The signals were passed through a high impedance amplifier (UN-03b, Syntech) and displayed on an oscilloscope and a chart recorder. Compounds were tested against five individual antennae. The stimulus (2 sec duration) was delivered into a purified airstream (1 liter/min) flowing continuously over the preparation. The delivery system, which employed a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). Samples of the standard solutions of test compounds (10^{-4}–10^{-6} g/10 μl) were applied to Whatman No. 1 filter paper strips, and the solvent was allowed to evaporate for 30 sec before the paper strip was placed in the cartridge. The control stimulus was 10 μl hexane. Fresh cartridges were prepared immediately prior to each stimulation. Compounds were presented twice to each preparation at intervals of 2–10 min, the exact interval being determined by the concentration of the previous stimulus.

Gas Chromatography (GC). Air entrainment volatiles were separated on either a 50 m × 0.32 mm ID methyl silicone bonded phase fused silica capillary column (HP-1) fitted in a Hewlett Packard 5880A gas chromatograph (HP-5880A) or a 30 m × 0.32 mm ID BP-20 capillary column fitted in an AI-92 gas chromatograph. The HP-5880A was equipped with a split/splitless injector, and the AI-92 was equipped with a cold on-column injection system. Both instruments were fitted with flame ionization detectors (FID) and the carrier gas in both cases was hydrogen. The oven temperature for the HP-1 column was maintained at 40°C for 5 min and then programmed at 5°/min to 150°C, then at 10°/min to 250°C. For the BP-20 column, the oven temperature was maintained at 40°C for 5 min and then programmed at 10°/min to 225°C. Quantification of the compounds in the air entrainment samples was carried out on both polar and nonpolar GC columns, using methyl salicylate (1 μg) as the internal standard.

Coupled Gas Chromatography–Electroantennography (GC-EAG). The GC-EAG system, in which the effluent from the capillary column GC is delivered simultaneously to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the air entrainment samples was achieved on an AI-93 gas chromatograph equipped with a cold on-column injector and an FID. The carrier gas was hydrogen, and the column (50 m × 0.32 mm ID HP-1) 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.

Gas Chromatography–Mass Spectrometry (GC-MS). A capillary column (50 m × 0.32 mm ID HP-1) fitted in a Hewlett Packard 5890 gas chromatograph was directly coupled to the mass spectrometer and integrated data system (70-250 VG Analytical and VG Autospec, Fisons Instruments). Ionization was by electron impact at 70 eV and 230°C. The gas chromatograph was maintained at 30°C for 5 min and then programmed at 5°/min to 180°C. Tentative identifications made by GC-MS were confirmed by comparison of the mass spectral data with those of authentic samples and by peak enhancement when coinjected with authentic compounds using GC, as above (Pickett, 1990).

Chemicals. The majority of chemicals were obtained commercially: 6-methyl-5-hepten-2-one, (*Z*)-3-hexen-1-ol, and (±)-linalool (from Aldrich); (*E*)-β-ocimene (from Quest International) and (*Z*)-3-hexen-1-yl acetate (from Lancaster Synthesis Ltd.). (*E*)-β-Farnesene was synthesized (Dawson et al., 1982). With the exception of (*E*)-β-ocimene, which was a 70:30 mixture of *E*- and *Z*-isomers, all compounds were ≥98% pure. Standard solutions of all compounds were diluted in purified hexane or ether for electrophysiological or behavioral studies.

RESULTS

In the Y-tube olfactometer, volatiles isolated from *V. faba* plants infested with *A. pisum* were significantly more attractive to *A. ervi* females than those isolated from undamaged plants (23 parasitoids responded to the plant/host complex, but only five responded to undamaged plants; $P < 0.01$). Furthermore, in the wind tunnel bioassays, volatiles from *A. pisum* infested plants elicited a significantly greater oriented flight response from naïve *A. ervi* females than did volatiles from uninfested plants ($P < 0.05$). Very few individuals succeeded in landing on the filter paper target, which provided only a weak visual cue (Figure 1).

Coupled GC-EAG on *A. ervi* females revealed a number of peaks associated with electrophysiological activity in both extracts, subsequently identified by GC-MS and confirmed by peak enhancement on coinjection with authentic samples on the two GC columns. The identifications add to those compounds pre-

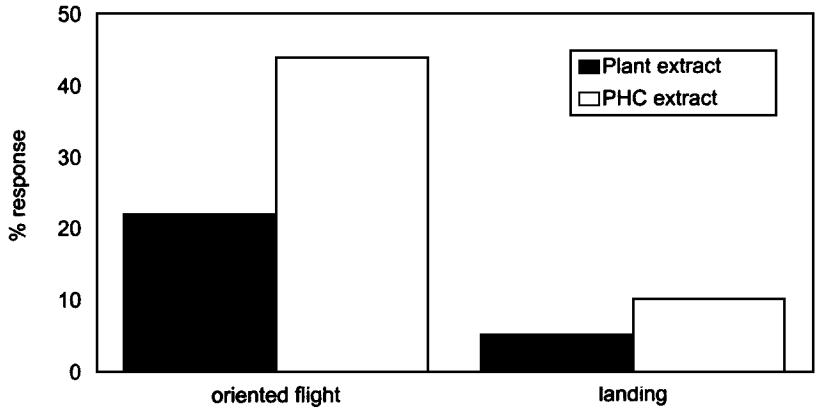

FIG. 1. Percentage of naïve *Aphidius ervi* females ($N = 40$) showing oriented flight and landing responses to a filter paper target, treated with an air entrainment extract (1.5 hr equivalent) of either an undamaged plant (*Vicia faba*) or a plant/host (*Acyrthosiphon pisum*) complex (PHC) in a wind tunnel ($\chi^2 = 4.54$, $P < 0.05$, for oriented flight and $\chi^2 = 1.13$, not significant, for landing response).

viously identified from *V. faba* (Johnson et al., 1971) and other related species (e.g., Takabayashi et al., 1994). These compounds were then tested by EAG at three concentrations (Table 1), confirming electrophysiological activity for (*E*)-β-ocimene, 6-methyl-5-hepten-2-one, linalool, (*E*)-β-farnesene, (*Z*)-3-hexenyl acetate, and (*Z*)-3-hexen-1-ol.

TABLE 1. ELECTROANTENNOGRAM RESPONSES OF NAIVE *Aphidius ervi* FEMALES TO SYNTHETIC COMPOUNDS IDENTIFIED IN GC-EAG STUDIES

Compound tested	EAG response (mV)[a]		
	10^{-6} g	10^{-5} g	10^{-4} g
(Z)-3-Hexen-1-ol	0.27 ± 0.05	0.28 ± 0.04	0.94 ± 0.19†
(Z)-3-Hexenyl acetate	0.29 ± 0.03	0.28 ± 0.04	0.84 ± 0.05*
Linalool	0.49 ± 0.07*	0.73 ± 0.10‡	1.73 ± 0.25‡
(E)-β-Ocimene	0.34 ± 0.07	0.20 ± 0.02	0.88 ± 0.13†
6-Methyl-5-hepten-2-one	0.33 ± 0.06	0.28 ± 0.05	0.92 ± 0.08‡
(E)-β-Farnesene	0.50 ± 0.09*	0.95 ± 0.11‡	Not tested
Hexane (Control)	0.26 ± 0.06		

[a]Responses are significantly different from control at $^*P < 0.05$, $^\dagger P < 0.01$, and $^\ddagger P < 0.001$ respectively (Student's *t*-test).

In the wind tunnel, all six electrophysiologically active compounds produced significantly more oriented flights than the solvent control ($P < 0.05$), which showed no activity (Table 2). Female parasitoids with oviposition experience gave significantly stronger flight responses than naïve females ($P < 0.05$) for all compounds except (*E*)-β-farnesene (Table 2). 6-Methyl-5-hepten-2-one appeared to be the most attractive to *A. ervi* females, eliciting significantly more oriented flights than all the other compounds except linalool ($P < 0.05$), and significantly more landing responses than all the other compounds except (*Z*)-3-hexenyl acetate ($P < 0.05$) (Table 2).

For some of the electrophysiologically active compounds, the air entrainment extracts of uninfested *V. faba* plants and those infested with *A. pisum* or the nonhost *A. fabae* showed marked differences in the amounts produced. Increased release of 6-methyl-5-hepten-2-one was induced by *A. pisum* but not by *A. fabae*, as was release of geranic acid. This compound was not noted as active by the coupled GC-EAG studies but will be investigated further. Time-course measurements for compounds showing variations associated with *A. pisum* infestation are given in Figure 2. Levels of (*E*)-β-ocimene remained constant for four days after infestation, but 6-methyl-5-hepten-2-one, linalool, geranic acid, and (*E*)-β-farnesene appeared for the first time (except as trace amounts) on the first day after infestation and then increased up to the fourth day, as did

TABLE 2. ORIENTED FLIGHT AND LANDING RESPONSES OF NAÏVE AND EXPERIENCED *Aphidius ervi* FEMALES TO COMPOUNDS (10 μg) APPLIED TO FILTER PAPER TARGETS (1 × 2 cm STRIPS) IN A WIND TUNNEL

Compound	Condition	Total tested	Taking off (*N*)	Oriented flight (%)	Landing (%)
(*E*)-β-Ocimene	naïve	50	46	10	0
	experienced	50	46	24	2
6-Methyl-5-hepten-2-one	naïve	50	49	28	4
	experienced	51	50	49	18
Linalool	naïve	76	61	11	0
	experienced	87	63	29	2
(*E*)-β-Farnesene	naïve	50	49	10	0
	experienced	51	43	12	4
(*Z*)-3-Hexen-1-ol	naïve	50	45	6	0
	experienced	50	47	22	2
(*Z*)-3-Hexenyl acetate	naïve	50	48	8	0
	experienced	50	44	30	8
Ether	naïve	48	42	0	0
	experienced	53	42	0	0

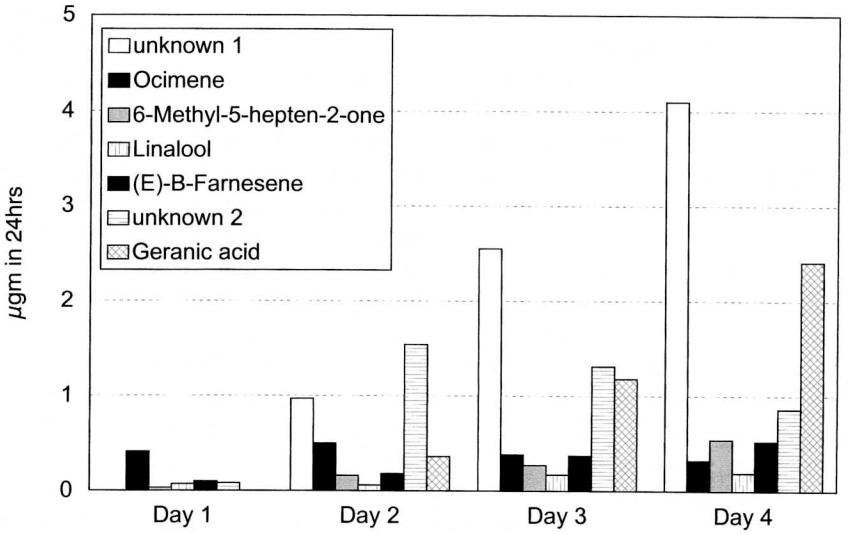

FIG. 2. Amounts of the main components present in air entrainment extracts of the plant/host (*Vicia faba/Acyrthosiphon pisum*) complex in successive 24 hr periods after aphid infestation.

the two apparently inactive unknown compounds **1** and **2**. *V. faba* plants infested with *A. fabae* showed a similar pattern but with some additional peaks, the identities of which will be reported elsewhere after further investigations.

DISCUSSION

Previous studies in this series (Guerrieri et al., 1993, 1997; Du et al., 1996, 1997) on tritrophic interactions between the parasitoid *A. ervi*, its aphid host *A. pisum*, and the aphid food plant *V. faba* showed increased attraction of the parasitoid not only to the plant/host complex, but also to plants damaged by aphids and then washed with water to remove exuviae and honeydew. In addition, plants with aphid infestations on the basal leaf, which was subsequently removed, remained attractive to parasitoids for up to 24 hr, even though aphids had not been in contact with the remaining part of the plant (Guerrieri et al., 1996 and unpublished data). This strongly suggested that feeding by the aphid altered the composition and/or ratios of volatiles released by the plant and that herbivore induced plant volatiles acted as synomones for the foraging parasitoid (Guerrieri et al., 1993; Du et al., 1996; Powell et al., 1998). Volatile extracts entrained from plants infested with *A. pisum* elicited stronger behavioral

responses from *A. ervi* females, in both olfactometer and wind tunnel bioassays, than extracts from uninfested plants, supporting the hypothesis that aphid induced plant volatiles attract foraging parasitoids. This is similar to the situation already reported for lepidopterous caterpillars and their parasitoids (Turlings et al., 1990a,b) and for spider mites and their acarine predators (Dicke and Sabelis, 1988; Dicke et al., 1990a). In this study, fewer volatiles were released from undamaged plants compared to the plants infested by aphids, although the difference was not as large as that reported in other systems (Turlings et al., 1990b, 1991). However, the aphid feeding action (i.e., sucking of phloem sap) is substantially different from that of leaf-chewing lepidopterous larvae since, once feeding commences, little further tissue damage occurs.

GC-EAG analysis of the volatile extracts from *A. pisum* infested plants, and EAG studies with synthetic compounds, indentified 6-methyl-5-hepten-2-one, linalool, (*E*)-β-ocimene, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, and (*E*)-β-farnesene as active components that could be involved in parasitoid attraction. The green leaf volatiles (*Z*)-3-hexenyl acetate and (*Z*)-3-hexen-1-ol and the terpenes linalool and (*E*)-β-farnesene were shown to be released by corn seedlings (*Zea mays*, Poaceae) in response to feeding by lepidopterous larvae (Turlings et al., 1991). (*Z*)-3-Hexenyl acetate, (*Z*)-3-hexen-1-ol, and the terpene (*E*)-β-ocimene were among the compounds released by Lima bean (*Phaseolus lunatus*, Fabaceae) and cucumber (*Cucumis sativus*, Cucurbitaceae) leaves in response to feeding by spider mites (Dicke et al., 1990a,b), the Lima beans also releasing linalool. In these other trophic systems, the green leaf volatiles were released from mechanically damaged plants, whereas the terpenes tended to be released in response to herbivore attack and so are more probable candidates as synomones for parasitoids and predators. It has been known for some time that herbivore damage to trees stimulates the release of terpenoid compounds that function as synomones for insect predators (Dicke et al., 1990b). However, linalool and (*E*)-β-farnesene were also released from corn plants that were not damaged by herbivores but had been cut off at the stem (Buttery and Ling, 1984).

In the wind tunnel bioassays, the strongest responses from *A. ervi* were obtained with 6-methyl-5-hepten-2-one, a compound that had not been recorded among the volatiles released by herbivore damaged corn, Lima bean, or cucumber. Moreover, as indicated by the GC analyses, its release from *V. faba* was increased on feeding damage by *A. pisum* but not by *A. fabae*. Linalool and (*E*)-β-farnesene were increased by both aphid species, while (*E*)-β-ocimene was already a dominant component in the volatile extract from uninfested plants. Therefore, 6-methyl-5-hepten-2-one is potentially one of the volatile components that allows this parasitoid to distinguish between plants infested with a host aphid, *A. pisum*, and the nonhost *A. fabae*, an ability that was demonstrated in previous wind tunnel studies (Du et al., 1996). For parasitoids with a limited

host range, the ability to distinguish, while in flight, between plants with appropriate hosts and those with closely related, but inappropriate, species would obviously be advantageous. However, *V. faba* is not the principal food plant of *A. pisum* under natural conditions, and it would be instructive to investigate the responses of *A. ervi* to volatiles released by other plant species infested with this aphid.

Previous experience with the plant/host complex increased the responses of female *A. ervi* to all the compounds tested, with the exception of (*E*)-β-farnesene. Increased flight responses following foraging experiences have previously been demonstrated for *A. ervi* in wind tunnel studies using undamaged *V. faba* plants (Du et al., 1997; Guerrieri et al., 1997). It has been proposed that the ability to modify responses to semiochemical cues, as a result of successful foraging experiences, endows a parasitoid with a degree of behavioral plasticity that allows it to respond to changing foraging opportunities and that this process is more likely to be associated with plant-derived synomones than with host-derived kairomones (Vet et al., 1990; Tumlinson et al., 1992; Vet and Dicke, 1992; Turlings et al., 1993; Vet, 1995). Although (*E*)-β-farnesene can be released by plants, it also comprises the aphid alarm pheromone for many aphid species (Pickett and Griffiths, 1980), and so may function as a host-derived behavioral cue in the case of aphid parasitoids. Therefore, responses to (*E*)-β-farnesene may be more fixed (innate) and so less likely to change as a result of learning processes (Vet and Dicke, 1992).

As little tissue damage occurs after the onset of aphid feeding, a systemic induction of semiochemical biosynthesis is implied, which is currently under investigation. Furthermore, differences between the plant responses induced by *A. pisum*, a host for *A. ervi*, and the nonhost *A. fabae* implies that induction involves aphid species-specific signals, which are also being investigated further.

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ISOLATION AND IDENTIFICATION OF FEMALE SEX PHEROMONE AND DEVELOPMENT OF A SEX ATTRACTANT FOR *Lacanobia subjuncta*

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Abstract—Extracts of terminal abdominal segments of female *Lacanobia subjuncta* contained (*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecenal, and (*Z*)-11-hexadecenol at a ratio of 1:0.2:0.02. (*Z*)-11-hexadecenyl acetate and (*Z*)-11-hexadecenal were most effective as a lure for trapping males when loaded in rubber septa at ratios near 3:1, respectively. The complete three-component blend was most effective as a lure for trapping males when loaded in rubber septa at a ratio of 3:1:0.08 for the acetate, aldehyde, and alcohol, respectively. In a comparison of doses of this three-component blend, greatest numbers of males were captured with 6.1 mg per rubber septum. These components, when loaded with an optimum ratio of components and suitable dose, should provide a useful lure for trapping *Lacanobia subjuncta* in agroecosystems, permitting monitoring of distribution, emergence, and flight periods.

Key Words—Attractant, trap, apple, cutworm, fruitworm, moth.

INTRODUCTION

Larvae of *Lacanobia subjuncta* (Grote and Robinson) (Lepidoptera: Noctuidae) are minor pests of apple in interior Washington and adjacent Oregon (Landolt, 1997, 1998). This insect occurs throughout temperate North America and feeds on a wide variety of plants, including several crops (McCabe, 1980). Although adults can be captured in blacklight traps and in traps baited with fermenting molasses (Landolt, 1998), an inexpensive and efficient method is needed to monitor *L. subjuncta* in apple orchards.

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Sex attractants and sex attractant pheromones have been identified for several species of *Lacanobia*, although not for *L. subjuncta*. These sex attractants include combinations of monounsaturated 14- and 16-carbon acetates, as well as related aldehydes (Mayer and McLaughlin, 1991). It was expected that *L. subjuncta* would possess a female pheromone comprised of similar compounds that would be useful as a lure for attracting males of this species.

We report here the isolation and identification of a pheromone produced by females and attractive to males of *L. subjuncta* and the optimization of a blend of these compounds as a lure for use in traps. This work included structural determination of suspect pheromonal compounds in extracts of female abdominal glands and field demonstrations of male attraction to synthetic blends of those compounds isolated and identified from female glands. An optimized formulated blend was developed as a lure for traps, based on male response in the field to different ratios of components and to different dosages of pheromone. Such a lure and trap should be useful as a means of monitoring this insect in orchards.

METHODS AND MATERIALS

Eggs were obtained from *Lacanobia subjuncta* moths collected in a light trap in August 1996 in a commercial apple orchard near Parker, Yakima County, Washington. Mated female moths that were placed overnight in 50-ml clear plastic vials deposited eggs in clusters on the vial walls. Larvae were reared in 3.6-liter glass jars on apple sucker foliage and then on potted greenhouse-grown apple seedlings (30–40 cm tall) held in large screened cages (60 × 60 × 60 cm) in a controlled environment room (24°C, 70% relative humidity). Mature larvae pupated in damp vermiculite (4 cm deep) within the cages or in soil of pots containing apple seedlings. Pupae were sorted by sex and placed in small screened cages (20 × 20 × 20 cm) in controlled environment chambers. Chambers included fluorescent lighting on a 14L:10D photoperiod, with lights off at 9:00 AM and lights on at 7:00 PM PDST. Pupae were moved daily to provide emerged adults of discrete age groups. Pairs of one male and one female moth were placed in plastic boxes (7 × 18 × 10 cm) with water, sugar water, and an apple seedling in order to obtain eggs for the rearing of subsequent generations. Four generations of *L. subjuncta* were raised in the laboratory for experiments reported here.

Gland Extractions. Virgin female moths 2–7 days old were removed from the environmental chamber 3–5.5 hr into the scotophase. They were transferred in a light-tight box to a darkened laboratory and were then placed individually in 50-ml plastic vials in a chilled ice chest for 5–20 min. The sex pheromone gland was exposed by squeezing the abdomen with forceps to extrude the ovipositor tip, which was then excised with scissors. The tip was transferred with

forceps to a glass microvial containing 30–50 μl of methylene chloride. Abdominal tips were accumulated in this solvent, allowed to soak an additional 5 min, and the solvent was then transferred with a 500-μl syringe to a clean 3-ml glass microvial for storage at −20°C. Three pooled samples of female tip extract were accumulated from three laboratory-reared generations of moths: from 66 females, 38 females, and 14 females. These extracts were used for chemical analyses.

Before analysis by GC-MS, aliquots of 1–5 female equivalents (FE) of the extract were concentrated by evaporation of solvent to 3–5 μl and the sides of the vial were rinsed with 2 μl of heptane. The sample was further concentrated to 1 μl, which was taken up in a 10-μl syringe for injection into the GC-MS.

Voucher specimens of adult laboratory-reared and trapped *L. subjuncta* were deposited in the M. T. James Entomological Collection, Department of Entomology, Washington State University, Pullman, Washington.

GC-MS Analysis. Analyses were done on a Hewlett Packard 5890 II gas chromatograph with a model 5971 mass selective detector (quadropole mass spectrometer for a detector). It was equipped with a DB-1 fused silica capillary column (J & W Scientific, Folsum, California), 0.25 mm × 60 m, and 25-μm film thickness. Chromatography was done with a temperature program of 80°C for 2 min, then increasing 20°C/min to a final temperature of 190°C. An extract was also analyzed on the same instrument with a J & W DB-wax column of the same dimensions with the same temperature program, but to a final temperature of 210°C. Spectra of eluting peaks were compared and matched to those of known compounds and by searching the National Bureau of Standards NBS75K library of compounds (Hewlett Packard's pn#G1033A).

Derivatization. Retention times and retention indices were studied for (*Z*)- and (*E*)-7-, (*Z*)- and (*E*)-9-, and (*Z*)- and (*E*)-11-hexadecenyl acetates. These standards were obtained from Farchan Inc. (Willoughby, Ohio), Chemical Sample Co. (Columbus, Ohio), or S. Voerman (IPO-DLO, Wageningen, Netherlands). Determination of the bond position and configuration of the major pheromone component was done by determining mass spectral fragmentation patterns of the dimethyl disulfide (DMDS) derivative, made according to the procedure of Buser et al. (1983).

To make the derivatives, all samples were brought up to 50 μl with hexane, then 50 μl of neat DMDS and 10 μl of an iodine solution (60 mg I_2/ml ether) were added. The vial was closed and warmed overnight in an oven at 40°C. The following day, the sample was diluted with 200 μl hexane. Iodine was removed by addition of 100 μl sodium bisulfite solution (5% in distilled water) and mixing until all color had disappeared. The organic phase was removed by syringe into a 3-ml tapered glass vial, concentrated to 20–50 μl, and analyzed within 30 min by GC-MS (DB-1 column). The instrument and conditions were as described previously, except the final temperature was 250°C. This procedure

was performed on a 5-FE aliquot of pheromone gland extract in 35 μl methylene chloride and 15 μl hexane, and standards of (*Z*)-11-hexadecenyl acetate (*Z*11-16:Ac) and (*E*)-11-hexadecenyl acetate (100 and 1000 ng in hexane). (*Z*)-11-Hexadecenal (*Z*11-16:Ald) was subsequently prepared as above for comparison to a derivatized minor component in the female extract.

Field Tests. Four field experiments were conducted to verify pheromone attractiveness to *L. subjuncta* males and develop an optimized trap lure. These experiments compared captures of males in traps baited with different ratios of two pheromone components and different ratios of the three pheromone components and compared captures of males in traps baited with different pheromone doses. Two-component ratios (*Z*11-16:Ac and *Z*11-16:Ald) were tested initially because it was considered that the (*Z*)-11-16-hexadecenol (*Z*11-16:Alc) may be present in the gland extract as a biochemical precursor rather than as a released pheromone component. Pheromone chemicals used in field experiments were analyzed by capillary gas chromatography to determine purity. The *Z*11-16:Ac was 98% pure with 1.6% *E*11-16:Ac. The *Z*11-16:Alc was 98% pure while the *Z*11-16:Ald was 97% pure.

Field tests were conducted in a pear orchard near Parker, Yakima County, Washington, known to have a population of *Lacanobia subjuncta*, during May, June, July, and August of 1997. Universal moth traps (Unitrap, Agrisene, Fresno, California) were baited with preextracted red rubber septa (West Co.) loaded with pheromone treatments. Septa had been double extracted with methylene chloride in a tumbler, air dried in a fume hood, and stored in a freezer. Pheromone dosages were applied in 200 μl of solvent. Loaded septa were aired 24 h in a laboratory fume hood prior to use in the field. Each trap contained a 4-cm^2 piece of Vaportape (Hercon Environmental Co., Emigsville, Pennsylvania) to kill captured moths. Traps were hung 2 m above ground in pear trees.

Statistical procedures were conducted using StatMost (DataMost 1995).

In the first experiment, five ratios of *Z*11-16:Ald and *Z*11-16:Ac were tested. Compounds were formulated for an estimated combined release rate of 100 ng/hr. Calculations of expected component release ratios and blend release rates were made using half-life findings for these compounds from McDonough et al. (1989) and McDonough (1991) and formulas of Heath et al. (1986). Load ratios (and load amounts) were 1:0 (5 and 0 mg), 42.5:1 (4.25 and 0 .1 mg), 17.5:1 (3.5 and 0.2 mg), 5:1 (2.0 and 0.4 mg), and 0:1 (0 and 0.7 mg) of *Z*11-16:Ac and *Z*11-16:Ald. A randomized complete block design was used, with each of five replicate blocks comprised of the five treatments (pheromone ratios). Treatments within a block were initially randomized. Each block of five traps was placed in a row of pear trees, with 31.5 m between traps and a minimum of 31.5 m between trap rows (blocks). Traps were checked every one to two days and treatments within a block were rotated one position each time traps were checked, until a complete rotation of treatments through the five trap

positions was accomplished. Data were subjected to an analysis of variance and, following a significant *F* value, treatment means were separated by Duncan's new multiple-range test (Duncan, 1955).

In the second experiment, ratios of Z11-16:Ald to Z11-16:Ac were re-evaluated to more precisely determine the optimum ratio of components for maximum captures of males in pheromone-baited traps. Six ratios of Z11-16:Ac and Z11-16:Ald were tested, formulated in septa for an estimated combined release rate of 100 ng/hr. Load ratios (and load amounts) were 17:1 (3.57 and 0.21 mg), 11:1 (3.06 and 0.28 mg), 7.3:1 (2.55 and 0.35 mg), 4.9:1 (2.04 and 0.42 mg), 3.1:1 (1.53 and 0.49 mg), and 1.8:1 (1.02 and 0.56 mg) of Z11-16:Ald and Z11-16:Ac. A randomized complete block design was used, with five replicate blocks, each comprised of the six treatments (pheromone ratios). Treatments within a block were randomized daily. Each block of six traps was placed in a row of pear trees, with 31.5 m between traps and a minimum of 31.5 m between trap rows (blocks). Traps were checked daily for four days. Trap catch data for pheromone treatments were subjected to ANOVA and, following a significant *F* value, differences between treatments were determined using Duncan's (1955) new multiple-range test.

In the third experiment, ratios of the third component, Z11-16:Alc to the two-component blend were compared as lures for males. Ratios evaluated (Ac, Ald, Alc) were 3:1:0 for 0%, 3:1:0.025 for 0.6%, 3:1:0.076 for 1.9%, 3:1:0.16 for 3.9%, 3:1:0.45 for 10.2%, and 3:1:1 for 21% Z11-16:Al. Load amounts of Z11-16:Ac, Z11-16:Ald, and Z11-16:Alc for these six treatments were 1530, 520, and 0 μg (0% Alc); 1510, 510, and 13 μg (0.6% Alc); 1480, 500, and 39 μg (1.9% Alc); 1440, 490, and 79 μg (3.9% Alc); 1300, 440, and 198 μg (10.1% Alc); and 1071, 360, and 396 μg (21% Alc). A randomized complete block design was used, with five replicate blocks, each comprised of the six treatments (pheromone ratios). Treatments within a block were randomized daily. Each block of six traps was placed in a row of pear trees, with 31.5 m between traps and a minimum of 31.5 m between trap rows (blocks). Traps were checked every three to four days for 21 days. Trap catch data for pheromone treatments were subjected to ANOVA and, following a significant *F* value, differences between treatments were determined using Duncan's new multiple-range test (Duncan, 1955).

In the fourth experiment, six doses of the three-component blend (Z11-16:Ac, Z11-16:Ald, Z11-16:Alc) at a 3:1:0.08 load ratio in rubber septa were tested for attractiveness to male *L. subjuncta*. Doses tested were 0, 55, 204, 673, 2020, and 6059 μg of pheromone. Per septum load amounts of Z11-16:Ac and Z11-16:Ald, Z11-16:Alc were 40, 14, and 1 μg (55-μg dose); 150, 50, and 4 μg (204-μg dose); 490, 170, and 13 μg (673-μg dose); 1480, 500, and 40 μg (2020-μg dose); and 4440, 1500, and 119 μg (6059-μg dose) for the five treatments with pheromone. Controls consisted of septa treated with

200 μl solvent and no pheromone. A randomized complete block design was used, with five replicate blocks, each comprised of the six treatments (pheromone doses). Treatments within a block were randomized daily. Each block of six traps was placed in a row of pear trees, with 50 m between traps and a minimum 50 m between trap rows (blocks). Traps were checked daily for four days, providing 20 data sets. Trap catch data were subjected to a regression analysis to determine if a significant relationship was evident between dose and trap catch.

RESULTS

Female Gland Extract. Preliminary GC and GC-MS analyses of the methylene chloride extracts of female *L. subjuncta* abdominal tips revealed the presence of three suspect pheromone compounds. Mean amounts per female of these compounds from the three pooled samples of female abdominal tip extract were 24.7 ± 10.7 ng for the *Z*11-16:Ac, 5.0 ± 4.4 ng for *Z*11-16:Ac, and 0.37 ± 0.15 ng for *Z*11-16:Alc. The ratio of major to minor components was 25:5:0.4 (1:0.2:0.02) based on amounts calculated from GC peak areas. These components coeluted with *Z*11-16:Ald, *Z*11-16:Ac, and *Z*11-16:Alc and revealed spectra comparable to library spectra and spectra of synthetic standards of these same three compounds. However, the isomer configurations could not be distinguished absolutely. The retention index on DB-1 for synthetic samples of both *E*11- and *Z*11-16:Ac was 1977 and the major component in the extract was also 1977. The minor components in the extract had retention indices of 1785 compared to 1784 for a synthetic standard of *Z*11-16:Ald and 1849 compared to 1848 for a synthetic standard of *Z*11-16:Alc. On DB-wax the major component had a retention index of 2349 compared to 2346 for synthetic *Z*11-16:Ac and 2339 for synthetic *E*11-16:Ac. The minor components in the extract had retention indices on DB-wax of 2193 compared to 2194 for synthetic *Z*11-16:Ald and 2428 compared to 2430 for synthetic *Z*11-16:Alc and 2421 for *E*11-16:Alc.

Mass spectral analysis of the DMDS derivatives resolved the isomer identification of the hexadecenyl acetate. Derivatized standards of *Z*11- and *E*11-16:Ac showed the following diagnostic peaks (Buser et al., 1983): for *Z*11-[M^+376 (18%), A^+117(57%), B^+259(100%)], at a retention time of 32.32 min; for *E*11- [M^+376(15%), A^+117(59%), B^+259(100%)] at a retention time of 32.70 min. The derivative of the major component from the pheromone extract gave diagnostic peaks of M^+376(19%), A^+117(58%), B^+259(100%) at retention time of 32.09 min, confirming its identity as *Z*11-16:Ac. The derivatized extract also showed a peak at 24.86 min, which had diagnostic peaks at M^+332(25%), A^+117(96%), B^+215(100%). A derivatized *Z*11-16:Ald stan-

dard gave peaks at $M^+332(22\%)$, $A^+117(100\%)$, $B^+215(92\%)$ and eluted 24.86 min, which support the identity of a minor component as Z11-16:Ald.

Field Tests. In the initial comparison of ratios of Z11-16:Ac and Z11-16:Ald as trap lures (ANOVA $F = 18.4$, $df = 124$, $P < 0.001$), greatest numbers of males of *L. subjuncta* were captured in traps baited with Z11-16:Ald and Z11-16:Ac at a load ratio of 5:1 (17.1% Z11-16:Ald) (Figure 1A). Significant numbers of males were also captured in traps baited with these compounds at a load ratio of 17:1 (5.6% Z11-16:Ald). At other load ratios, captures of males were nearly 0 and were not significantly greater than the captures of males in unbaited traps. The total number of *Lacanobia subjuncta* males captured in this test was 352.

In the second comparison of ratios of Z11-16:Ac and Z11-16:Ald (ANOVA $F = 4.1$, $df = 119$, $0.01 < P < 0.001$), with load ratios of from 17:1 to 2:1, respectively, the greatest numbers of male *L. subjuncta* were captured in traps baited with a load ratio of 4:1 (Figure 1b). However, captures of males in traps baited with the two pheromone components at load ratios of 8:1, 6:1, 4:1, and 2:1 were statistically similar, and all were significantly higher than captures of males in traps baited with pheromone at load ratios of 12:1 and 17:1. Total number of male *L. subjuncta* captured in this experiment was 314.

In the third experiment, comparing captures of males in traps baited with different percentages of Z11-16:Alc in the three-component blend (ANOVA $F = 6.0$, $df = 179$, $P < 0.001$), the greatest numbers of males were captured in traps baited with 1.9% Z11-16:Alc, which was significantly greater than numbers of males captured in traps baited with 0%, 10.1%, or 20% Z11-16:Alc, but not significantly greater than numbers of males captured in traps baited with 0.6 or 3.2% Z11-16:Alc (Figure 2). Almost no moths were captured in traps baited with 20% Z11-16:Alc, significantly less than those in traps baited with the two component blend missing the alcohol. A total of 823 male *L. subjuncta* were captured in this test.

In the fourth experiment, the greatest numbers of male *L. subjuncta* were captured in traps baited with the greatest dose of the three-component blend tested (6.1 mg/septum) (Figure 3). Each dose of pheromone was significantly higher than any lower doses by Duncan's multiple-range test at $P < 0.05$. Regression analysis also revealed a significant effect of pheromone dose on trap catch ($F = 17.1$, $df = 5$, $P = 0.01$, $r^2 = 0.81$). A total of 3026 male *L. subjuncta* were captured in this test.

DISCUSSION

Results of these analyses and experiments revealed that the three compounds (Z)-11-hexadecenal, (Z)-11-hexadecenyl acetate, and (Z)-11-hexa-

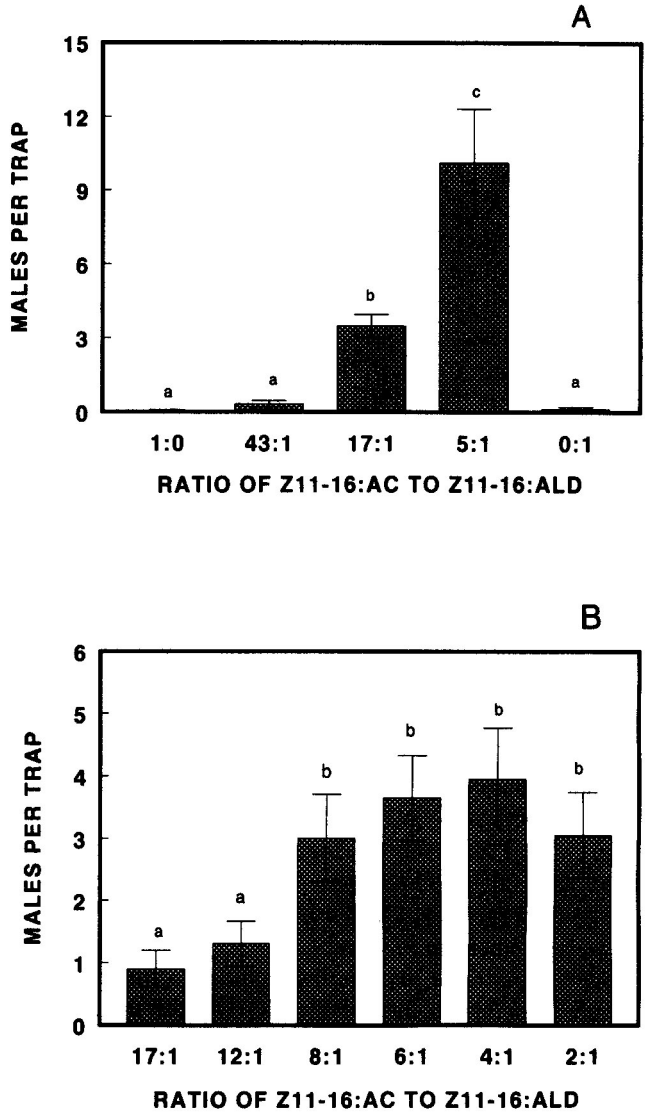

FIG. 1. Mean (±SE) numbers of male *Lacanobia subjuncta* moths captured in traps baited with blends of Z11-16:Ac and Z11-16:Ald differing in the load ratios of the components. The first comparison (A) consisted of a broader range of ratios, while the second comparison (B) consisted of a range of ratios closer to that found in female gland extracts. Bars with the same letter are not significantly different by Duncan's new multiple-range test.

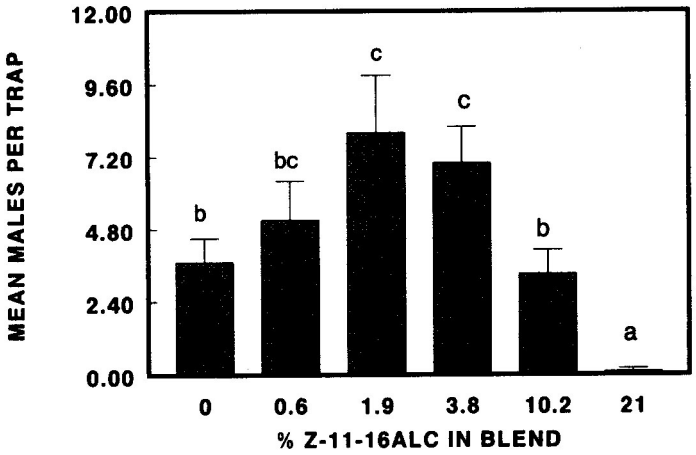

FIG. 2. Mean (±SE) numbers of male *Lacanobia subjuncta* moths captured in traps baited with different percentages of Z11-16:Alc in the three component pheromone blend. Bars with the same letter are not significantly different by Duncan's new multiple-range test.

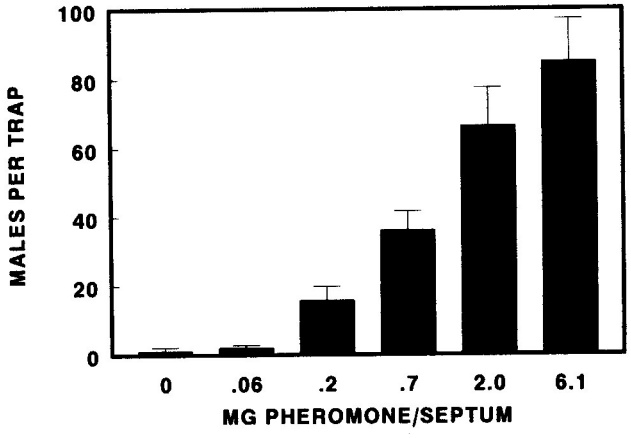

FIG. 3. Mean (±SE) numbers of male *Lacanobia subjuncta* moths captured in traps baited with different doses of the three component blend comprised of Z11-16:Ac, Z11-16:Ald, and Z11-16:Alc.

decenol comprise the sex pheromone of female *Lacanobia subjuncta*. These three compounds were found in solvent extracts of female abdominal tips. When in combination the two compounds Z11-16:Ac and Z11-16:Ald, or the three compounds Z11-16:Ac, Z11-16:Ald, and Z11-16:Alc, were attractive to males in field trials, making them both sex attractants and sex pheromones for this species.

Sex attractants are known for other species of *Lacanobia*, based on field screening of likely sex pheromone compounds (Roelofs and Comeau, 1970; Steck et al., 1980, 1982a,b). These sex attractants are combinations of (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, (Z)-11-tetradecenyl acetate, Z11-16:Ac, and corresponding aldehydes. Descoins et al. (1978) isolated and identified a sex pheromone from pheromone glands of females of *Lacanobia oleracea* that consists of Z11-16:Ac and (Z)-11-hexadecen-1-ol. The two compounds Z11-16:Ald and Z11-16:Ac that we report here as part of the sex pheromone of *L. subjuncta* also were reported as a sex attractant for the related *Lacanobia atlantica* (Grote) by Steck et al. (1980, 1982a) and for *Lacanobia nevadae* (Grote) (Steck et al., 1982b). Steck et al. (1982a) reported capturing males of *Papestra ingravis* (Small) with a combination of Z11-16:Ac and (Z)-11-tetradecenyl acetate. Z11-16-carbon compounds appear to be common constituents of sex pheromones and sex attractants for noctuids in the subfamily Hadeninae (Steck et al., 1982b), of which *Lacanobia subjuncta* is a member.

This blend of compounds should provide a useful means for monitoring the emergence and flight periods of this minor pest of apple and other fruits. The compounds can be formulated in rubber septa to provide long-lasting lures. Of the doses and ratios tested, a load dose of 6.1 mg of Z11-16:Ac, Z11-16:Ald, and Z11-16:Alc at a load ratio near 3:1:1 provided the greatest captures of male *L. subjuncta*. However, additional experiments must be conducted to determine what doses are optimum for use in monitoring traps. At high population levels, the higher doses tested may be too attractive, requiring frequent emptying of traps and costly maintenance schedules. All experiments reported here were conducted in orchards in which no damage to foliage or fruit was evident, indicating relatively low population levels. Under very low population levels and in detection programs, it may be advantageous to use higher doses to increase the sensitivity of the lure.

Very few moths of other species were captured during these experiments, although our testing extended through much of the summer season, from mid-May to late August. During May and the first-generation flight of *L. subjuncta* in Washington, several male *Egira rubrica* (Harvey) (Noctuidae) were captured in traps baited with Z11-16:Ac and Z11-16:Ald. During late August, a small number of male glassy cutworms, *Crymodes devastator* (Brace), were captured in traps baited with 0.2- and 0.7-mg dosages of the three component blend.

With higher doses and at recommended component ratios, there should then be no interference from additional species of moths.

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COMMUNAL FORAGING BEHAVIOR AND RECRUITMENT COMMUNICATION IN *Gloveria* sp.

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Abstract—The caterpillars of *Gloveria* sp. mark trails with a pheromone they deposit by dragging the ventral surface of the tip of the abdomen along branch pathways as they move between their communal nest and distance feeding sites. The threshold sensitivity of the caterpillar for an extract prepared from the secretory site was approximately 0.5×10^{-3} caterpillar equivalents/cm of trail. Bioassays show that *Gloveria* follows neither authentic trails of *Malacosoma americanum* nor artificial trails prepared from 5β-cholestane-3-one, a chemical previously reported to elicit trail following from other social caterpillars. Although our observations show that fed caterpillars mark heavily as they return to their nest, we found no evidence that individual caterpillars are able to recruit hungry nestmates to new food finds. In this species, recruitment to food occurs only after many caterpillars have reinforced a trail to a newly discovered food source. In contrast, hungry caterpillars of the confamilial species *M. americanum*, tested under identical conditions, responded strongly to the postprandial trails of individual caterpillars and rapidly abandoned depleted sites in favor of new food finds. We postulate that the difference in the efficiency with which these two species recruit nestmates to food evolved in response to differences in the spatial distribution of their food supplies.

Key Words—*Gloveria, Eucheira, Malacosoma*, trail pheromone, recruitment, foraging, Lasiocampidae.

INTRODUCTION

Studies of tent caterpillars (*Malacosoma* spp.) show that the larvae secrete a trail pheromone from the ventral surface of the tip of the abdomen (Fitzgerald,

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1995 and references therein). The best known of these species, *M. americanum*, utilizes a system of recruitment communication in which successful foragers alert tent mates to the discovery of their food finds. Comparatively little is known of the foraging ecology of the additional 300 or so species of caterpillars that live in groups (Costa and Pierce, 1997), and we know correspondingly little of the antecedant behavioral and ecological factors that may have led to the evolution of recruitment communication in *Malacosoma*. Nonetheless, one factor thought to influence the evolution of recruitment in caterpillars is the mode of communal foraging. Fitzgerald and Peterson (1988) argued that colonies of caterpillars that launch forays from a fixed central location are more likely to evolve advanced systems of recruitment communication than colonies that forage in other ways. Those species of *Malacosoma* that recruit to food are central-place foragers and so are most ants and termites, taxa in which trail-based recruitment communication is particularly well developed.

A recent study of *Eucheira socialis* (Pieridae) showed the spatial distribution of the food supply may also affect the evolution of recruitment systems. Although *E. socialis* forages from a central location, it utilizes a relatively simple system of trail marking and does not recruit to food (Fitzgerald and Underwood, 1998). Comparison of the foraging ecology of this species with that of *M. americanum* showed that while the two species are similar in most respects, the caterpillars of *Eucheira* are much less selective in their feeding habits, leading Fitzgerald and Underwood (1998) to suggest that the patchiness of the food supply and the concomitant need to search extensively for food may have led to the fine-tuning of the foraging system of *M. americanum*. *Eurcheira* and *M. americanum*, however, are only remotely related, and it can be reasonably argued that prediposing factors based on phylogeny rather than food distribution may underlie differences in their trail-based systems of communication. A better test of the potential role of food patchiness and feeding selectivity in the evolution of recruitment communication among central place foragers would involve comparisons of congeneric or confamiliar species whose foraging systems differ with respect to this factor. We report here the results of one such study involving the caterpillar *Gloveria* sp. (Lepidoptera: Lasiocampidae), a montane species found in the pine forests of Mexico.

The taxonomy of the Central American *Gloveria* is poorly resolved and the specific status of the caterpillar we report on here is unknown (personal communication, F. W. Stehr). It is referred to hereafter by the generic name only. The larval biology of *Gloveria* has not been previously documented, but our observations show that colonies consisting of up to several hundred caterpillars construct a hanging silk nest in the branches of pine trees. The larvae leave the nest en masse to feed at distant sites on the needles of the tree once or twice each night, always under the cover of darkness. They return to the structure immediately after feeding and rest there until the onset of the next bout of

foraging. The overt features of the foraging ecology of *Gloveria* are very similar to those of *M. americanum*, but, unlike the latter, our observations show that the caterpillars of *Gloveria* feed nonselectively, consuming both 1-year-old needles and the current year's growth. Moreover, pines bear an abundant and dense food supply, so the caterpillars rarely need to forage further than a few meters from their nest to obtain adequate food.

METHODS AND MATERIALS

Insect Collection and Rearing. Caterpillars for laboratory studies were collected from pine forests along Highway 40 in the Sierra Madre Occidental Mountains in Sinaloa and Durango states, Mexico. Caterpillars were found on four species of pine in the subgenus *Diploxylon*, all of which have relatively soft needles (Perry, 1991). Preliminary laboratory feeding tests showed that of the species of pine found in the vicinity of Cortland, New York, the caterpillars preferred white pine (*Pinus strobus*), and colonies were provided with branches of this species during our studies. A total of 18 field-collected colonies were maintained in the laboratory. Since the caterpillars feed only in darkness, all tests involving whole colony movement patterns were conducted under light provided by a 25-W red bulb during a colony's normal activity period.

Bioassays. Although *Gloveria* caterpillars mark the branches they follow with conspicuous silk trails, previous studies of lasiocampid caterpillars in the genus *Malacosoma* showed that an extra silk trail marker secreted from the ventral surface of the tip of the abdomen is primarily responsible for eliciting trail following (Fitzgerald, 1995 and reference therein). Observations were made of *Gloveria* caterpillars as they moved over branches to determine if they also marked trails by lowering and dragging the tip of their abdomen. In addition, we observed the response of caterpillars to tip extract prepared by cutting the terminal 5 mm of the tip of the abdomen from 60 caterpillars, then extracting the tips in hexanes. After soaking for 24 hr, the tips were removed and the solvent concentrated to 0.5 ml. The concentrated extract was serially diluted and the dilute extract bioassayed to determine the threshold sensitivity of the caterpillar. Bioassays were conducted by placing 5 μl of the extract in a 2-cm-long straight line on a paper card. A first-instar was placed at one end of the line and its response noted. A bioassay was considered positive if a caterpillar walked to the distant end, turned, and retraced its pathway. As a control, we tested similar trails prepared from solvent alone.

We observed the response of first-instars allowed to select between an extract trail at threshold strength and one 10 times stronger. These bioassays were conducted with paper Y mazes, the design of which is described elsewhere (Peterson and Fitzgerald, 1991). Five-microliter quantities of extract at the dif-

ferent concentrations were applied to the 2-cm-long arms of the maze. The stem of the maze was treated with the stronger of the two concentrations.

Potentially pheromonally active chemicals were tested by swiping the venter of the abdominal tip of a fifth-instar along a 4-cm-length of a 5-mm-diameter wooden dowel. The responses of fifth instars to the trail were observed during their normal foraging bouts. In addition, we recorded the response of fifth instars to tip-extract trails prepared as above and to 5β-cholestane-3-one, a compound previously shown to elicit trail following from *M. americanum* (Fitzgerald, 1995 and references therein) and the larvae of the pierid butterfly *Eucheira socialis* (Fitzgerald and Underwood, 1998). Artificial trails were prepared by placing the candidate material on a wooden dowel at the approximate rate of 2.5 μl/cm. Trails prepared from solvent alone were used as controls. The responses of caterpillars to the trails of laboratory colonies of *M. americanum* laid down on dowels during their normal activity bouts were also observed. Bioassays of all dowel trails were conducted with fifth instars of *Gloveria* as they were returning to the nest after feeding. Preliminary observations indicated that fed caterpillars are more cautious and selective than hungry caterpillars and they were used exclusively for all bioassays involving mature caterpillars. A failure to respond within 3 min was considered a negative response. All bioassays were replicated with 10 different caterpillars, each time with a new candidate trail.

The responses of fifth instars to silk pulled from the spinneret and wound onto wooden dowels were tested. The procedure for obtaining silk in this manner has been published elsewere (Fitzgerald, 1993). In addition, a series of Y-choice tests were conducted in which caterpillars were allowed to choose between silk trails so prepared and extract trails prepared as noted above. The alternate arms of the Y consisted of either silk or extract; the stem was made from a section of wooden dowel the colony had marked as the caterpillars moved back and forth between their nest and food and thus consisted of both silk and tip marker. To test the response of a caterpillar to the two trail types, the stem of the maze was placed in its pathway as it returned from feeding during a normal activity bout. Sides on which the treatments were presented were alternated for each successive test to preclude a positional bias.

We conducted studies to determine the conditions under which *Gloveria* larvae mark trails with the tip of their abdomen. Colonies were maintained on wooden stands having wooden dowel bridges connecting their nest to a food plant. Colonies expanded the nest as the caterpillars grew and made once or twice daily en masse movements to food during the scotophase of a 12L:12D laboratory light cycle. After allowing the caterpillars a minimum of one week to attach their nest to the stand and establish a regular feeding schedule, we made nightly observations to their marking behavior by using the following procedure. At the onset of a feeding bout, a new section of doweling was abutted to the end of the bridge bearing the well-established trail that connected the nest

to the food plant. As the caterpillars moved to food, we recorded the number that marked the substrate by dragging the tip of their abdomen. Observations were made of caterpillars as they moved over their established trail and over the new bridge section. The marking behavior of caterpillars that turned back to the nest without reaching the food plant was also recorded, as was the marking behavior of caterpillars that fed and were returning to the nest. Each set of observations was replicated four to five times with three to four different colonies. To facilitate observations, we used magnifying glasses with red-light illuminators. We saw no indication that the red light in any way affected the behavior of the caterpillars.

We conducted an investigation to determine if caterpillars recruited nestmates to food finds as previously shown for *Malacosoma* (Fitzgerald, 1995 and references therein). Colonies were allowed to move to food for two consecutive nights by crossing a new dowel bridge. The bridge consisted of a proximal stem section and a distal arm. The arm was attached to the end of the stem at an angle of 15°. At the start of the third night, a second arm was joined to the end of the stem to form a Y, the arms of which were separated by an angle of 30°. The food supply was then moved from its original position to the end of the new arm. Caterpillar activity on the bridge was monitored the next night or over the next two nights by videorecording at a rate of one frame per second. Recordings were analyzed to assess the role of communication in the process that led to the establishment of a trail to the new site and the abandonment of the old site. The study was replicated eight times with different colonies.

Although recruitment communication has been well documented for *M. americanum* (Fitzgerald, 1995 and references therein), the circumstances under which previous studies were conducted differed significantly from the experimental protocol used in our study of *Gloveria*. For comparative purposes, we therefore conducted four replicates of the above study with four different colonies of *M. americanum*. Colonies were reared from egg masses collected locally. Caterpillars were provided with branches of black cherry, *Prunus serotina*, as a food supply.

Statistics. Statistical analyses were conducted with Sigmaplot or Prostat statistical software.

RESULTS

Our observations show that *Gloveria* caterpillars drag the tip of their abdomen against the substrate when marking trails. In 10 tests, caterpillars maintained in colonies readily moved onto dowels bearing artificial trails prepared either from material obtained by swiping the tip of the abdomen or from tip extract. In contrast, none of 10 caterpillars moved onto solvent-treated dowels

that served as controls. The threshold sensitivity of a caterpillar for tip extract, as determined by bioassay, was approximately 0.5×10^{-3} caterpillar equivalents/cm of trail. When allowed to choose between alternate arms of a Y maze treated with pheromone applied at threshold strength or one order of magnitude greater, 18 of 20 caterpillars selected the stronger trail ($P < 0.01$, chi-square test). When presented with dowels on which silk had been wound, all of 10 fifth instars ventured onto the dowels, but showed marked hesitancy. When caterpillars were allowed access to either a dowel bearing wound silk or tip extract in Y-choice tests, 12 of 15 chose the tip extract over the silk ($P = 0.02$, chi-square test). Collectively, these tests indicate that an extra-silk trail pheromone secreted from the region of the abdomen lying between the anal prolegs is primarily responsible for eliciting the trail following response. Silk, the conspicuous component of the trail, plays a minor role and most likely functions to facilitate traction on smooth bark and leaf surfaces.

In 10 tests, *Gloveria* caterpillars failed to move onto dowels bearing either trails of the eastern tent caterpillar or artificial trails prepared from 5β-cholestane-3-one.

The results of observations of the trail-marking behavior of *Gloveria* caterpillars as they moved to and from food over established trails and over previously unmarked sections of bridging are shown in Table 1. As hungry caterpillars move to food, they mark previously unmarked branches more than they overmark established trails. Caterpillars that turn back to the nest after failing to find food overmark new and established trails little if at all. Caterpillars

TABLE 1. PERCENT OF FED AND UNFED *Gloveria* sp. CATERPILLARS MARKING AS THEY MOVED OVER A BRANCH MARKED DURING PREVIOUS FORAYS (ESTABLISHED TRAIL) AND A BRANCH NOT MARKED DURING PREVIOUS FORAYS (NEW BRANCH)

Condition and direction of travel	Substrate crossed	Colonies (*N*)	Replicates (*N*)	Caterpillars observed (*N*)	Percent marking (mean ± SE)[a]
Unfed					
Outbound	Established trail	4	5	310	62.6 ± 10.0a
	New branch	4	5	403	89.0 ± 5.2b
Turn-back	Established trail	4	5	296	8.8 ± 2.3c
	New branch	3	4	146	7.2 ± 4.8c
Fed					
Inbound	New branch	4	5	353	90.0 ± 3.9b
	Established	3	4	258	22.0 ± 4.4c

[a]Means followed by the same letter are not significantly different ($P > 0.05$). Student-Newman-Keuls test of arcsine $\sqrt{P}$ transformations.

that find food and feed overmark the newly established trail heavily as they follow it back to the nest, but mark little after they move onto the well-established portions of their trail system. In no case did a caterpillar continue to mark on the surface of the nest.

Y-choice tests to determine the effectiveness of fed caterpillars in recruiting nestmates to their food finds showed that hungry caterpillars were slow to abandon the established trail even after numerous caterpillars had fed at the new site and returned to the nest. Table 2 and Figure 1 show the number of choices for alternate pathways that the caterpillars made beginning immediately after the first fed caterpillar returned to the nest. In only one of eight tests did caterpillars favor the new branch over the established trail during the first half of the foray following the return of the first fed caterpillar, despite the fact that many fed caterpillars returned to the nest during this period. Test results for the eight replicates were significantly heterogeneous, indicating that during this half of the foray the new and established trails were not different enough for larvae to consistently distinguish between them. In contrast, larvae showed a significant preference for the trail leading to the new food source over the previously established trail during the second half of the tests (Table 2). Collectively, the results indicate that the choice of caterpillars is not strongly affected by the trail pheromone deposited by individual caterpillars as they return to the nest after feeding. The shift to the new trail occurs only after many caterpillars have reinforced the trail both as they move to food and again as they return to the nest. For colonies 5, 6, and 7 (Table 2), we also recorded the foray on the second night after shifting the position of the food. In all three cases, the colonies significantly favored the new trail over the old ($P < 0.01$, chi-square test) with $81.7 \pm 6.4\%$ (SE) of the choices made by the caterpillars during the foray in favor of the new trail (Figure 1). This indicates that trails deposited on the new branch during the previous evening's foray persisted into the following night, enabling the caterpillars to move efficiently to the food find.

In contrast, comparative tests show that *M. americanum* caterpillars are able to distinguish between a trail that leads to food and one that leads to a depleted feeding site after as few as one fed caterpillar has marked the new trail (Table 3, Figure 2). For the first half of the choices, all tests were significant in favor of the new trail, all were significantly homogeneous, and pooled tests were significant. The second half of the experiment had similar results except that individual trials were more heterogeneous; however, all were significant with the same polarity.

DISCUSSION

It is estimated that of the approximately 300 species of caterpillars that are known to forage in groups, 46% show low mobility and typically feed within

TABLE 2. NUMBER OF CHOICES FOR ORIGINAL AND NEW BRANCHES MADE BY *Gloveria* sp. CATERPILLARS FOLLOWING RETURN OF FIRST FED CATERPILLARS AFTER FOOD WAS SWITCHED FROM END OF ORIGINAL BRANCH TO END OF NEW BRANCH

	Total				First half					Second half				
Colony	New	Original	G^a	P	New	Original	Fed-back[b]	G	P	New	Original	Fed-back	G	P
1	25	41	3.92	0.05	13	20	8	1.50	0.22	12	21	41	2.49	0.11
2	32	31	0.02	0.90	13	19	16	1.13	0.29	19	13	43	1.13	0.29
3	101	38	29.62	**	52	18	69	17.23	**	49	20	96	12.57	**
4	26	114	59.70	**	6	64	13	56.09	**	20	50	50	13.28	**
5	84	63	3.01	0.83	31	43	26	1.95	0.16	53	20	112	15.47	**
6	75	51	4.60	0.03	29	33	40	0.26	0.61	46	18	94	12.67	**
7	68	65	0.07	0.80	27	39	27	2.19	0.14	41	26	110	3.38	0.07
8	100	72	4.58	0.32	41	45	11	0.19	0.67	59	27	102	12.20	**
Total	511	475	105.51	**	212	281		80.54	**	299	195		73.21	**
Heterogeneity			104.19	**				70.85	**				51.15	**
Pooled			1.31	0.25				9.69	**				22.06	**

[a] Replicated *G*-test statistic.
[b] Fed-back column indicates the total number of caterpillars that had fed and returned to the tent by the end of the first and second half of the observations. $^{**}P < 0.01$.

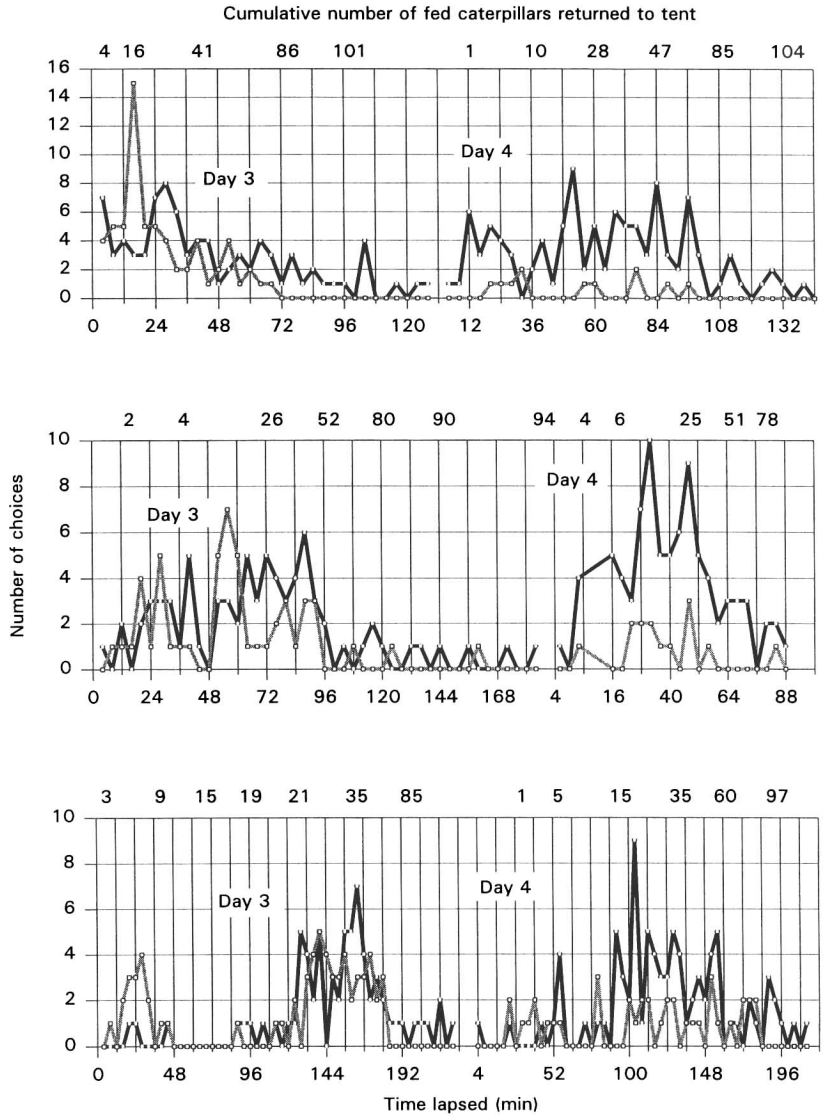

FIG. 1. Choices by *Gloveria* caterpillars during their first and second overnight foraging bouts after the food source was switched from the end of a branch they had previously marked (gray line) to the end of a new, previously unmarked branch (black line). Data for the first night show the distribution of responses beginning immediately after the first fed caterpillar returned from the food find. The entire foraging bout is recorded for the second night. The cumulative number of caterpillars that fed and returned to the nest is shown at the top. Replicates from top to bottom are for colonies 5–7 in Table 2.

TABLE 3. NUMBER OF CHOICES FOR ORIGINAL AND NEW BRANCHES MADE BY *Malacosoma* CATERPILLARS FOLLOWING RETURN OF FIRST FED CATERPILLAR AFTER FOOD WAS SWITCHED FROM END OF ORIGINAL BRANCH TO END OF NEW BRANCH

	Total				First half					Second half				
Colony	New	Original	G^a	P	New	Original	Fed-back[b]	G	P	New	Original	Fed-back	G	P
1	73	14	43.84	**	34	10	3	13.83	**	39	4	32	33.00	**
2	223	117	33.60	**	109	61	5	13.74	**	114	56	10	20.19	**
3	189	108	22.37	**	91	40	1	20.39	**	98	68	2	5.45	0.02
4	70	25	22.19	**	35	13	4	10.47	**	35	12	27	11.75	**
Total	555	264	122.01	**	269	124		58.43	**	286	140		70.39	**
Heterogeneity			16.32	**				3.64	0.30				19.32	**
Pooled			105.68	**				54.78	**				51.06	**

[a] Replicated G-test statistic.

[b] Fed-back column indicates the total number of caterpillars that had fed and returned to the tent by the end of the first and second half of the observations. $^{**}P < 0.01$.

a single patch of leaves throughout their larval life, 43% are nomadic, moving from one patch to the next; and 11% are central place foragers, mounting intermittent forays between their resting site and distant patches of food (Costa and Pierce, 1997). Knowledge of the role of trail marking in cooperative foraging is based on studies of fewer than 3% of these species. The few representatives of patch-restricted foragers studied indicate that an arena or substrate marker intimately associated with silk plays a major role in holding aggregates together (Capinera, 1980; Roessingh, 1989, 1990; Fitzgerald, 1993). Recruitment to food has not been demonstrated in these species. The single nomadic forager studied, *Malacosoma disstria*, marks trails in the manner of *M. americanum* but recruits to bivouacs rather than to food finds (Fitzgerald and Costa, 1986). Besides *Gloveria*, the only central-place foragers for which we have detailed knowledge are *E. socialis* (Fitzgerald and Underwood, 1998), *M. americanum* (Fitzgerald, 1995), and *M. neustrium* (Peterson, 1988). Attributes of the foraging and trail-marking system of *Gloveria* identified during this study are compared with those of *M. americanum* and *E. socialis* in Table 4 and are discussed below.

Caterpillars of *Gloveria, Malacosoma*, and *Eucheira* all mark trails with a pheromone deposited from a ventral secretory site located at the tip of the abdomen. The motor pattern associated with trail marking in *Gloveria* is identical to that reported for *Malacosoma*, but *E. socialis* uses a distinctly different pattern, marking during only part of a locomotive cycle and depositing a discontinuous trail. Fitzgerald and Underwood (1998) suggested that marking with the tip of the abdomen in caterpillars is derived from advance–withdrawal conflict behavior associated with movement into new terrain. The motor pattern associated with this ambivalent behavior brings the ventral surface of the tip of the abdomen into contact with the substrate and sets the stage for its evolutionary conversion to a trail-marking organ. To the extent that this scenario is correct, the motor patterns associated with marking in *Eucheira* more nearly resemble the ancestral form, while those of *Gloveria* and *Malacosoma* are derivative patterns, emancipated from their original motivational basis and devoted more clearly to the trail-marking process.

Marking by dragging the abdomen is also the dominant mode of trail laying employed by termites and ants, although in the latter trail pheromones are frequently delivered from the tip of the extended stinger (Wilson, 1971). While ants and termites lack anterior secretory sites that might serve in this capacity, this is clearly not the case in caterpillars. Most caterpillars, including the three species discussed here, lay continuous strands of silk secreted from a spinneret located just below the mouthparts. The silk of some patch-restricted foragers contains a pheromone that serves as an arena marker, and it would seem no less feasible that the silk of the central place foragers considered here might contain a trail marker. It is unclear why trail marking evolved as a separate function from silk secretion in these species, but one reason may be that their silk serves

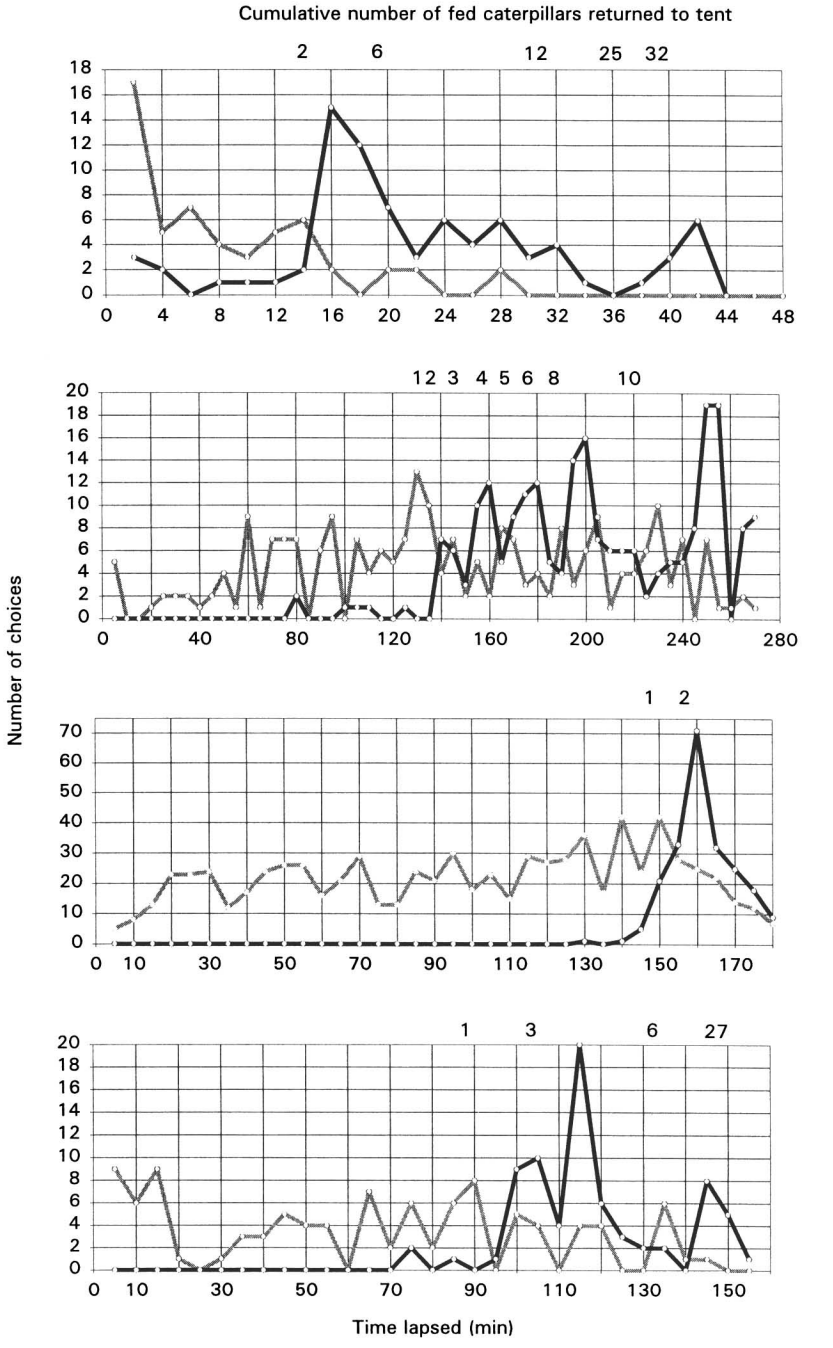
Cumulative number of fed caterpillars returned to tent
2
6
12
25
32
12 3 4 5 6 8
10
1 2
1
3
6
27
Number of choices
Time lapsed (min)

TABLE 4. COMPARISON OF FORAGING AND TRAIL-MARKING BEHAVIOR OF *Gloveria* sp., *Malacosoma americanum*, AND *Eucheira socialis*

Trait	*Gloveria*[a]	*Malacosoma*[b]	*Eucheira*[c]
Trails marked with tip of abdomen	yes	yes	yes
Marking motor pattern	continuous	continuous	cyclic
Marking new branches	yes	yes	yes
Marking on turn-back when searching for food	no	no	yes
Marking after feeding	yes	yes	no
Marking onto nest after feeding	no	yes	no
Recruitment to trails	yes	yes	yes
Bilevel trail system	no	yes	no
Recruitment to food	weak	strong	none
Individuals recruit to food	no	yes	no
Sensitivity to differences in trail strength	high	high	high
Role of silk in trail following	minor	minor	minor
Response to 5β-cholestane-3-one	−	+	+
Patchiness of food supply	low/occasional	high/chronic	low/occasional
Selective foraging	no	yes	no
Collective flexibility	low	high	low

[a] This study.
[b] Fitzgerald (1995, and references therein).
[c] Fitzgerald and Underwood (1997).

other functions, precluding its unambiguous use in a communicative context that goes beyond mere arena marking. The central-place foragers discussed here secrete silk in copious quantities, both to construct their communal shelters and to provide purchase on the smooth surfaces of the host plant. A second reason may be that marking from the posterior end of the body enables caterpillars to assess alternative pathways at branch junctures by advancing nearly a full body length onto them without simultaneously and indiscriminantly marking them.

Laboratory bioassays of trail markers show that *Gloveria, M. americanum*, and *E. socialis* respond to quantitative increases in pheromone laid down on a pathway, preferring stronger trails over weaker ones. Thus, marks left by caterpillars as they move over branches increase the probability that others will

FIG. 2. Choices by *M. americanum* caterpillars during their first foraging bout after the food source was switched from the end of a branch they had previously marked (gray line) to the end of a new, previously unmarked branch (black line). The distribution of responses for the entire foraging bout is recorded. The cumulative number of caterpillars that fed and returned to the nest is shown at the top. Replicates from top to bottom are for colonies 1–4 in Table 3.

follow. All three species thus recruit to trails. *Gloveria* and *Malacosoma*, but not *Eucheira*, also mark trails as they return from feeding and thus recruit to food. However, the recruitment system of *Gloveria* is less efficient than that of *Malacosoma*, allowing colonies of the latter to abandon a trail to an exhausted feeding site more rapidly than the former. The evolutionary refinement of trail-based communication leading to a colony's ability to redirect foraging activity from one site to another has been previously described in ants and termed "collective flexibility" (de Biseau et al., 1992). Once a trail to a food find is established, species of ants that have low collective flexibility continue to forage at the site even if a richer food source has been discovered elsewhere (Beckers et al., 1990; de Biseau et al., 1992). In contrast, species of ants that are collectively flexible override the attraction of an established trail by varying the amount of trail marker laid down or by using different chemicals and/or leading small groups of colony mates to the food source (Beckers et al., 1990; de Biseau et al., 1992, 1994). We found no evidence that colonies of *Gloveria* exhibit the collective flexibility necessary to efficiently abandon an established feeding site in favor of a newer and richer food find. The trail deposited by a larva of *Gloveria* as it returns to the nest after feeding does not appear any more effective in eliciting trail following than a trail deposited by a hungry caterpillar searching for food. In contrast, *M. americanum* employs a trail marking system that allows colonies to quickly abandon one site in favor of another. Moreover, unlike *Gloveria*, fed, returning caterpillars of *Malacosoma* lay trails that extend without interruption from their feeding site to the surface of their tent, thus rendering the structure a communication center. As is the case with these confamilial species, studies of ants have also shown that the evolution of recruitment does not necessarily follow phylogenetic lines but is associated with ecological parameters and colony size (Traniello, 1989; Peeters, 1997).

Hölldobler (1976) suggested that the evolution of group foraging and the ability to recruit in the ant genus *Pogonomyrmex* may be related to the dispersion of food. The species that use a patchily distributed food (*P. barbatus* and *P. rugosus*) have more efficient recruitment systems than those species that harvest dispersed seeds (*P. maricopa*). Similarly, our study suggests that the trail-marking system of *M. americanum* may be fine tuned relative to those of *Gloveria* and *E. socialis* to allow the caterpillars to deal with food as a chronically scarce resource. *M. americanum* prefers to feed on a tree's youngest leaves, and the caterpillars search extensively to find them (Fitzgerald, 1995). In addition, caterpillars often eclose from their egg mass before leaf flush, and the first instars must comb the tree in search of the first few leaves to appear (Fitzgerald and Willer, 1983). Although both *E. socialis* and *Gloveria* may occasionally need to deal with a patchily distributed food supply under conditions of high population density, our observations show that they forage with little or no discrim-

ination on leaves that are typically abundant and close at hand relative to their needs.

Comparison of the results of the Y-choice experiments for *Gloveria* reported here with similar Y-choice experiments reported for *E. socialis* (Fitzgerald and Underwood, 1998) shows that even though *E. socialis* does not recruit to food, the two species differ little in the efficiency with which they abandon an exhausted feeding site in favor of a new food find. Our Y-choice tests simulated conditions caterpillars are likely to encounter early in the life of a colony when nearly any branch explored leads to food. The test provided only two alternative pathways, whereas on natural host trees there would be many alternate choices. The performance of the two species could be expected to differ even less under more natural conditions, particularly when the host experiences heavy defoliation, as can occur when multiple colonies infest trees. Under such conditions, the habit of *Gloveria* to mark branches that lead to food more heavily than those that do not would be expected to channel hungry caterpillars to food discoveries more efficiently than the less selective marking system of *E. socialis*.

Despite the responsiveness *E. socialis* to the trails of *M. americanum* and to 5β-cholestane-3-one (Fitzgerald and Underwood, 1998), the compound is not a universal caterpillar pheromone. In addition to *Gloveria*, the caterpillars of the gregarious tent building species *Archips cerasivoranus* (Tortricidae) and *Hyphantria cunea* (Arctiidae) (T.D.F. personal observation) show no response to the material.

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MICROORGANISMS AND CELLULOSE DIGESTION IN THE GUT OF THE WOODLOUSE *Porcellio scaber*

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Abstract—In the common woodlouse *Porcellio scaber* different parts of the gut were observed with respect to microbial counts, cellulose activity, and degradation of cellulose. Cellulose is mainly digested in the anterior part of the hindgut, as was indicated by the distribution of cellulolytic activity and the decrease of cellulose content inside the gut. The cellulases woodlice utilize for the degradation of litter are mainly produced by endosymbiotic bacteria in the hepatopancreas rather than by microorganisms ingested with the food. Microorganisms ingested with the litter are digested in the anterior part of the hindgut and may provide an important food source. In the posterior hindgut, bacterial proliferation ensures microbial colonization of feces.

Key Words—Terrestrial isopods, Oniscidea, *Porcellio scaber*, gut microbes, obligate endosymbionts, hepatopancreatic bacteria, cellulase activity, cellulose digestion, litter degradation, decomposition.

INTRODUCTION

Terrestrial isopods play an important role in the decomposition of leaf litter (Biwer, 1961; Cameron and LaPoint, 1978), and leaf litter is one of their most important food sources (Hassall and Rushton, 1984). Consequently, they need cellulolytic enzymes for the digestion of cellulose (Kozlovskaja and Striganova, 1977).

Hartenstein (1964) suggested that cellulases are produced endogenously in *Oniscus asellus* Linnaeus. Later, other investigators observed that isopods depend on the cellulolytic enzymes of ingested microorganisms (Hassall and Jennings, 1975; Kozlovskaja and Striganova, 1977), or at least utilize acquired fungal

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enzymes such as xylanase and cellulase (Kukor and Martin, 1986). The dependence of woodlice on microbial activity of the leaf litter, measured in terms of cellulase activity, was demonstrated subsequently (Uesbeck and Topp, 1995; Zimmer and Topp, 1997a). However, the distribution of cellulase activity and microbial counts inside the gut of *Porcellio scaber* Latreille has led to several contradictions, since parts of the gut exhibiting high cellulolytic activity contained comparably few microorganisms (Zimmer and Topp, 1998). Consequently, the aims of this study were to determine the nutritional significance of cellulolytically active microorganisms ingested with the litter to *P. scaber* and to localize the source of the cellulases *P. scaber* utilizes for cellulose digestion.

METHODS AND MATERIALS

Adults of *P. scaber* were collected in a mixed alder–poplar forest near Cologne, Germany. In the laboratory, the woodlice were kept separately in small Petri dishes, the bottoms of which were covered with Plaster of Paris to maintain sufficient humidity. Feeding experiments were carried out at 15°C and a photoperiod of 16L:8D. Each measurement was conducted with 15 specimens.

Prior to the experiments, specimens were fed with litter originating from the forest where the isopods had been collected. Under these conditions, microbial counts of different gut sections were determined and compared to the corresponding values of the food. Coprophagy during the experiment was minimized by removing fecal pellets every day. The investigated sections of the gut were the secretory lobes of the midgut (hepatopancreas) and the hindgut, which was subdivided into anterior and posterior parts (Hassall and Jennings, 1975; Hames and Hopkin, 1989).

Additionally to studies on isopods feeding on natural food, further experiments were performed with a chemically defined artificial diet that was rich in small carbohydrates and cellulose, to which was added 1% (dry wt) antibiotics (a mixture of penicillin, streptomycin, and amphotericin) or 5% (dry wt) tannic acid (Sigma, St. Louis, Missouri), respectively, to alter the microbial colonization and activity of the gut (Zimmer, 1998).

The content of glucose, cellulose, and cellulase activity was measured in the food, the gut, and the feces. All samples were treated in the same manner. Small samples (10–20 mg of food, 2–4 mg dissected parts of the gut, and 5–10 mg feces) were homogenized in 500 μl double-distilled water.

Cellulose content was measured as the amount of glucose released by hot acidic hydrolysis when a homogenate was boiled for 5 hr in 750 μl of 1.5 N HCl. After neutralizing (750 μl of 1 N NaOH), cooling, and centrifuging (10 min; 17,500*g*), the glucose content of the supernatant solution was determined with an enzymatic test for the "determination of glucose and fructose" (Boehringer, Mannheim, Germany). The amount of cellulose in the sample was cal-

culated by using a standard curve of hydrolyzed α-cellulose (Sigma). The measurement of cellulase activity in terms of the amount of released glucose after a previous incubation of the homogenized sample with α-cellulose in a phosphate buffer (pH 5.5) was conducted by using the same enzymatic test (Skambracks, 1996).

Counts of platable bacteria, actinomycetes, and fungi of the litter and the gut sections were determined as described in Treves and Martin (1994) by using nutrient agar (Sigma) for bacteria and actinomycetes and potato dextrose agar (Sigma) for fungi. Replicate plates were cultured under anaerobic conditions ("anaerocult", Merck, Darmstadt, Germany) to estimate the proportion of facultatively anaerobic microorganisms. The proportion of cellulolytically active microorganisms was determined by the overlay technique and subsequent staining with Congo red according to Teather and Wood (1982), by using crystalline α-cellulose as a substrate. Total microbial counts were estimated with the acridine orange method (Francisco et al., 1973).

Since most of the results were not normally distributed, data are given as median and median absolute deviation (M $\pm$ MAD). Multiple comparison of samples was performed with the Kruskall-Wallis H test. Subsequently, significant differences were localized using the Mann-Whitney U test. Prior to ANOVA, data were transformed to normality.

RESULTS

The content of cellulose in ingested litter decreased as it passed through the alimentary canal to the feces (Figure 1a). The secretory lobes of the midgut (hepatopancreas) contained only small amounts of cellulose, and the cellulose content in the anterior hindgut equaled that of the litter ($P > 0.2$). A significant decrease of the cellulose content was observed from the anterior to the posterior hindgut ($P < 0.05$). The same percent of cellulose as measured in the posterior hindgut was determined in the feces ($P > 0.3$).

The chemical breakdown of cellulose requires the activity of cellulolytic enzymes. Comparing the different gut sections, cellulase activity was significantly higher in the anterior hindgut ($P < 0.01$), but did not differ between the hepatopancreas and the posterior hindgut ($P > 0.3$; Figure 1b). Cellulose degradation results in the release of glucose (Figure 1c). The amount of enzymatically released glucose was significantly higher in the gut than on the litter and the feces ($P < 0.001$). The glucose content in the litter and the feces did not differ from each other ($P > 0.5$). Coinciding with the distribution of cellulolytic activity, the anterior hindgut contained the highest glucose concentration ($P < 0.01$). Hepatopancreas and posterior hindgut did not differ concerning the amount of free glucose ($P > 0.1$).

Litter was colonized by about 5×10^8 microbial cells/mg (total microbial

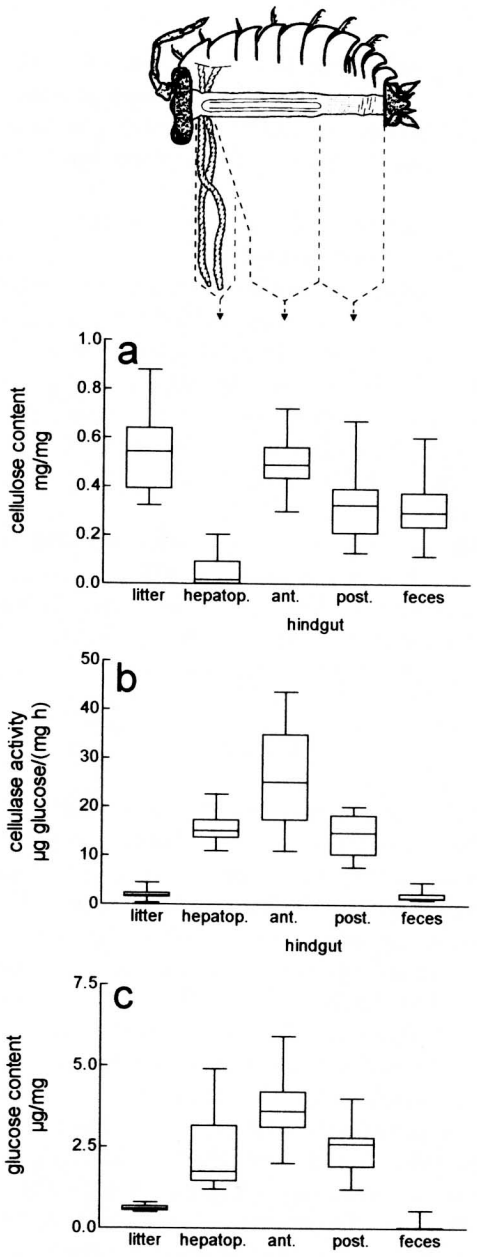


FIG. 1. Characteristics of cellulose digestion in the gut of *P. scaber*. Cellulose content (a), cellulase activity (b), and glucose content (c) of the food (litter). Sections of the gut are: hepatopancreas (hepatop.), anterior hindgut (ant.), and posterior hindgut (post.). Each box plot represents data from 15 specimens.

counts; Figure 2a). About 5×10^3 actinomycetes/mg (Figure 2b), 10^6 bacteria/mg (Figure 2c), and 10^5 fungi/mg (Figure 2d) were cultivated on agar. Thus, the proportion of platable microorganisms was about 0.1–1% of the total microbial counts. High total microbial counts of about 10^8–10^9 cells/mg were observed in the hepatopancreas (Figure 2a). However, neither bacteria nor fungi could be cultivated from these midgut lobes with the described methods (Figure 2b–d).

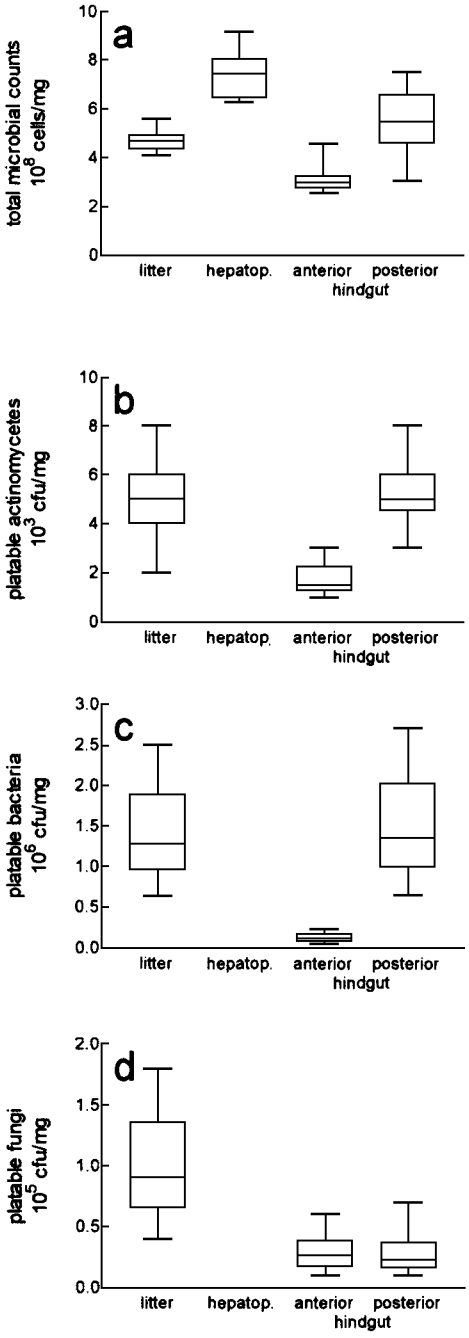
Inside the hindgut, the relation of platable and total microbial counts changed remarkably compared to the litter (Figure 2). In the anterior hindgut, counts of platable actinomycetes, bacteria, and fungi were strongly reduced compared to the litter (20–35%; $P < 0.001$), while total microbial counts decreased less strikingly (25–100%; $P < 0.05$). Consequently, the proportion of platable microbes in the anterior hindgut dropped to about 0.01–0.05%.

The posterior hindgut was characterized by a significant increase of both platable (except fungi; $P > 0.5$) and total microbial counts ($P < 0.001$). In this hindgut section, platable actinomycetes and bacteria reached values that equaled those of the litter ($P > 0.05$). Total microbial counts in the posterior hindgut slightly exceeded those of the litter ($P < 0.1$; Figure 2a). Due to the increase in counts of platable microbes, their percent on total microbial counts rose to values of about 0.1–0.5% in the posterior hindgut.

The number of platable cellulolytic microbes was the same on the litter and in the anterior and posterior hindgut of the specimens observed (2×10^3 cells/mg). Thus, due to the described decrease in the number of platable microorganisms in the anterior hindgut, the proportion of cellulolytic microbes in this section of the gut (4–5%) was about 20-fold higher than on the litter and in the posterior hindgut (0.1–0.2%). Comparing these data to the results shown in Figure 2, fungi that probably have been ingested with the litter are responsible for the high numbers of cellulolytic microorganisms.

About 30–60% and 80–90%, respectively, of the platable bacteria and fungi in the anterior hindgut were determined to be facultative anaerobes by the technique of replica plating. Similarly, we observed 60–90% and 70–80%, respectively, of facultatively anaerobic bacteria and fungi in the posterior hindgut. No actinomycetes from the gut grew under anaerobic conditions.

After feeding on artificial diet, microbial counts inside the hindgut generally decreased. About 20%, 15%, 10%, and 70% of the values of platable actinomycetes ($P < 0.01$), bacteria ($P < 0.01$), fungi ($P < 0.001$), and total microbial counts ($P < 0.05$), respectively, obtained from litter-feeding isopods were found in the anterior hindgut. In the posterior hindgut, the number of platable actinomycetes increased (160%; $P < 0.01$). By contrast, total microbial counts remained unchanged (110%; $P > 0.4$), while bacteria (85%, $P < 0.05$) and fungi (45%; $P < 0.01$) were significantly reduced when feeding on artificial diet.


a
total microbial counts
10^8 cells/mg
10
8
6
4
2
0
litter
hepatop.
anterior
posterior
hindgut
b
platable actinomycetes
10^3 cfu/mg
10
8
6
4
2
0
litter
hepatop.
anterior
posterior
hindgut
c
platable bacteria
10^6 cfu/mg
3.0
2.5
2.0
1.5
1.0
0.5
0.0
litter
hepatop.
anterior
posterior
hindgut
d
platable fungi
10^5 cfu/mg
2.0
1.5
1.0
0.5
0.0
litter
hepatop.
anterior
posterior
hindgut

Total microbial counts in the hepatopancreas were not influenced when isopods fed on artificial diet (99%, $P > 0.8$). However, adding antibiotics to the diet led to strongly reduced microbial counts in the midgut glands (38%; $P < 0.05$). Similar effects were observed after feeding on tannic acid (34%; $P < 0.01$). Platable actinomycetes, bacteria, and fungi in the anterior hindgut (25%, <1%, <1%, respectively; $P < 0.001$) and the posterior hindgut [<1%, <1% ($P < 0.001$), 70% ($P < 0.05$), respectively] were strongly affected by the used antibiotics. Furthermore, total microbial counts were reduced by 45% ($P < 0.05$). Tannic acid also strongly reduced the counts of platable microbes in the anterior hindgut [<1%, 10% ($P < 0.001$), 50% ($P < 0.05$), respectively] and the posterior hindgut [<1%, 10% ($P < 0.001$), 86% ($P < 0.05$), respectively].

To investigate the reasons for the contradiction between cellulolytic activity (Figure 1b) and the number of gut microbes (Figure 2), we performed analyses on the food sources described above. The variance of cellulase activity in the anterior hindgut could only be explained by the number of bacteria in the hepatopancreas (ANOVA: $P < 0.001$), but did not correlate with either of the other determined microbial counts. Similarly, cellulolytic activity in the hepatopancreas was explained by the total microbial counts in this region ($P < 0.05$). The posterior hindgut exhibited cellulolytic activity that also correlated with the number of hepatopancreatic bacteria ($P < 0.001$).

DISCUSSION

The observed numbers of platable actinomycetes and bacteria in the hindgut of *P. scaber* coincide with results presented by Reyes and Tiedje (1976) in *Trachelipus rathkii* (Brandt). In contrast to Griffiths and Wood (1985), who described a greater density of microorganisms in the hindgut than on the food, we cultivated only about 30%, 10%, and 30% of actinomycetes, bacteria, and fungi, respectively, from the anterior hindgut, compared to the litter the isopods fed on. These values suggest extensive digestion in this hindgut region and, presumably, nutritional utilization of microorganisms ingested with the food (cf. Reyes and Tiedje, 1976; Gunnarsson and Tunlid, 1986).

Multiplication of the remaining bacteria may result in an increase in the number of microorganisms in the posterior hindgut (Coughtrey et al., 1980;

FIG. 2. Microbial counts on litter and in distinct sections of the hepatopancreas (hepatop.), and anterior and posterior hindgut of *P. scaber*: (a) total microbial counts; (b) counts of platable actinomycetes (colony forming units, cfu); (c) counts of platable bacteria (cfu); and (d) counts of platable fungi (cfu). Each box plot represents data from 15 specimens, with three replicates each.

Gunnarsson and Tunlid, 1986). This hindgut section is characterized by pH levels of 6.0–6.5 (Zimmer and Topp, 1997b). Under these conditions, extensive bacterial growth and multiplication were observed. By contrast, the number of platable fungi did not increase in the posterior hindgut. The increase in total microbial counts (Krištufek et al., 1995) and the number of platable bacteria and fungi (Krištufek et al., 1992) have been observed in saprophagous earthworms (Lumbricidae) with a gut passage of 6–8 hr (Daniel and Anderson, 1992), in isopods (Reyes and Tiedje, 1976; Márialigeti et al., 1984) and in other soil animals (e.g., Hanlon, 1981). In terrestrial isopods, gut passage takes about 13–17 hr [Hartenstein (1964) in *Oniscus asellus*], 10–13 hr [Alikhan (1969) in *Porcellio laevis* Latreille], or 4–6 hr [Grünwald (1987) in several species]. Artificial diet as used in this study remained in the gut of *P. scaber* for 5–7 hr at 15°C (Zimmer, 1998).

Compared to counts of platable microbes, relatively high total microbial counts were observed in the anterior hindgut. One reason for high numbers of unplatable microbes may be the transport of hepatopancreatic bacteria to the anterior hindgut. There is a filter system at the connection of the hepatopancreas to the hindgut (Storch, 1987; Storch and Štrus, 1989), preventing an influx of particles larger than 1.2 μm into the hepatopancreas (Wood and Griffiths, 1988); we assume this to be a one-way filter. If multiplying microbial populations are maintained in the hepatopancreas as endosymbionts (Wood and Griffiths, 1988), there must be a possibility to excrete the surplus cells. This assumption is supported by the observation of numerous microbial cells in the anterior hindgut after feeding on artificial diet that was only weakly colonized by microorganisms (data not shown). In the anterior hindgut, hepatopancreatic bacteria may be digested or stay active, grow, and multiply in the posterior region of the hindgut. Inoculation of juveniles feeding on the feces of adults with hepatopancreatic bacteria would be possible in this way.

The distribution of cellulose in the observed sections of the gut of *P. scaber* confirms the function of the filter system (Storch, 1987; Storch and Štrus, 1989) in preventing cellulosic particles from entering the hepatopancreas. Furthermore, our results indicate that cellulose digestion occurs in the hindgut of *P. scaber*. Comparative analyses of the amount of ingested and egested cellulose revealed that 40–70% of the ingested cellulose is digested in *P. scaber* feeding on artificial diet. Similar values (about 40%) have been observed in a phytophagous terrestrial crab (Greenaway and Linton, 1995).

Remarkable amounts of glucose were found in the hepatopancreas, where absorption of nutrients takes place (e.g., Hassall and Jennings, 1975; Hames and Hopkin, 1989). Cellulose degradation and the release of glucose take place in the anterior hindgut, where hepatopancreatic enzymes are mainly active (Hassall and Jennings, 1975; Lane, 1988). Glucose may be drawn from the digestive

hindgut to the absorptive lobes of the hepatopancreas via the typhlosole canals (Hames and Hopkin, 1989) or may permeate the gut cuticle (Zimmer, unpublished observations) and reach the hepatopancreas through the ectocuticular space (sensu Lane, 1988). The epithelium of the anterior hindgut is involved in absorption of nutrients as well (e.g., Schmitz and Schultz, 1969) and may forward nutrients directly to the hepatopancreatic epithelium. Whatever mechanism is involved, the glucose content is supposed to drop from the anterior to the posterior hindgut.

Generally, terrestrial arthropods are assumed not to possess endogenous cellulases, but to utilize microbial enzymes ingested with the food (Martin, 1983; and Hassall and Jennings, 1975; Kozlovskaja and Striganova, 1977; Kukor and Martin, 1986, for terrestrial isopods). By contrast, Ray and Julian (1952) and Ray (1959) presented evidence that the marine wood-boring isopod, *Limnoria tripunctata* Menzies produces cellulases in the midgut lobes. More recent studies suggested that there may be at least some endogenous cellulolytic enzymes in saprophagous and xylophagous invertebrates (Slaytor, 1992; Scrivener and Slaytor, 1994; Treves and Martin, 1994; Skambracks, 1996). These enzymes do not act like extracellular fungal cellulases but are similar to bacterial cellulases (Slaytor, 1992). Consequently, an enzymatic distinction between endogeneous cellulase, produced by the animal, and cellulases exhibited by bacterial endosymbionts may be difficult.

Our results on the correlation between cellulolytic activity in the anterior hindgut and the number of bacteria in the hepatopancreas suggest an involvement of these endosymbionts in cellulose degradation. This assumption is supported by the correlation between the corresponding microbial counts and cellulolytic activity in the midgut lobes. Cellulase activity in the posterior hindgut that statistically depended on the number of hepatopancreatic bacteria may be unimportant from a nutritional point of view. The posterior hindgut exhibited only half as much cellulase activity as the anterior section, and the pH level in the posterior hindgut is higher than the pH level in the anterior section (Zimmer and Topp, 1997b). The latter maintains optimal conditions for cellulase activity (pH 5.5–6.0: Zimmer and Topp, 1997a). Cellulose degradation mainly takes place in the anterior hindgut. No typhlosole canals, which are thought to support fluid transport from the anterior hindgut to the hepatopancreas (Hames and Hopkin, 1989), can be found in the posterior hindgut, and the cuticle of this hindgut section is nearly impermeable to molecules the size of glucose (Zimmer, unpublished observations). Hence, glucose derived from cellulose digestion in the posterior hindgut can hardly be drawn to the absorptive region of the hepatopancreas. Thus, the decrease of the glucose content from the posterior hindgut to the feces is probably due to assimilation by multiplying microorganisms in this section of the gut.

In contrast to studies that suggest the utilization of ingested extracellular cellulases, we conclude from our results that the major proportion of active cellulases in the anterior hindgut of *P. scaber* is produced by the hepatopancreatic bacteria. With respect to the assumption of "endogenous" cellulases in *O. asellus* (Hartenstein, 1964) or *P. scaber* (Zimmer and Topp, 1998), the present results suggest "functionally endogenous" cellulases produced by obligately endosymbiotic (Wood and Griffiths, 1988) bacteria. With symbiosis as an evolutionary motor (Margulis, 1981), this association, presumably derived from a parasitic relation (Price, 1991), may have made the terrestrialization of isopods possible in terms of facilitating the digestion of vascular plant material.

Hepatopancreatic bacteria probably produce extracellular cellulases that are drawn from the hepatopancreas to the hindgut, together with other "digestive fluids". On the other hand, bacteria are possibly drawn to the hindgut and attack cellulose directly by attaching closely to the cellulose fibers, as is known from cellulolytic bacteria (Wood and Garcia-Campayo, 1990). Our hypothesis does not exclude the ingestion of extracellular microbial cellulases with the food. Prior to their proteolytic digestion, these enzymes that originate from the "external rumen" (Hassall and Rushton, 1985) are active inside the gut (Hassall and Jennings, 1975; Kukor and Martin, 1986), where favorable conditions are maintained (e.g., appropriate pH level) (Zimmer and Topp, 1997b). However, we suspect the proportion of ingested cellulases to be insignificant with respect to the total gut cellulases.

The significant dependence of woodlice on the microorganisms they ingest with their food (e.g., Uesbeck and Topp, 1995; Zimmer and Topp, 1997a) may be due to direct effects. Ingested microorganisms may deliver essential nutrients (Ullrich et al., 1991) or support the isopods in maintaining gut conditions concerning the pH level (Zimmer and Topp, 1997b) or may aid in osmoregulatory features.

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LOSS OF TANNINS AND OTHER PHENOLICS FROM WILLOW LEAF LITTER

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Abstract—A litter bag experiment was conducted to determine the fate of condensed tannins during the initial phase of willow leaf decomposition. Chemical methods and HPLC were used to identify and quantify the phenolics in aqueous acetone extracts of the leaves during the first eight weeks after leaf fall. Extractable phenolics and tannins were lost rapidly from the leaves and had half lives of about 2.4 weeks. Lower molecular weight tannins were lost more rapidly than higher molecular weight tannins, suggesting that the primary route for loss of tannins is leaching. There was no evidence for decomposition of the tannin during the leaf decay process. After leaching, the tannin apparently sorbs tightly to the soil.

Key Words—*Salix*, condensed tannin, proanthocyanidin, tannin, decomposition, leaching, soil chemistry.

INTRODUCTION

Leaf litter decomposition is a critical process in nutrient cycling (Attiwill and Adams, 1993). After leaves fall, they may be decomposed by physical processes, by abiotic chemical processes, and by biotic processes (Facelli and Pickett, 1991). The rate of litter decomposition is determined by factors such as climate, soil chemistry, and microbial and invertebrate activity. Leaf litter quality is also a critical determinant of the rate of decay (Melillo et al., 1982). Nitrogen, phosphorus, cellulose, lignin, and tannin levels have been used as indices of litter quality (Vitousek et al., 1994; Flanagan and Van Cleve, 1983).

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In general, it is believed that tannins retard the rate of litter decay. Tannins may form recalcitrant complexes with other substrates such as cellulose or protein (Horner et al., 1988). Tannin-containing litter may be unpalatable to detritus feeders (Staaf, 1987) or inhibit fungal decomposers (Harrison, 1971). Despite these likely mechanisms for the negative effect of tannins on the decay process, negative correlations between tannin level and decay rate have been obtained in only some studies (Tian et al., 1992; Gallardo and Merino, 1993). In other studies, tannin content was unrelated or was positively correlated with rate of decay (Gallardo and Merino, 1993; Meentmeyer, 1978).

An understanding of the lifetime and fate of tannin in decaying leaf matter is required so that specific stages of the process that might be affected by tannin content can be targeted for further study. However, it is difficult to monitor tannin content of leaf litter specifically. Many species of plants contain complex mixtures of nontannin phenolics, condensed tannins, and hydrolyzable tannins. The widely used Folin-based method for analyzing "tannin" in leaf litter (Tian et al., 1992; Gallardo and Merino, 1992, 1993; Fox et al., 1990; Constantinides and Fownes, 1994) actually provides a broad measure of all readily oxidized compounds including tannins, nontannin phenolics, and nonphenolic compounds such as ascorbic acid. More specific measures of tannins are needed for detailed studies of the role of tannins in leaf litter decay.

Our goal was to describe the loss of tannin during the initial stages of willow leaf litter decomposition. We selected sandbar willow leaves (*Salix exigua* Nutt.) after establishing that they contain simple condensed tannin comprised of epicatechin and catechin monomers (procyanidin) and do not contain hydrolyzable tannnin (Schofield, 1995). Willow contains nontannin phenolic glycosides (Julkunen-Tiitto, 1986), which we did not monitor.

METHODS AND MATERIALS

Sample Collection. The experiment was performed in the Miami Research Area on the campus of Miami University in Oxford, Ohio. Willow leaves were obtained from a stand of sandbar willow, *Salix exigua* Nutt., on the eastern bank of Four Mile Creek just south of the dam on State Route 73. The soil at the site was fine sandy loam (Appendix A, Schofield, 1995). Rainfall during the period totalled 16.7 cm (Klink, 1998).

Leaves were collected on plastic tarpaulins beneath the willow stand starting on the first day of leaf fall (October 20, 1994). Leaves were collected daily for four consecutive days and stored in a large nylon mesh bag near the site after discarding leaves trapped in puddles of water during the collection period. At the end of the collection period, the leaves were gently mixed and randomly divided into samples weighing approximately 1.5 g (wet weight). Each sample

was weighed exactly and placed inside a nylon mesh litter bag (1 mm screen) that was closed by stapling.

Macrocosms for containing soil and leaf material were made from PVC pipe and lids. The top part of the apparatus (10.2 cm wide × 12.7 cm high) held soil supported by nylon mesh, and the bottom part (same dimensions) was a 750 ml chamber for trapping rain water leachate. About 150 ml loosely packed top soil from the field site was placed in the top chamber of each assembled apparatus. After partial burial of the entire apparatus so that only 2–3 cm of the top chamber protruded above the surrounding soil, a litter bag was placed on the soil surface. Control pots were either empty or contained soil but no leaves. A random number table was used to assign each apparatus to a sampling date, with $N = 5$ for week 0 and $N = 9$ for weeks 2, 4, 6, and 8. The $t = 0$ materials were immediately returned to the laboratory for analysis. The remainder of the site was covered with chicken wire to minimize disturbance by large animals, and was checked at least every 48 hr to remove debris such as twigs.

On the assigned sampling date macrocosms were returned to the lab. Leaves and soil were weighed before and after freeze drying, which preserves phenolics more effectively than oven or air drying (Hagerman, 1988). The water leachate was freeze dried to concentrate it before analysis.

Chemical Analysis. Phloroglucinol, catechin, and epicatechin were purchased from Sigma Chemical Company (St. Louis, Missouri). Condensed tannin was purified from *Sorghum* grain and from sandbar willow leaves according to Hagerman and Butler (1980a). Epicatechin phloroglucinol was prepared as an HPLC standard by using the method of Koupai-Abyazani et al. (1992). The reaction was carried out with 80 mg of phloroglucinol and 125 mg of *Sorghum* tannin, and the products were separated by chromatography on Sephadex LH-20 with ethanol as the eluant. Phloroglucinol, catechin, and epicatechin phloroglucinol studied in that order from the column; the epicatechin phloroglucinol was freeze dried and characterized by ^{13}C NMR (Porter, 1989).

Total phenolics were extracted from the samples of freeze-dried willow leaves (Hagerman, 1988). In brief, the 100-mg samples were extracted three times in 5.0 ml of 70% acetone [acetone–water, 70:30, v/v] at 4°C. After removing a subsample of the pooled extracts for total phenolic analysis, the remainder of the extract was adjusted to 0.001 *M* ascorbic acid to minimize oxidation and was stored at 4°C until analysis. Leaf residues were immediately analyzed with the acid butanol method to determine levels of unextracted tannin.

Total extractable phenolics in the crude extracts were measured with the Prussian blue method (Graham, 1992), with gallic acid as the standard, and were also monitored with HPLC and the column and solvents described below.

Condensed tannin was measured with the acid butanol method (Porter et al., 1986) and with the sulfuric acid method (Bae et al., 1993) standardized with purified *Sorghum* condensed tannin. Unmodified polymeric condensed tannins

cannot be directly analyzed with HPLC because they sorb too strongly to stationary phases. However, condensed tannins can be indirectly monitored with HPLC after reaction with phloroglucinol–HCl to yield the phloroglucinol derivative of the tannin extender unit (Koupai-Abyazani et al., 1992). A 500-μl sample of crude extract (or 1 mg of purified tannin) was evaporated to dryness under nitrogen and redissolved in 150 μl of freshly prepared ethanol–HCl (99:1, v/v) containing a 5.0 mg/ml phloroglucinol. After incubation at room temperature overnight, the solution was evaporated under nitrogen and the residue dissolved in 50 μl water, which was then extracted three times with 150 μl ethyl acetate. The pooled ethyl fractions were evaporated under nitrogen and redissolved in 75 μl of ethanol–water (70:30, v/v) for analysis by HPLC. Chromatography was performed at ambient temperature with a C-18 column (Beckman Ultrasphere 5 μm, 4.6 × 25 cm, Anspec, Ann Arbor, Michigan) protected by a 4 × 4 mm C-18 guard column. Separations were performed at a flow rate of 1 ml/min with a linear gradient of solvent A (1% acetic acid in water) and solvent B (60% methanol, 40% solvent A): 100% solvent A going to 60% solvent B over 60 min, then to 100% solvent B over 5 min. The eluate was monitored at 280 nm, and data were collected and integrated with HP Chemstation software A.00.33 (Hewlett Packard Company, Wilmington, Delaware).

The average molecular weight of condensed tannins was determined by using HPLC analysis of the phloroglucinol reaction products. For our instrumental conditions, the relationships between the area under individual peaks and the phenolic concentration for commercial catechin and epicatechin phloroglucinol prepared from *Sorghum* tannin were:

$$\text{peak area} = 1.16\ (\pm\ 0.066) * \mu\text{mol catechin}\ (R^2 = 0.915)$$

$$\text{peak area} = 0.39\ (\pm\ 0.027) * \mu\text{mol epicatechin phloroglucinol}\ (R^2 = 0.879)$$

For purified tannins the standard curves were used to directly calculate the ratio of extender unit to terminal unit in the tannin sample, providing an average chain length or molecular weight. In order to calculate molecular weights for tannins in crude extracts it was necessary to correct the total catechin peak areas to account for free catechin.

Soil samples and soil amended with known amounts (up to 0.2% by weight) of *Sorghum* tannin were analyzed with methods described by Rice and Pancholy (1973), Webster et al. (1993), Whitehead et al. (1982), and Quarmby and Allen (1989). We tried to modify our HPLC methods to detect phenolics in soil samples, and attempted to develop a protein-binding method to determine soil-bound tannins.

Statistical Procedures. Leaf weights and chemical measures were analyzed by one-way ANOVAs with follow-up analysis based on Tukey multiple comparisons (Neter et al., 1990). Decay models were generated with linear regres-

sion of log-transformed responses. Individual means were compared with t tests for samples with unequal variances. All tests were performed by proc GLM and proc REG in SAS (1986).

RESULTS

Leaf Chemistry. The dry weight of the litter samples did not change during the eight-week experimental period. The average dry weight of leaf samples at the beginning of the experiment was 1.25 ± 0.02 g, and at the end was 1.17 ± 0.13 g ($t = 1.59$, $P < 0.074$).

The total extractable phenolic content of the leaf litter decreased exponentially with time (Figure 1). The data fit a first-order decay model {in [phenolic] = 1.22 − 0.286(t)}, which yielded a half-life for total phenolics of 2.42 ±

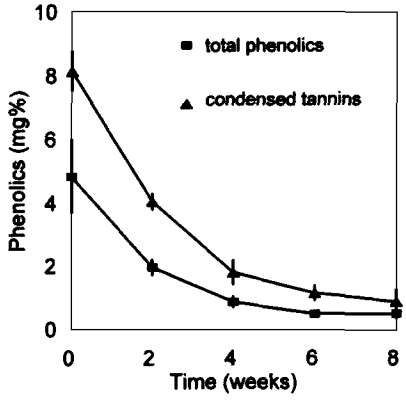

FIG. 1. Phenolic content of leaf litter samples over time. Total phenolics in the leaf extracts were determined with the Prussian blue method standardized with gallic acid and are expressed as weight percent gallic acid. Condensed tannins in the leaf extracts were determined with the acid butanol method standardized with *Sorghum* condensed tannin and are expressed as weight percent condensed tannin. The points shown are means with the bar showing SD, $N = 9$ except for $t = 0$ where $N = 5$. A test of the equality of mean total phenolic levels across weeks was rejected by one-way ANOVA ($F_{4,36} = 119$, $P < 0.001$). Follow-up analysis with Tukey's multiple comparison procedure showed that the mean phenolic levels at weeks 0, 2, and 4 differed from each other; the levels at weeks 4, 6, and 8 did not differ from each other. A test of the equality of mean tannin levels across weeks was rejected after one-way ANOVA ($F_{4,36} = 427$, $P < 0.001$). Follow-up analysis with Tukey's multiple comparison procedure showed that the mean tannin levels at weeks 0, 2, 4, and 6 differed from each other; the levels at weeks 6 and 8 did not differ from each other.

0.17 weeks. Similar results were obtained with HPLC of unmodified crude extracts. There were numerous peaks in the chromatograms of extracts prepared from $t = 0$ leaves, but there were essentially no peaks in extracts of $t = 4$ weeks samples (Figure 2). The disappearance of the peak corresponding to catechin fit a first-order decay model {ln [catechin] = $7.67 - 0.399(t)$}, which yielded a half-life of 1.74 ± 0.13 weeks.

Levels of condensed tannins also declined rapidly. Loss of condensed tannins as measured with the acid butanol method fit the first-order model {ln [condensed tannin] = $1.93 - 0.288t$) with a half-life of 2.40 ± 0.14 weeks (Figure 1). Condensed tannins were more specifically measured by HPLC analysis of the phloroglucinol reaction products. There was a large amount of epicatechin phloroglucinol in the reaction products from the $t = 0$ samples (Figure 3a) but none in the reaction products of the $t = 8$ weeks samples (Figure 3b). The data for loss of tannin measured as epicatechin phloroglucinol products fit a first-order decay and yielded a half-life of 2.41 ± 0.14 weeks.

We confirmed that the phloroglucinol degradation method could be used to determine condensed tannin molecular weight by comparing tannin purified from *Sorghum* grain with that purified from sandbar willow leaves. Based on the ratio of epicatechin phloroglucinol to catechin in the reaction products, we calculated that the *Sorghum* tannin is comprised of 17 epicatechin extender units per terminal catechin (molecular weight 4898 g/mol), while the willow tannin was 5.9 epicatechin extender units per terminal catechin (molecular weight 1690 g/mol). We obtained an average chain length for condensed tannins in the crude phenolic extracts of $t = 0$ willow leaves of 6.31 ± 0.88 epicatechin extender units per terminal catechin. The similarity of the values obtained for the purified tannin and the crude extracts validates the method for assessing tannins in crude extracts.

The average chain length of condensed tannin in the crude extracts increased during the experiment. Tannin in extracts from the $t = 6$ weeks samples had 15.5 ± 3.8 extender units per terminal unit ($t = 0$ sample vs. $t = 6$ weeks sample, $t = 4.00$, $P < 0.002$). The chain length at eight weeks was similar to that at six weeks, but the error in the determination was very large because peak areas were so small.

There was no evidence that condensed tannins are incorporated into the insoluble fraction of the leaf litter during decay. The residue left after extracting all soluble phenolics from $t = 0$ leaves was 0.16 weight percent condensed tannin, while the residue from $t = 8$ weeks samples contained only 0.08% condensed tannin. Levels of condensed tannin in the residue decreased with time throughout the experiment ($F_{4,36} = 109$, $P < 0.001$).

Soil and Leachate. It was not possible to detect tannin in any soil sample. Untreated soil samples, air dried soil samples, and samples amended with up to 0.2% (by weight) purified *Sorghum* tannin were extracted by protocols like those used to extract tannins from leaves (Hagerman, 1988) or from feces (Rob-

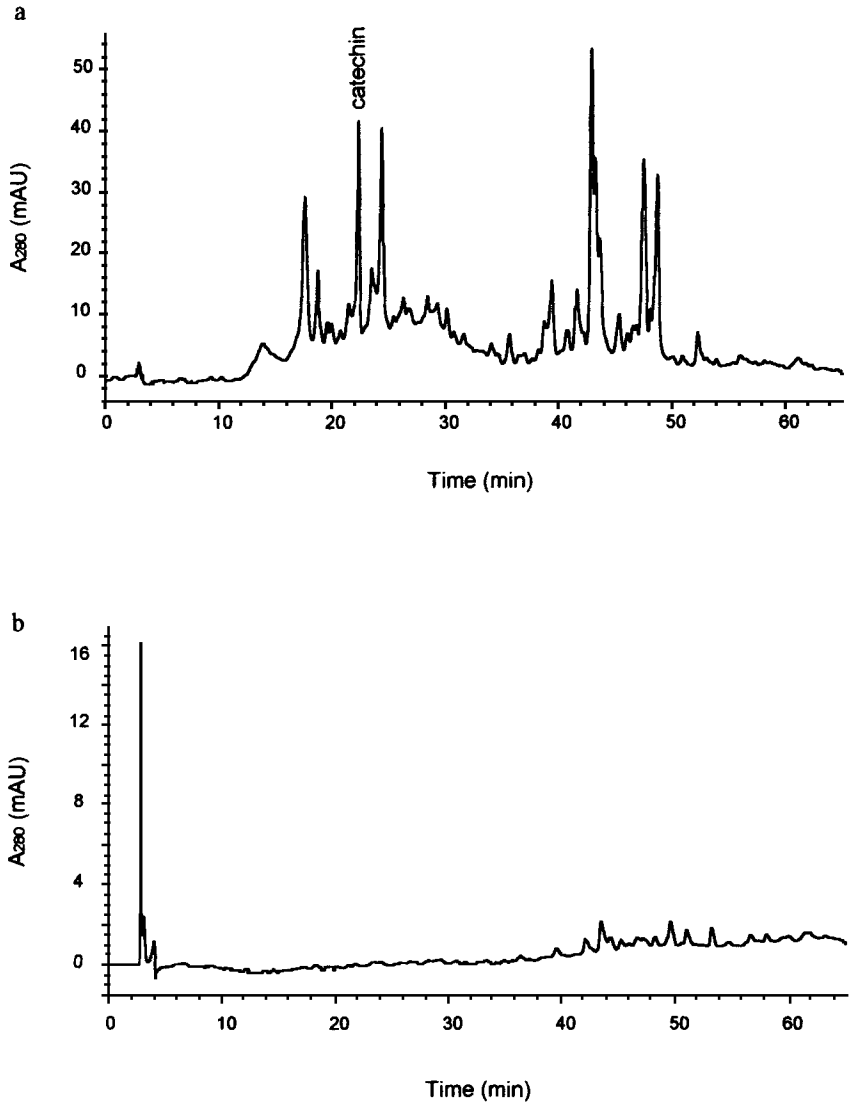

FIG. 2. HPLC of leaf litter extracts for samples at $t = 0$ (a) and $t = 4$ weeks (b). Extracts were run on RP-HPLC and phenolics detected by absorbance at 280 nm. The peak corresponding to catechin is indicated on the chromatogram. Other peaks are unidentified phenolics. Note that the absorbance scale is different in the two chromatograms.

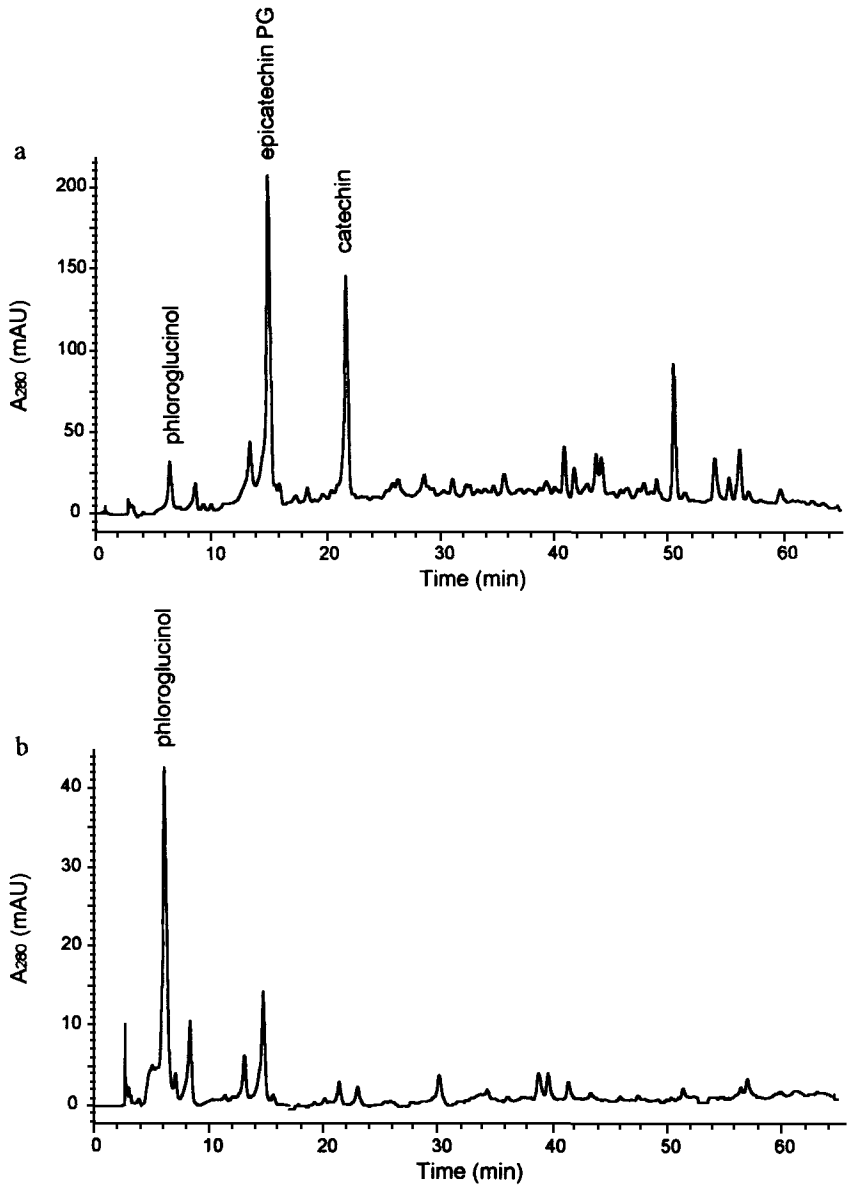

FIG. 3. HPLC of the phloroglucinol HCl reaction products of litter extracts for samples at $t = 0$ (a) and $t = 8$ weeks (b). Extracts were reacted with phloroglucinol HCl, run on HPLC, and detected by absorbance at 280 nm. Peaks corresponding to excess phloroglucinol, epicatechin–phloroglucinol (epicatechin PG), and catechin are indicated on the chromatograms. Note that the absorbance scale is different in the two chromatograms.

bins et al., 1991). Extraction with basic solutions (Rice and Pancholy, 1973; Webster et al., 1993; Whitehead et al., 1982), boiling methanol (Quarmby and Allen, 1989), or strong hydrogen bonding solvents such as *N*,*N*-dimethyl formamide (Hagerman, 1992) did not release tannin from the soil. Tannins were not detected when soil was directly analyzed with the Prussian blue method, with the acid butanol method, or with the sulfuric acid method, which is less susceptible to interference than the acid butanol method (Bae et al., 1993). We attempted several novel methods for analysis of tannins in soils, including HPLC to analyze soil extracts or reaction products of soil samples with phloroglucinol, but peaks corresponding to tannins or phenolics were not detected. An attempt was made to measure tannin indirectly by monitoring its ability to bind radioiodinated protein (Hagerman and Butler, 1980b), but the soil was quite sorptive, and tannin-amended soil did not absorb more protein than the control samples. Attempts to analyze tannins in the soil were abandoned after all these unsuccessful attempts.

Water that leached through leaves and soil during the decomposition process was collected in the bottom of the apparatus. The leachates did not react with acid butanol or sulfuric acid, suggesting that they did not contain condensed tannin. The leachates did not form a colored complex with iron(III) in alkaline solution, suggesting the absence of ortho-substituted phenolics. Although the leachates did react in the Prussian blue method, we subsequently found that the glue used to construct the apparatus gave false positives in this test.

DISCUSSION

There was little loss of dry matter from willow leaves during the first eight weeks after litter fall. This is consistent with several similar studies in which 1-mm mesh bags were used (Lousier and Parkinson, 1976; Flanagan and Van Cleve, 1983; Gallardo and Merino, 1993). Dry matter disappearance appears to be more rapid when leaves are placed in bags with larger mesh (>3 mm) (Slapokas and Granhall, 1991; Staaf, 1987). Particles are more easily lost from bags with larger mesh, and larger derivatives are able to participate in decomposition.

The small changes in dry weight are in contrast to the changes noted in chemical composition of the willow leaves. Total phenolics and tannins disappeared quickly from the leaves and were essentially gone from the leaves by six weeks after leaf fall. Rapid loss of soluble leaf constituents (Gallardo and Merino, 1993), phenolics (Kuiters and Sarink, 1986), or tannins (Slapokas and Granhall, 1991) has been reported in several other systems.

Monitoring the loss of individual phenolic components showed that rate of loss was related to the molecular weight of the compound. Catechin (molecular weight 290 g/mol) disappeared more rapidly than total phenolics or tannins.

Shorter chain length tannins were lost more quickly than longer chain length tannins, so that the molecular weight average of the tannin shifted from 1818 g/mol at $t = 0$ to 4465 g/mol at six weeks. This pattern of disappearance suggests that leaching is an important pathway for loss of phenolics from the leaves. In general, it is expected that lower molecular weight constituents will be more soluble, and thus will leach more rapidly. Other secondary products, such as monoterpenes, are leached from leaves during litter decomposition (Wood et al., 1995).

Phenolics are apparently tightly sorbed to soil after they are leached from the leaf litter, since they were not detected in the leachate. The high affinity of tannin for soil was unexpected but was confirmed by experiments in lab. Purified tannin added to soil samples could not be recovered or detected with any of the extraction protocols or tests employed. Similar retention of hydrolyzable tannin by soils has been reported recently (Kennedy et al., 1996).

An alternate fate of tannin could be complexation with leaf protein (Gallardo and Merino, 1992) or with plant cell wall polysaccharides (Beart et al., 1985) during litter decay. Analysis of leaf residues after extraction of soluble phenolics and tannins suggests that this hypothesis is not correct for willow leaves, since the amount of tannin in the residue decreased slightly during decomposition of the leaves.

Other routes for loss of tannins from the leaves are degradation or oxidation. It was clear that tannin was not degraded into smaller phenolics during the course of leaf decay, since HPLC of the total extractable phenolics demonstrated that new small phenolics did not appear during decay. Potential oxidative products of tannin decay were not directly monitored, but absence of new peaks in chromatograms suggested that oxidation products were not formed. DeMontigny et al. (1993) reported similar NMR spectra for the tannins in salal (*Gualtheria* sp.) leaves and in salal-derived litter, supporting the idea that condensed tannin is not chemically modified during leaf decay.

The effect of tannins on processes at different stages of decay can be predicted from our results. For example, the direct effect of tannins on processes occurring in the leaves is likely only during the first few weeks of decay. Later in decay, after tannins have left the leaf, they may affect metal ion or nitrogen availability to microbes or other decomposers (Scalbert, 1991; Bending and Read, 1996). Soil-sorbed tannins could also be toxic or inhibitory to microbial or invertebrate decomposers.

The HPLC method described here provides a useful tool for determining molecular weights of condensed tannins. It complements NMR methods that require large amounts of purified tannin (Porter, 1989). Mass spectral analysis is useful for small proanthocyanidins (Self et al., 1986), but neither fast atom bombardment (FAB) nor matrix-assisted laser desorption (MALDI) techniques have been useful for large proanthocyanidins such as those purified from *Sorghum* or willow (Hagerman, Cassady, and Jainhuknan unpublished observations).

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Erratum

In the paper "Solid-Phase Microextraction and Cuticular Hydrocarbon Differences Related to Reproductive Activity in Queenless Ant *Dinoponera quadriceps*" (Volume 24, No. 3) by Thibaud Monnin, Christian Malosse, and Christian Peeters, the explanation of the symbols in the captions for figures 4 and 5 appearing on pages 484 and 486, should have read: filled circle, mated alphas (N = 7); open circle, virgin alphas (N = 19); filled triangle, sterile workers less than one month-old (N = 9); and open triangle, older sterile workers (N = 22).

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REPELLENCY OF ROSEMARY OIL AGAINST *Myzus persicae* IN A LABORATORY AND IN A SCREENHOUSE

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Abstract—The repellencies of 13 labiate essential oils against *Myzus persicae* were investigated with a linear track olfactometer. Rosemary, thyme, peppermint, lavender, and spearmint oils repelled aphids at a dose of 10 μl. Rosemary and thyme oils repelled at a dose of 1 μl. The repellent actions of 13 components of rosemary oil were also evaluated. Among these components linalool, *d*,*l*-camphor, and α-terpineol had repellent action. The repellency of rosemary oil in a screenhouse was investigated. Aphids were released in a screenhouse and allowed to choose between tobacco plants in an area permeated with rosemary oil odor and plants in a control area. The number of aphids in the treatment area was about 70% of that in the control area. These results indicated that the landing of *M. persicae* on host plants was influenced by odors and that it may be possible to control aphids with repellents.

Key Words—*Myzus persicae*, green peach aphid, repellent, rosemary oil, labiate, essential oil, olfactometer, field-test.

INTRODUCTION

The behavior of some aphid species, such as *Aphis fabae* Scopoli (Nottingham et al., 1991; Isaacs et al., 1993; Nottingham and Hardie, 1993) and *Brevicoryne brassicae* (L.) (Nottingham et al., 1991), is influenced by plant odors in experiments with an olfactometer. In a field study with traps, *Cavariella aegopodii* (Scopoli) was attracted by carvone, one of its host plant volatiles, and this attractancy was masked by linalool (Chapman et al., 1981). In a field trial conducted on barley in Sweden, Pettersson et al. (1994) succeeded in reducing the population of *Rhopalosiphum padi* (L.) by releasing methyl salicylate from an emulsifiable concentrate sprayed on the crop or from slow-release vials. They

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also reported that populations of *Sitabion avenae* (Fabricius) and *Metopolophium dirhodum* (Walker) on wheat were reduced by methyl salicylate released from polyethylene bags in a field trial conducted in the United Kingdom. Thus, in the field, repellents can be effective for the control of aphids.

Myzus persicae (Sulzer) (Homoptera: Aphididae), which is polyphagous and attacks about 100 plant species in Japan, is an important agricultural pest. Although the EAG response of this aphid to plant volatiles has been reported (Visser et al., 1996), its behavior toward plant volatiles or nonhost plant volatiles has not been thoroughly examined. In this study, we screened essential oils from 13 labiate plants as repellents against *M. persicae* and investigated the repellent effect of rosemary oil on the aphid in a screenhouse.

METHODS AND MATERIALS

Aphids. Adult alates of *M. persicae* virginoparae, derived from a stock culture maintained on *Nicotiana tabacum* in a greenhouse at 20°C and a photoperiod of 16L:8D, were used for all experiments.

Chemicals. Hyssop, oregano, and patchouli oils were supplied by Takasago Int. Co. (Tokyo, Japan). The other essential oils were supplied by Soda Aromatic Co. (Tokyo, Japan). 1,8-Cineole, α-pinene, β-pinene, *d*-(+)-limonene, α-terpineol, borneol, *p*-cymene, and bornyl acetate were supplied from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). *d,l*-Camphor, myrcene, and linalool were supplied by Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). (+)-Camphene was supplied by Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin). (−)-*trans*-Caryophyllene was supplied by Sigma Chemical Co. (St. Louis, Missouri).

Olfactometer. A linear track olfactometer (Figure 1), modified from an original design by Sakuma and Fukami (1985), was used in all the laboratory tests. It was made of transparent acrylic tubing and steel rods. The rods formed a T-junction at the point where airstreams carrying treatment and control airflows met from two side arms in the olfactometer. Aphids chose the direction by chemotaxis at the T-junction. Air was drawn through the apparatus at a rate of 1.0 liter/min in all the tests. Thirty adult alate virginoparae were placed in a pot installed at the base of the central vertical tube in the olfactometer. The inner walls of the three vertical tubes at the center and both sides, including the pot, were coated with talcum powder (Wako Pure Chemical Ind.) to prevent the aphids from climbing the wall and thus forcing them to climb the rod only. All tests were carried out in a darkroom at 22–24°C to remove the influence of light. The number of aphids in the traps on the treatment or control side was counted 2 hr after the start of the test. The olfactometer was washed with soapy water and the treatment and control sides were alternated after every trial. Each

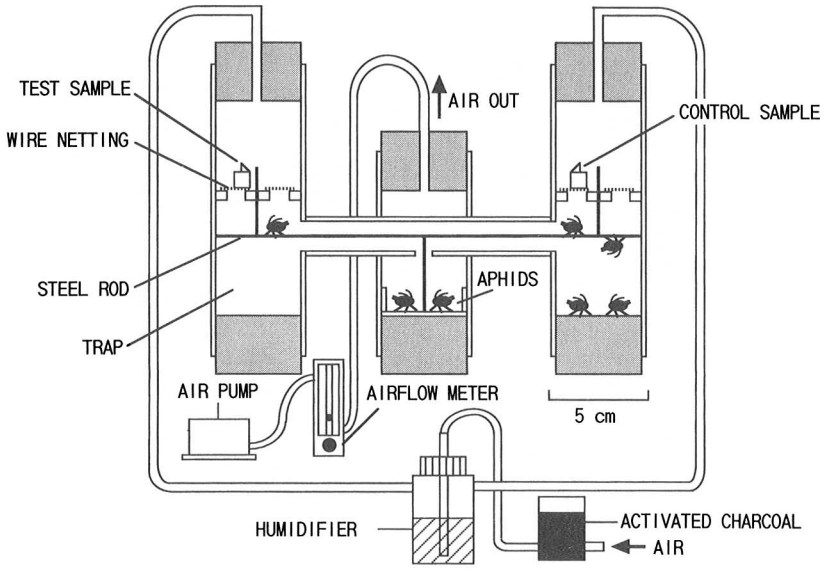

FIG. 1. Linear track olfactometer (side view).

experiment was replicated 12 times, and the results were analyzed by paired *t* tests; the number of aphids in the treatment trap was compared with that in the control trap. If tested aphids showed little activity, the experiment was terminated and the aphids were changed. Aphids were starved for 20 hr prior to each test.

Application of Essential Oils and Chemicals in Olfactometer Tests. In tests with liquid samples, a 10-μl sample on a piece of filter paper (Advantec, No. 2, 10 × 20 mm) was placed in the treatment side, and filter paper without treatment was placed in the control side. For doses of 0.01, 0.1, and 1 μl, 10 μl of an acetone solution of each sample was applied to filter paper and compared with acetone controls. In tests with solid samples, 40 μl of an acetone solution of each sample was applied to each filter paper and compared with acetone controls. Both treatment and control filter papers were allowed to dry to remove acetone before they were placed in the olfactometer.

Repellency of Rosemary Oil in a Screenhouse. Aphids were released in an outdoor screenhouse (12 × 12 m, mesh: #30, Figure 2A) and allowed to choose between the plants in the treatment area or those in the control area. Potted *Nicotiana tabacum* (pots: 15 cm diameter, 13 cm high), grown at 25°C for two months after seeding in the greenhouse, were used in the test. Six pots were placed in each corner of the screenhouse (Figure 2B). An open vessel (34 mm

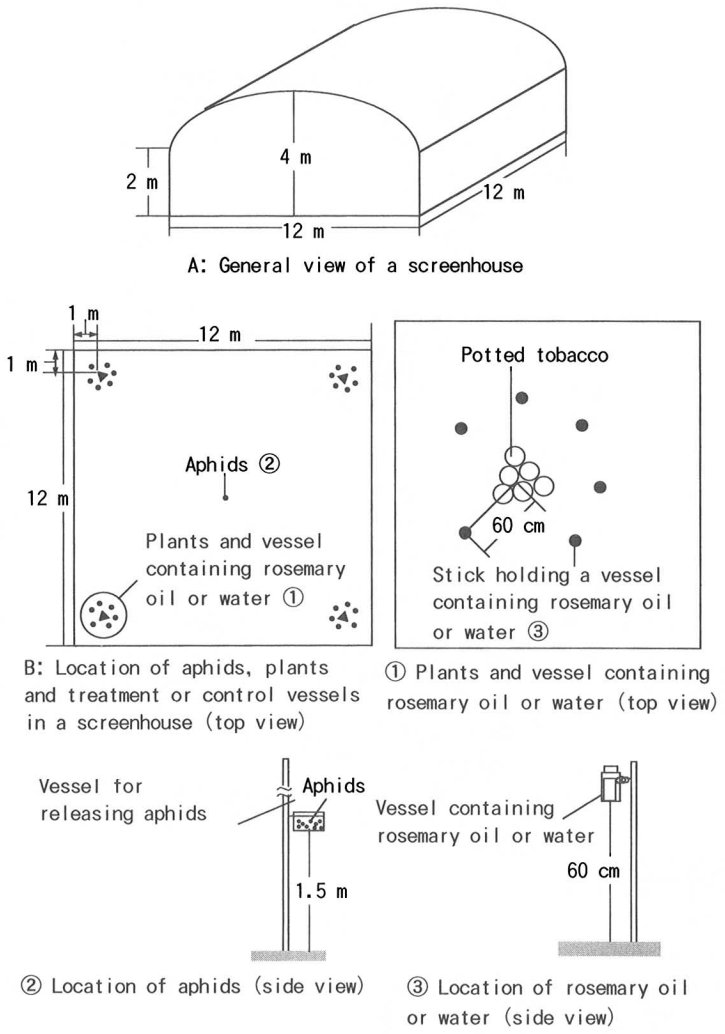

FIG. 2. The general view of a screenhouse and the location of aphids, plants, and vessels containing rosemary oil or water in the screenhouse.

diameter, 77 mm high) containing rosemary oil or water (40 ml) was set in a Teflon bottle (60 mm diameter, 133 mm high) and the bottle was held 60 cm above the ground on a stick (Figure 2, part 3). The bottle had 90 vent holes (ϕ 4 mm diameter) on its side through which vapor of rosemary oil or water was released. Each vessel placed in the two treatment corners contained rosemary

oil, and the two in the control corners contained water. The mouth of each open vessel was plugged with absorbent cotton (1g), with a wick in contact with the rosemary oil or water. Six bottles were placed around the potted tobacco plants in each corner. One thousand aphids starved 4 hr prior to release were placed in an open plastic vessel (78 mm diameter, 45 mm high) at the center of the screenhouse. Aphids were released at 3:00–4:00 PM and the number of aphids on tobacco plants was counted at 1:00–2:00 PM on the next day. The experiment was conducted at our laboratory between August 23 and October 3, 1996.

The sum of the number of aphids on tobacco plants in the two treatment corners was compared with that in the other two control corners by a paired *t* test. Twenty inspections were made and each inspection was accepted as one replicate. The treatment and control corners were alternated in each replicate.

RESULTS

Repellencies of Labiate Essential Oils Against M. persicae *in Olfactometer Tests.* The repellencies of 13 labiate essential oils against *M. persicae* were evaluated by using the olfactometer. Rosemary and thyme oils repelled aphids at doses of 1 and 10 μl, showing highly significant differences between the mean numbers of aphids trapped in the treatment area and those in the control area (Table 1). Although peppermint oil repelled at a dose of 10 μl, no significant repellent action was observed at 1 μl. Lavender and spearmint oil repelled at a dose of 10 μl ($P < 0.05$). No significant repellency was observed with basil, hyssop, marjoram, mint, oregano, patchouli, pennyroyal, and sage oil.

Repellencies of Rosemary Oil Components Against M. persicae *in Olfactometer Tests.* Thirteen components were identified in rosemary oil by the comparison of GC retention times to those of the standards and by GC-MS data (Hori and Komastu, 1997): 1,8-cineole (percent of total components: 48%); α-pinene, *d,l*-camphor (12%); β-pinene (7%); (+)-camphene (5%); borneol (4%); (−)-*trans*-caryophyllene (3%); *d*-(+)-limonene, α-terpineol, *p*-cymene (2%); linalool, myrcene, and bornyl acetate (1%). The repellent action of these 13 components was investigated in the olfactometer. Linalool, *d,l*-camphor, and α-terpineol repelled *M. persicae* at a dose of 10 μl or 10 mg (Table 2). The other components did not show significant repellency against *M. persicae*.

Repellent Action of Rosemary Oil in Screenhouse. The release rate of rosemary oil was about 2 g/vessel/day. Rosemary oil repelled the aphids even in the screenhouse. The difference between the mean number of aphids landing on tobacco plants in the treatment area and on these in the control area was small (20.4 vs. 28.8), because of large variation in the number of aphids landing in different replicates. However, there was a significant difference ($P < 0.0004$) in the number of the aphids on the tobacco plants in the treatment area and on those in the control area by paired *t* test.

TABLE 1. REPELLENCY OF LABIATE ESSENTIAL OILS AGAINST ALATE VIRGINOPARAE OF *Myzus persicae*[a]

Essential oils (μl)	Aphids trapped (*N*)		
	Treatment	Control	P^b
Rosemary oil			
(10)	5.5 ± 0.93	12.8 ± 1.25	0.01
(1)	6.3 ± 0.92	11.8 ± 1.15	0.01
(0.1)	9.7 ± 1.22	11.3 ± 0.95	NS
(0.01)	6.6 ± 0.74	8.8 ± 0.82	NS
Thyme oil			
(10)	6.3 ± 1.04	13.3 ± 1.47	0.01
(1)	7.7 ± 0.84	15.0 ± 1.90	0.01
(0.1)	12.4 ± 1.00	10.6 ± 1.41	NS
(0.01)	8.0 ± 0.81	7.4 ± 1.02	NS
Peppermint oil			
(10)	4.4 ± 0.60	9.9 ± 1.12	0.01
(1)	8.4 ± 0.71	9.8 ± 0.93	NS
Lavender oil (10)	5.7 ± 0.86	9.1 ± 1.05	0.05
Spearmint oil (10)	4.8 ± 0.64	8.1 ± 0.70	0.05
Basil oil (10)	6.5 ± 1.09	9.3 ± 1.10	NS
Hyssop oil (10)	7.5 ± 0.98	11.6 ± 1.73	NS
Marjoram oil (10)	6.7 ± 0.78	8.3 ± 1.07	NS
Mint oil (10)	8.5 ± 1.16	8.3 ± 1.48	NS
Oregano oil (10)	10.2 ± 1.70	11.8 ± 2.04	NS
Patchouli oil (10)	7.7 ± 0.80	9.3 ± 1.82	NS
Pennyroyal oil (10)	8.3 ± 0.70	11.8 ± 1.20	NS
Sage oil (10)	6.5 ± 0.85	8.3 ± 0.94	NS

[a] Values are means ± standard error.
[b] NS, no significant difference in paired *t* test ($N = 12$).

DISCUSSION

In this study, rosemary oil as well as thyme oil strongly repelled *M. persicae*. Rosemary oil also repelled other aphid species such as *Aphis gossypii* Glover, *Macrosiphum euphorbiae* (Thomas) (Hori, unpublished), and *Neotoxoptera formosana* (Takahashi) (Hori and Komatsu, 1997). The main component in rosemary oil is 1,8-cineole, and it strongly repelled *N. formosana*, but did not repel. *M. persicae*. *M. persicae* was repelled by linalool. *d,l*-camphor, and α-terpineol. *d,l*-Camphor and α-terpineol also repelled *N. formosana* but linalool did not. While rosemary oil repelled the aphids at a dose of 1 μl, its components did not repel them at a dose of 1 μl or 1 mg. The repellent action of rosemary oil may be caused by synergism of its components.

TABLE 2. REPELLENCY OF ROSEMARY OIL COMPONENTS AGAINST ALATE VIRGINOPARAE OF *Myzus persicae*

Chemical	Aphids trapped (*N*)		
	Treatment	Control	P^b
Linalool			
(10 μl)	7.4 ± 0.89	13.1 ± 1.43	0.01
(1 μl)	9.8 ± 1.19	11.6 ± 1.13	NS
d, *l*-Camphor			
(10 mg)	8.3 ± 1.02	12.9 ± 1.38	0.01
(1 mg)	8.3 ± 1.16	9.5 ± 1.07	NS
α-Terpineol			
(10 μl)	7.7 ± 1.62	15.8 ± 1.77	0.05
(1 μl)	8.3 ± 1.44	11.8 ± 1.21	NS
Borneol (10 mg)	11.2 ± 1.13	14.6 ± 1.36	NS
Bornyl acetate (10 μl)	7.3 ± 1.21	9.3 ± 0.80	NS
(+)-Camphene (10 mg)	10.7 ± 1.36	13.8 ± 1.45	NS
1,8-Cineole (10 μl)	7.3 ± 1.27	10.9 ± 1.57	NS
p-Cymene (10 μl)	11.6 ± 1.32	11.1 ± 1.08	NS
d-(+)-Limonene (10 μl)	12.4 ± 1.63	14.9 ± 1.62	NS
Myrcene (10 μl)	11.2 ± 1.12	14.8 ± 1.18	NS
α-Pinene (10 μl)	8.6 ± 0.84	9.0 ± 0.71	NS
β-Pinene (10 μl)	12.1 ± 1.32	11.7 ± 1.30	NS
(−)-*trans*-Caryophyllene (10 μl)	11.6 ± 1.05	12.3 ± 1.50	NS

[a] Values are means ± standard error.
[b] NS, no significant difference in paired *t* test ($N = 12$).

Early studies (e.g., Kennedy et al., 1959) suggested that olfactory cues play no part in host plant selection by aphids prior to their landing, but several olfactometer tests have confirmed that the behaviors of some aphid species are influenced by plant odors (Nottingham et al., 1991; Isaacs et al., 1993; Nottingham and Hardie, 1993; Hardie et al., 1994). However, there is little knowledge of whether the behavior of *M. persicae* is influenced by plant volatiles. This study proved that *M. persicae* senses plant volatiles and that their behavior is influenced by these volatiles.

In the field, landing of aphids is influenced by plant volatiles as proved in the case of *C. aegopodii* (Chapman et al., 1981), *R. padi*, *S. avenae*, and *M. dirhodum* (Pettersson et al., 1994). In the present study, landing of *M. persicae* was inhibited by rosemary oil in a screenhouse. This result indicates that *M. persicae* senses plant volatiles in the field and probably uses the volatiles as olfactory cues in host plant selection, but the effect of rosemary oil on *M. persicae* in the screenhouse was too weak for practical use in the field.

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BEHAVIORAL EFFECTS AND SENSORY DETECTION OF DRIMANE DETERRENTS IN *Myzus persicae* AND *Aphis gossypii* NYMPHS

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Abstract—Eleven synthetic drimane compounds were tested for their deterrency to nymphs of *Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover) (Homoptera: Aphididae). In general, *A. gossypii* nymphs were less sensitive to the drimanes than *M. persicae* nymphs. Warburganal (**1**) and polygodial (**2**), with a β-dialdehyde configuration and double bond at C7–C8, were highly active as deterrents and/or feeding inhibitors against both species. One of the synthetic compounds (**10**) showed relatively high activity against *A. gossypii*, while it was not active at all against *M. persicae*, indicating that synthetic analogs of natural deterrents can be highly selective feeding deterrents. From 24-hr interval observations and ablation studies, it is concluded that nymphs of both *M. persicae* and *A. gossypii* detect polygodial and possibly the other drimanes tested with contact chemosensilla at the tips of their antennae. The ablation studies also showed that in both species no tarsal, labial, or epipharyngeal sensilla are involved in detecting polygodial within 24 hr.

Key Words—*Myzus persicae*, *Aphis gossypii*, Homoptera, Aphididae, behavior, feeding, sensory detection, antennae, deterrents, drimanes.

INTRODUCTION

Sesquiterpene drimanes, such as warburganal and polygodial, are secondary plant compounds that can be isolated from several plant species, e.g, *Warburgia ugandensis* (Kubo et al., 1976). They have been shown to protect these plants by toxic or deterrent activity against herbivorous organisms (Jansen, 1993; Mabry and Gill, 1979). Much of the research on drimanes aims at using them as plant

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protection agents against insects (Frazier and Chyb, 1995). The feeding deterrent activity of drimanes has been demonstrated against insects belongong to many different orders, e.g., *Spodoptera* and *Heliothis* species (Blaney et al., 1987), *Myzus persicae* (Asakawa et al., 1988), *Leptinotarsa decemlineata* (Gols et al., 1996), and *Pieris brassicae* (Messchendorp et al., 1996).

Research on structure–activity relationships (SAR) for deterrents may lead to the discovery of more effective molecules. In most studies dealing with insect feeding deterrents, the test compounds are applied to the surface of leaf discs or artificial diets. This strategy has also been used for aphids with significant effects in field tests (Pickett et al., 1987) and laboratory tests (Asakawa et al., 1988; Lowery and Isman, 1993; Gutiérrez et al., 1997; Powell et al., 1997).

It has previously been shown in short-term behavioral tests that adult *M. persicae* can detect the drimane polygodial (compound **2**; Figure 1), applied to Chinese cabbage leaf discs and to plastic and glass surfaces, via contact chemosensilla located at their antennal tips (Pickett et al., 1992; Powell et al., 1995). More knowledge about the mechanism(s) of action of deterring compounds against aphids and the sensilla involved in detecting them could assist in utilizing such compounds as crop protection agents.

In this work, 11 synthetic drimane compounds, applied to the surface of artificial diet sachets, were tested for their deterring activities on nymphs of two aphid species, *Myzus persicae* and *Aphis gossypii*. Both species are important pests on many crops throughout the world (Eastop, 1983; van Steenis, 1995), especially *A. gossypii*, which rapidly exhibits resistance to many insecticides

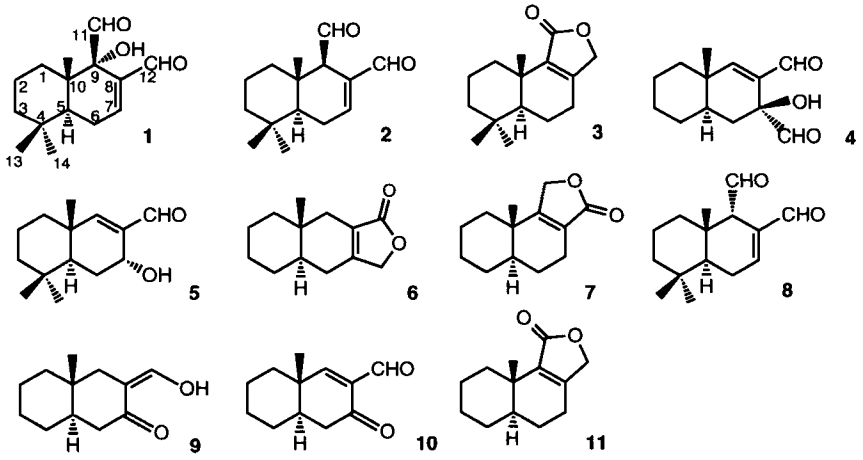

FIG. 1. Chemical structures of the drimanes. Trivial names: warburganal (**1**), polygodial (**2**), isodrimenin (**3**), polygonal (**5**), and isotadeonal (**8**).

(Kerns and Gaylor, 1992). Using both *M. persicae* and *A. gossypii* nymphs, we studied how drimanes influence the distribution pattern of the nymphs over artificial diet test-rings in time and which sensilla the aphids use to detect the drimanes.

METHODS AND MATERIALS

Aphids. Parthenogenetically reproduced, apterous *M. persicae* (clone WMp3) (Reinink et al., 1989) were reared on 3- to 4-week-old oil seed rape plants (*Brassica napus*, cultivar Olymp) in a greenhouse at 16 ± 2°C (night), 20 ± 5°C (day) and a 16L:8D photoperiod. Field collected *A. gossypii* aphids were obtained from the Centro de Ciencias Medioambientales, CSIC, Madrid, Spain. Parthenogenetically reproduced, apterous nymphs were reared on 5- to 8-week-old cucumber plants (*Cucumis sativus* L., cultivar Chinese slangen) in a greenhouse, under the same conditions as *M. persicae*. The 5- to 6-day-old *M. persicae* and 1- to 3-day-old *A. gossypii* nymphs reared on plants were used in the dual-choice tests. For the other experiments, nymphs reared on artificial diet (Harrweijn, 1983) were used.

Chemicals. Eleven synthetic drimanes (Figure 1) were obtained from the Department of Organic Chemistry, Wageningen Agricultural University (Jansen, 1993; C. T. Bouwman, unpublished). All compounds are racemic (only one of the two enantiomers is shown in Figure 1). Although compounds **1**, **2**, **3**, **5**, and **8** can be isolated from plants (Jansen, 1993), in all experiments (0.1% (1 g/liter) synthetic drimane solutions (ca. 4–5 mM) in ethanol (>99%) were used.

Dual-Choice Test. Artificial diet test rings were prepared by stretching a layer of parafilm (ca. 5 × 5 cm) over a plastic ring (2.7 cm diameter and 1.7 cm high) and painting half of the lower surface of the parafilm in the ring with 10 μl drimane solution (ca. 3.5 μg drimane/cm^2) and the other half with 10 μl ethanol. Two drops (ca. 30–50 μl) of artificial diet (Harrewijn, 1983) were kept between two layers of parafilm that were sealed together in two half circles (ca. 1–2 cm diameter), thereby forming two sachets containing artificial diet (for control and treated half). After the first layer was dried, the sachets containing artificial diet (for control and treated half) were stretched over the first layer on the plastic ring. We used artificial diet to prevent possible phytotoxic effects of the drimanes (Asakawa et al., 1988; Perczel, 1994), that could influence the test results. We applied the solutions to a separate layer of parafilm because it was previously found in our laboratory that solvents necessary to dissolve the drimanes, such as ethanol, diglyme, or Tween-80, penetrate into the diet when directly applied to the sachet surface and subsequently have deterring and/or toxic effects themselves (Perczel, 1994), which could influence the test results

as well. Twenty to 25 nymphs reared on plants were put on the inside surface of the ring, and the ring was placed on a plastic Petri dish with the diet on top. Nymphs, instead of adults, were used because previous work had shown that the latter are less sensitive to drimanes (Perczel, 1994) and because adults keep producing progeny, which could result in early depletion of the diet. Each drimane was tested with 20 replicates in a climate chamber at 22°C with continuous illumination (two high-frequency Philips PL-L 24 W, color 84 fluorescent lamps) from above. Thin filter paper was attached just below the lamps to make the light more diffuse. After 24 hr, most *M. persicae* nymphs had moved to the diet surface to penetrate into the diet and the number of nymphs on the control and treated half was counted. *A. gossypii* nymphs were still relatively restless at 24 hr and were therefore counted at 48 hr. Data on test rings with fewer than 10 nymphs on the diet surface were omitted. A deterrency index (DI) was calculated for each ring: DI = (C − T)/(C + T) (C = number of nymphs on control half; T = number of nymphs on treated half). Wilcoxon's matched pairs signed rank test was used to assess statistical significance.

Observations at 24 Hours of Dual-Choice Tests. The same diet test rings and methods as described for the dual-choice test were used. Aphids were reared on diet to avoid transition effects following the change from plants to diet at the start of the test. The aphids on the lower surfaces of the diet sachets were video recorded during the 24 hr of the test [Panasonic CCD camera (WV-CD 20) equipped with Ernitec 8-16 mm zoom lens, connected to a Panasonic time lapse video cassette recorder (AG 6010)]. *A. gossypii* nymphs that were reared on diet were less restless than nymphs reared on plants and settled within 24 hr in these tests. We counted the number of aphids that walked and that remained stationary on the control and treated sides and on the middle line (the seal between control and treated diet sachet) every hour. *M. persiace* was observed on test rings treated with polygodial (**2**) and *A. gossypii* on test rings with polygodial (**2**) and isotadeonal (**8**). There were 6–10 replicates.

Observations at 24 Hours of Dual-Choice Tests After Ablation of Distal Antennae Parts. Aphid nymphs reared on artificial diet were anaesthetized for ca. 1–2 min with CO_2 and from both antennae the distal parts (1/3–2/3 of the total length) were cut with a razor blade to remove the contact chemosensilla at the tip of the flagellum (Anderson and Bromley, 1987). The olfactory sensilla on the distal parts of the antennae that were also removed by the operation are not likely to play a role in the detection of the drimanes, since these compounds exhibit very low volatilities. Control nymphs were anesthetized with CO_2 for 1–2 min. After ablation the nymphs were left on artificial diet rings for 24 hr to recover. The same methods as described for the 24-hr interval observations of the dual-choice tests were used. Control and treated groups of *M. persicae* and *A. gossypii* nymphs were video recorded on dual-choice test rings treated with polygodial (**2**). There were seven to eight replicates.

Observations at 48 Hours of Tests with A. gossypii *Nymphs Reared on Plants.* The same methods as described for the 24-hr observations of the dual-choice tests were used. *A. gossypii* nymphs reared on plants were video recorded during the 48-hr dual-choice test with polygodial (**2**). Two more sets of nymphs were video recorded during 48 hr on control diet rings and on rings closed with a parafilm layer but without diet sachets. There were six replicates.

RESULTS

Dual-Choice Test. In general, *A. gossypii* was less sensitive to the drimanes than *M. persicae* (Figure 2). The highest deterring indices were found in response to the dialdehydes warburganal (**1**) and polygodial (**2**) in *M. persicae*. Three compounds with a lactone substitution (**3**, **6**, and **7**), the dialdehyde (**4**), and the monoaldehyde (**5**) are also effective deterrents. Surprisingly, most *A. gossypii* nymphs reared on plants were found dead within the first 24 hr of the tests with warburganal (**1**) and polygodial (**2**), while nymphs reared on artificial diet stayed alive and were significantly deterred by polygodial (hatched bar) in a 24-hr test (see results of the 24-hr interval observations). When these compounds were tested at 0.05%, the majority of the nymphs was dead by 24 hr; however, when tested at 0.01%, no deterring effects occurred and most nymphs survived. (data not shown). Of the other compounds, **6**, **8**, and **10** had significant activity against *A. gossypii*. Nymphs reared on artificial diet (hatched bars, see also results of the 24-hr interval observations) seemed to be less sensitive to polygodial (**2**) (*M. persicae*) and isotadeonal (**8**) (*A. gossypii*) than nymphs reared on plants (black bars), but no significant differences were obtained.

Observations at 24 Hours of Dual-Choice Tests. In both species between one third and one half of the total number of nymphs entered the parafilm membrane within the first hour (Figure 3). During the next 10–15 hr of the tests, the total number of nymphs on the diet surface gradually increased. After the first hour, the mean number of nymphs of both species on the polygodial-treated sides hardly increased. The mean number of nymphs on the control sides did increase at a similar rate to that of the nymphs on the diet surface. The mean number of *A. gossypii* nymphs on the isotadeonal-treated sides increased during the first 3 hr to remain constant afterwards. The number of *M. persicae* nymphs that walked during the tests was very low (0–1), while the number of walking *A. gossypii* nymphs was slightly higher (0–3). The deterrent indices of the tests at 24 hr (mean $\pm$ SE) are given in Figure 2 (hatched bars).

Observation at 24 Hours of Dual-Choice Tests After Ablation of Distal Antennae Parts. The day after the ablation of the distal parts of the antennae $>90\%$ of the nymphs had survived. During the dual-choice tests with polygodial, the nonablated, anesthetized nymphs of both species showed a distri-

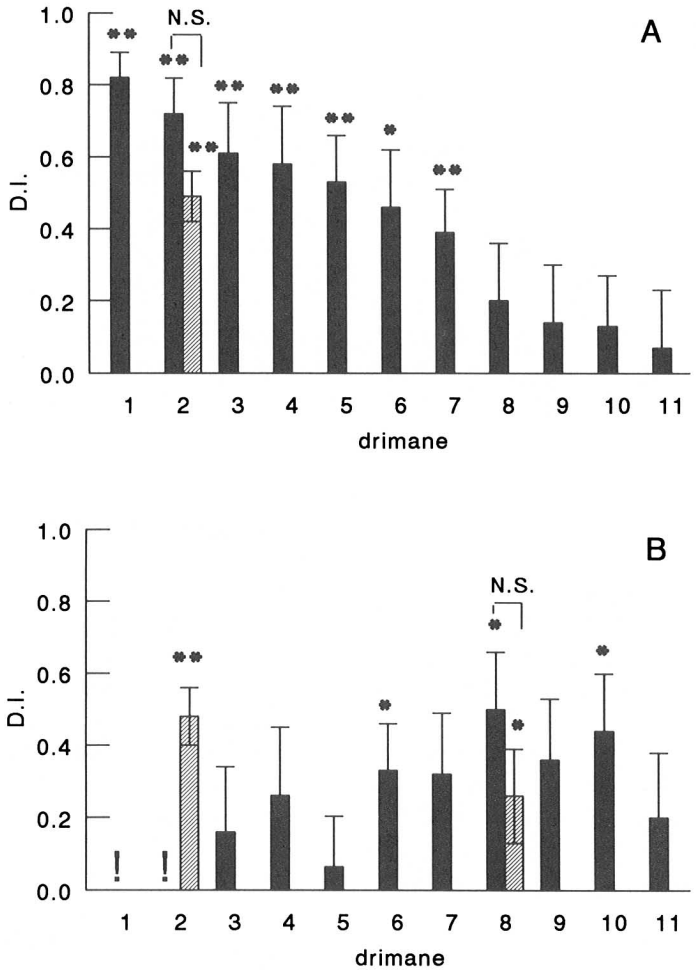

FIG. 2. Deterrency indices (DI) (means ± SE) of the dual-choice tests with nymphs reared on plants: (A) *M. persicae*, $N = 14$–20, calculated at 24 hr; (B) *A. gossypii*, $N = 19$–20, calculated at 48 hr. DIs of nymphs reared on artificial diet (hatched bars) are also given (see caption to Figure 3). The exclamation mark indicates the majority of the nymphs in these tests were dead at 48 hr. Statistics: Wilcoxon's matched pairs signed rank test (for deterrency indices) and Mann-Whitney U (to compare the results of nymphs reared on plants and nymphs reared on diet); $*P < 0.05$; $**P < 0.01$.

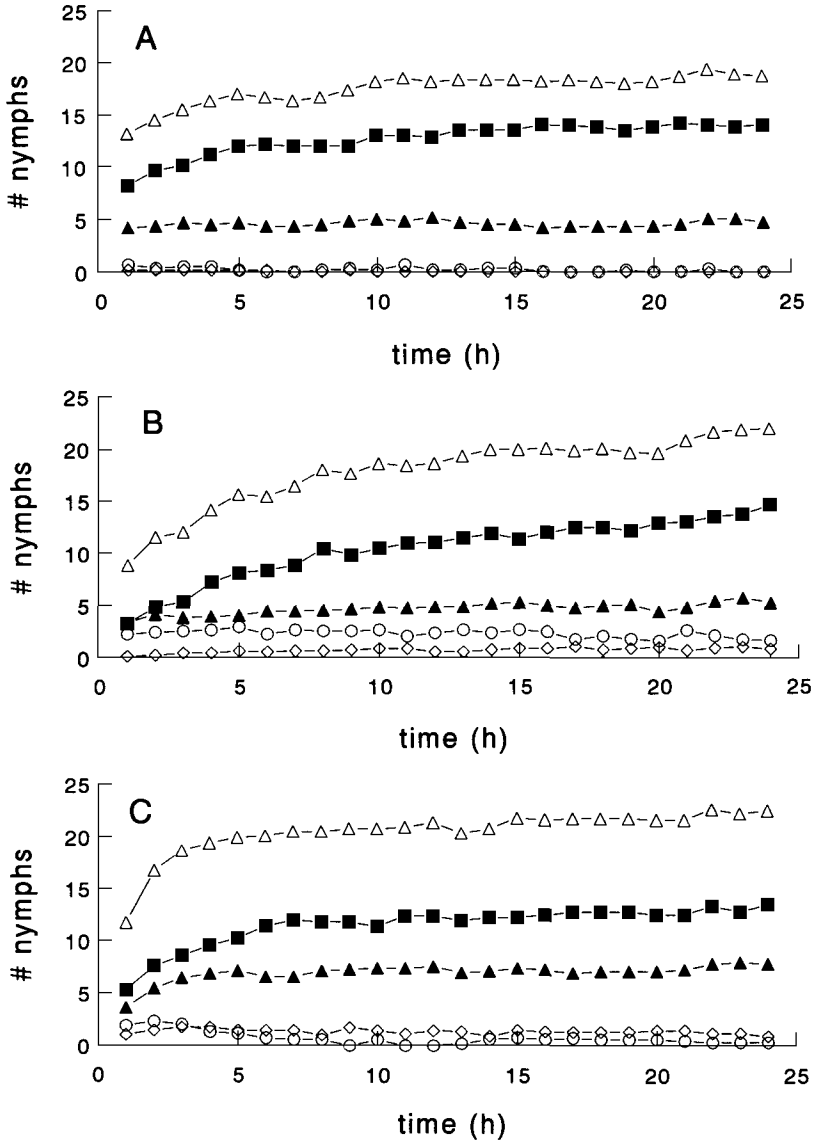

FIG. 3. The 24-hr distribution pattern during the dual-choice test of nymphs of (A) *M. persicae* on polygodial ($N = 6$); (B) *A. gossypii* on polygodial ($N = 10$), and (C) *A. gossypii* on isotadeonal ($N = 7$). Indicated are the mean numbers of nymphs of 20–25 introduced in total (△), walking (○), and stationary on the control (■) and treated (▲) surface, and on the middle line (◇). Standard errors ranged between 0 and 1.8. The deterrency indices of the tests at 24 hr are given in Figure 2.

bution pattern (Figure 4) similar to the pattern of nonanesthetized nymphs (Figure 3). At 24 hr, the deterrent indices of these tests were (mean ± SE) 0.55 ± 0.09 ($P < 0.01$) and 0.50 ± 0.1 ($P < 0.01$) for *A. gossypii* and *M. persicae*, respectively. The ablated nymphs of both species spread evenly over the control and treated sides of the diets throughout the tests (Figure 4). After 13 hr, some *A. gossypii* nymphs moved from the treated to the control sides, but no significant difference between numbers on treated and control sides developed within 24 hr (Wilcoxon's matched pairs signed rank test). The mean deterrent indices at 24 hr were 0.32 ± 0.2 (NS) and 0.31 ± 0.2 (NS) for *A. gossypii* and *M. persicae*, respectively.

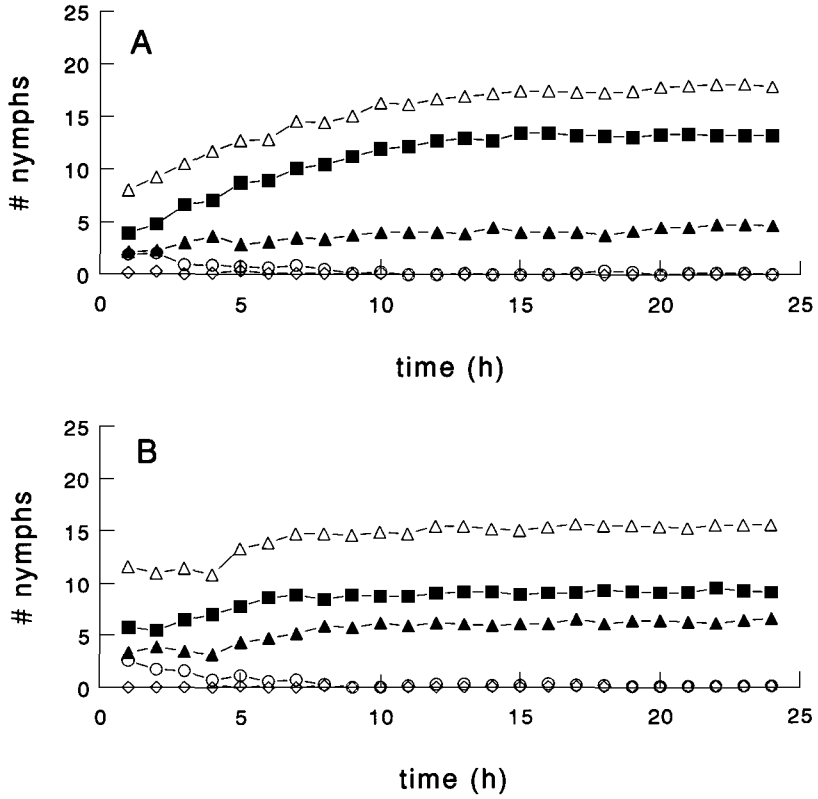

FIG. 4. The 24-hr distribution pattern during the dual-choice test after ablation of the distal parts of the antennae or anesthetization only of nymphs of *M. persicae* (A) control, $N = 8$; (B) ablation, $N = 8$, and *A. gossypii* (C) control, $N = 8$; and (D) ablation, $N = 7$). Symbols and SE as in Figure 3 legend.

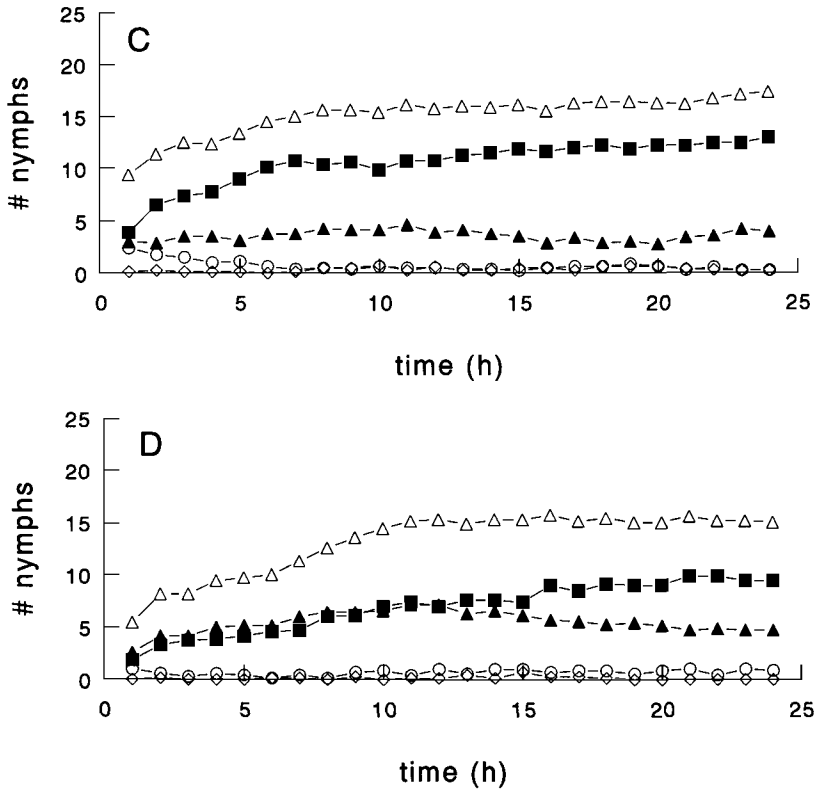

FIG. 4. Continued.

Observations at 48 Hours of Tests with A. gossypii *Nymphs Reared on Plants.* To investigate the cause of the mortality of *A. gossypii* nymphs reared on plants after transfer to polygodial dual-choice test rings, we examined the distribution pattern of the nymphs during 48 hr and also examined the distribution pattern of nymphs that were transferred to control diet and of nymphs without diet. Because the distribution patterns of the nymphs on the diets remained relatively stable after the first 24 hr, we show only the first day of the observation (Figure 5).

After being transferred from plants to control diet, the mean number of walking nymphs decreased during the first 15 hr from ca. 10 to ca. 0–3 nymphs, while the mean total number of nymphs on both halves of the diet increased. At 48 hr, most nymphs survived. When transferred to polygodial dual-choice rings, the distribution pattern of the nymphs was similar, but the number of

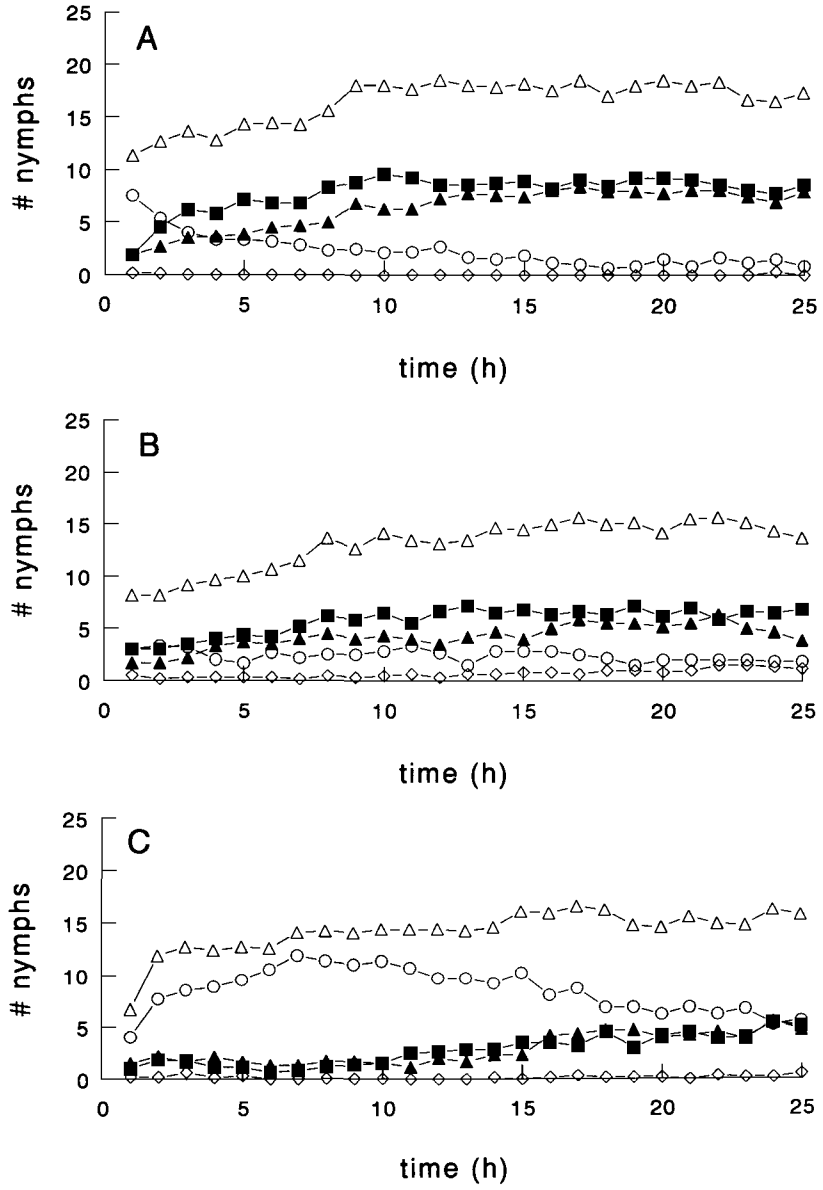

FIG. 5. The 24-hr distribution pattern of *A. gossypii* nymphs reared on plants during three tests: (A) control diet on both sides, (B) polygodial (dual choice); and (C) no diet test. $N = 6$ for all tests. Standard errors ranged between 0 and 2.9. Symbols as in Figure 3 legend; for the tests without diet and with control on both sides C and T indicate the two (indifferent) halves of the diet.

walking nymphs remained low throughout the test. The nymphs showed no preference for either the control or treated side. At 48 hr, ca. 75% of the nymphs were dead. From the video recordings we could not deduce when they had died because most dead aphids remained on the parafilm and could not be distinguished from penetrating aphids. Nymphs transferred to rings without diet walked more during the first 10 hr than nymphs on the control or polygodial dual-choice diet. After ca. 10 hr the mean number of stationary aphids on both halves conversely increased with the decrease in mean number of walking aphids. After ca. 40 hr, no further walking occurred. At 48 hr, ca. 85% of the nymphs were dead.

DISCUSSION

Figure 2 shows that the SAR of the drimanes for the two aphid species differ. Warburganal (**1**) and polygodial (**2**), with a β-dialdehyde configuration and double bond at C7–C8, are highly effective as deterrents and/or feeding inhibitors against both species. It is clear that not only the presence of substituents on the double ring drimane structure, but also their configuration and combination with other substituents (compare, e.g., compounds **1**, **2**, **4**, and **8** or compounds **3**, **6**, **7**, and **11**) determine the activity of the drimanes. Several authors (e.g., Blaney et al., 1987; Asakawa et al., 1988; Gols et al., 1996) have shown earlier that a C9 β-configured dialdehyde, combined with a C7–C8 double bond gives highly effective drimane antifeedants, which is confirmed by our results. Remarkably, the relatively simple synthetic compound **10** shows fairly high deterrent activity against *A. gossypii*, while it was not effective at all against *M. persicae*, *L. decemlineata* (Gols et al., 1996) and *P. brassicae* (Messchendorp et al., 1996), which we tested earlier. This result indicates that synthetic analogs of natural deterrents could be developed into highly selective feeding deterrents.

The 24-hr interval observations of the dual-choice tests (Figure 3) show that the number of walking nymphs remained low throughout the tests, while the total number of nymphs on the diet surface increased after the onset of the tests. We conclude that the majority of the nymphs of both species settled within an hour after having entered the diet surface and subsequently remained immobile for the greater part of the time. From the 24-hr ablation studies (Figure 4), it is clear that nymphs with ablated antennae do not differentiate between control and polygodial treated surfaces throughout the test. After ca. 12 hr, some *A. gossypii* nymphs switched from the treated to the control surface, but no significant difference developed within 24 hr. We conclude that nymphs of both *M. persicae* and *A. gossypii* detect polygodial and possibly also the other drimanes with the contact chemosensilla located at the tips of their antennae. This is in agreement with the results of Powell et al. (1995), who showed that adult

M. persicae, in short-term behavioral tests, detect polygodial with the contact chemosensilla at the tips of their antennae. The ablation studies also show that in both species no epipharyngeal, tarsal, or labial sensilla are involved in detecting polygodial (when applied to the parafilm surface) within 24 hr (Figure 5), which is in agreement with the hypothesis that among the latter two types of sensilla no chemoreceptors are present in aphids (Tjallingi, 1978). Since aphids wave their antennae and contact the surface while walking (Powell et al., 1995) and then fold their antennae backwards when penetrating or remaining immobile, the choice for the control side must be made before penetrating.

The fact that *A. gossypii* nymphs reared on plants, in contrast to nymphs reared on diet, do not survive in the dual-choice tests with warburganal (**1**) and polygodial (**2**) is rather surprising. In the 48-hr test on polygodial, the nymphs remained relatively immobile, in contrast to nymphs that were kept in test rings without diet, and spread evenly over control and treated side (Figure 5). It is possible that, because nymphs reared on plants experience a more extreme difference in taste, in comparison with their previous diet, than nymphs reared on artificial diet, the deterrent action of warburganal and polygodial is so strong that the nymphs are inhibited from ingesting any of the diet, resulting in death. This phenomenon could be related to a "central nervous system inhibitory state" caused by extreme deterrency, in which case the insect does not respond to those feeding stimuli that are present (Jermy, 1971). Earlier, with *Aphis fabae* (Hardie et al., 1992) and *M. persicae* adults (Powell et al., 1993), it was found that a 24-hr preexposure to leaves or green paper painted with polygodial caused a reduction in the subsequent number of penetrations and an increase in the mean duration of these penetrations. These effects of polygodial on adult aphids and the effects on nymphs in our experiments could have a similar origin.

The ecological role of deterrents on the leaf surface in the process of food plant selection by aphids is not yet fully elucidated (Klingauf, 1987; Niemeyer, 1990; Pickett et al., 1992; Tjallingii, 1995). The feeding habit of aphids by penetrating the plant and feeding from phloem sap means that they contact leaf surface compounds as well as compounds inside the plant. Instances of influences of leaf surface compounds (e.g., Klingauf et al., 1978) as well as phloem components (Tjallingii, 1995) on aphid feeding behavior are known. The relative importance of feeding behavior modifying compounds at both locations is not known and might differ between aphid species. Our study shows that both *M. persicae* and *A. gossypii* nymphs detect drimane deterrent compounds, applied to the surface of artificial diets with the contact chemosensilla at the tips of their antennae. It is possible that these sensilla also play a role in the selection of host plants by these and other aphid species. The difference in the sensitivity of the two species for the drimanes possibly reflects differences in the perception of deterrents at the molecular level in the antennal sensilla. This research shows that drimane compounds, applied to surfaces of artificial diets, can have strong, species-specific, deterring and feeding inhibiting effects on *M. persicae* and

A. gossypii aphids. This indicates that spraying plants with deterring compounds, through interference early in the host-plant selection sequence by aphids, could be a promising way to protect plants against aphid infestation.

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PREDICTION OF RESISTANCE IN SUGARCANE TO STALK BORER *Eldana saccharina* BY NEAR-INFRARED SPECTROSCOPY ON CRUDE BUDSCALE EXTRACTS: INVOLVEMENT OF CHLOROGENATES AND FLAVONOIDS

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Abstract—Predictive models based on data acquired by near infrared (NIR) spectrophotometry suggest that components in extracts from sugarcane nodal budscales contribute towards resistance to the stalk borer *Eldana saccharina*. NIR spectra for 60 sugarcane clones varying in resistance to *E. saccharina* indicated that chlorogenates and flavonoids might be involved in the NIR calibration and also in the interaction between the insect and sugarcane. Two extreme types of flavonoid profile subsequently were revealed, one associated with resistance and the other with susceptibility. Incorporation of the susceptible type profile into a defined synthetic diet increased feeding initiation and subsequent survival of first instar larvae compared with the resistant type profile. NIR calibrations proved capable of predicting the resistance ratings of validation sets.

Key Words—Near infrared, *Eldana saccharina*, sugarcane, host-plant resistance, flavonoids, Lepidoptera, Pyralidae.

INTRODUCTION

The stalk borer *Eldana saccharina* Walker is the major pest of sugarcane (*Saccharum* spp. hybrids) in South Africa (Atkinson et al., 1981). Since 1979 clonal resistance to *E. saccharina* has been a selection criterion in the plant breeding program (Bond, 1988). However, field-based selection is largely restricted to one of five selection sites where *E. saccharina* numbers are relatively high, and the effectiveness of the selection program is further limited by the presence of low levels of the pest during years of high rainfall (Anon, 1984). Screening

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trials with potted plants are restricted to late selection stages in which few clones are present because of space and cost limitations. Late-stage screening often results in the loss of otherwise elite clones because of susceptibility to *E. saccharina*. Measurements taken in these trials are frequently characterized by coefficients of variation as high as 50% (Keeping, personal communication). In many instances clones have to be screened several times before a reliable estimate of resistance rating can be made. The development of rapid, laboratory-based techniques, applicable to early selection stages when many clones are present, is desirable.

Newly hatched *E. saccharina* larvae spend about one week browsing on the stalk surface before survivors attempt to bore into the stalk. Observed differences in sugarcane resistance therefore might be partly explained by biochemical influences of the stalk surface on larval behavior and survival. Progress towards the use of near-infrared (NIR) spectrophotometry as a rapid means of biochemically profiling internode surface wax to classify sugarcane clones as resistant, intermediate, or susceptible has been reported by Rutherford and van Staden (1996).

In this work the possible contribution of a second stalk surface characteristic towards resistance was investigated. The nodal bud is a favored site of entry for *E. saccharina*, and entry frequently follows some browsing of the protective budscales (Leslie, 1993).

As in the earlier work on stalk surface wax (Rutherford and van Staden, 1996), the NIR technique was used in preference to traditional chromatographic component profiling methods because of three specific advantages: (1) the technique is extremely rapid, since no chromatographic separation is required; (2) multiple relevant components can be profiled simultaneously; and (3) no prior knowledge of the identity of any active components is necessary.

The objectives of this study were to determine whether NIR spectral data of sugarcane budscale extracts could be significantly correlated with resistance to *E. saccharina*, to gain some insight from the NIR data as to the identity of any likely active components, to demonstrate cause and effect, and to compare calibration methods. If successful in predicting resistance, the ultimate aim is to develop a fast, low-cost NIR technique for the prediction of budscale based resistance in unclassified clones.

METHODS AND MATERIALS

Extraction of Budscales. Budscales were peeled intact from the top three buds on seven stalks of each clone (21 budscales in total). Budscales were immersed (0.2 g fresh mass per 8 ml) in an 8:4:3 mix of cold, helium degassed

MeOH; $CHCl_3$; and H_2O, and were allowed to stand overnight at 4°C before being ground. The following volumes are all per 0.2 g fresh mass of budscales, volumes being adjusted accordingly for actual mass.

The homogenate was filtered through MeOH wetted Whatman No. 1 filter paper, which was rinsed sequentially with 4 ml of a 4:1 mixture $CHCl_3$; and MeOH and 2 ml of a 3:2 mixture of potassium-phosphate buffer (50 mM, pH 8) and MeOH. After brief shaking the extracts separated into an upper, predominantly aqueous phase, and a lower, predominantly $CHCl_3$ phase. The upper phase was recovered (approximately 5 ml). A further 1 ml of the 3:2 mix was added and again the upper phase was recovered (approximately 3 ml) and pooled with that collected previously. The latter step was repeated and then the pooled aqueous phase was acidified with metaphosphoric acid to approximately pH 4 and evaporated to dryness at 60°C under a stream of nitrogen. The dried extract was redissolved in 400 μl of MeOH and 600 μl of H_2O per 0.2 g of fresh mass and any particulates were spun down.

Determination of Resistance Ratings. The resistance ratings of sugarcane clones used in this study were derived from results obtained in the routine plant breeding screening and selection program. These screening trials are carried out as follows:

Plants are grown in 30-liter buckets for nine months before being stressed through reduced watering followed by inoculation with *E. saccharina* eggs as described by Nuss and Atkinson (1983). Each clone is replicated four to eight times, depending upon the number of clones in the trial, and five standard clones are included in each replicate. Five hundred day-degrees (with a developmental threshold of 10°C) after inoculation, stalks are split and larval survival, larval mass, and percentage of damaged internodes are assessed. Each clone is rated using each of the above measures and an average rating for each clone within the trial is obtained. Clones are rated as resistant (1, 2, and 3), intermediate (4, 5, and 6), and susceptible (7, 8, and 9), based on comparisons with the five standard clones.

During the course of the plant breeding screening and selection process each clone is assessed two or more times. Sixty-one clones, which showed consistent classification (resistant, intermediate, or susceptible) in at least two screening events, were selected from those available.

Near Infrared. An NIRSystems 5000 scanning monochromator (NIRSystems, Silver Spring, Maryland) was used to acquire transmission spectra (1-mm light path) with near-infrared radiation from 1100 to 2500 nm for 1 ml extracts from 61 sugarcane clones. Infrasoft International (ISI) NIRS 3 software was used for spectral analysis and calibration development.

Statistical Methods. Despite the success of full spectrum methods of calibration such as partial least squares regression, the use of multiple linear regres-

sion (MLR) with a small number of selected wavelengths is still a useful approach, particularly when the ultimate aim is to implement the application on a fast, low-cost instrument (Fearn et al., 1998).

In forward stepwise MLR 40 clones were used in equation development and 20 were reserved for validation. The SELECT spectral selection algorithm was evaluated for construction of the calibration and validation sets but, for reasons discussed later, its use was avoided. Instead samples were first split into three subsets of 20 using, as far as possible, a stratified random sampling technique (e.g., one of three clones with resistance rating 1 was randomly selected for subset 1). Subsets 1 and 2 were then combined to form the calibration set, while subset 3 was reserved for validation.

The spectral structure of the data was checked by principal component analysis (using the CENTER procedure) and any spectral outliers (i.e., a Mahanalobis H distance greater than 3 from the mean spectrum) were removed. In subsequent calibration, the critical H outlier value was set at 50 in order to retain all of the remaining spectral variability. Two outlier passes were permitted. This means that the ISI NIRS 3 software attempts to remove outliers twice before completing the final calibration. The critical T outlier value was fixed at 2.5. The maximum number of wavelengths to be used was set at one for every 10 samples in the calibration set as recommended in the ISI NIRS 3 User Manual (Schenk and Westerhaus, 1992). Resultant calibration equations were validated by predicting the ratings of subset 3, and a comparison was made between predicted and known ratings for this subset.

The second calibration procedure tested was modified partial least squares (MPLS) regression. MPLS is recognized as a very powerful tool for developing regression models from spectroscopic data and some advantages over classical MLR have been shown (Schenk and Westerhaus, 1991). MPLS, by reducing the large set of raw spectral data into a small number of orthogonal factors, is particularly efficient when the data are significantly intercorrelated. In studies using a limited number of samples, it is not normally possible to select an independent set of samples for validation of MPLS regression equations. Thus, standard error of prediction (SEP) was estimated by cross-validation, which is preferable to limiting the number of samples in the training set (Meuret et al., 1993).

In essence the approach used was as follows: The data set was split into three segments by the ISI CALIBRATION software corresponding to the same subsets outlined above. Two segments were used for the development of a prediction equation containing one MPLS factor. The performance of this equation was then evaluated on the remaining segment to produce a SEP. This exercise was repeated two further times using different combinations of subsets for calibration development and evaluation, such that three equations were produced utilizing one MPLS factor. The standard error of cross-validation (SECV)

was then derived from the above three SEP values by calculating the square root of the mean of the SEP squared values. This exercise was repeated for models containing 2, 3, 4, . . . , n factors and for each a SECV value was obtained. Cross-validation determines the optimum number of factors and minimizes overfitting; the best model being the one where the SECV first reaches a plateau or else is minimized. This procedure was repeated with four, five, and six cross-validation segments.

Results generated by MLR and MPLS regression equations were compared on the basis of the coefficient of determination (r^2), standard error of prediction corrected for bias [SEP(C)], the ratio of SEP(C) to the standard error of calibration (SEC), i.e., SEP(C)/SEC, regression intercept value (a) and regression gradient (b).

HPLC Separation of Flavonoids. Budscale extracts were concentrated under vacuum to 200 μl, and 20-μl aliquots were analyzed by HPLC. HPLC conditions were as follows: C18 Brownlee ODS-224 220 mm × 4.6 mm column; temperature 30°C, flow rate 1 ml/min; detection UV 345 nm; eluents A = pH 2 water (metaphosphoric acid), B = 7:5 acetonitrile–MeOH; gradient 10% B for 10 min; to 15% B in 10 min; to 35% B in 30 min; to 80% B in 10 min.

Dietary Incorporation of Flavonoids. Extracts from 1 g budscale samples from the five clones exhibiting the most resistant type flavonoid profiles were bulked (5 g total), dried, and redissolved in H_2O. This process was repeated for a susceptible bulk. Bulked extracts were adsorbed onto a 10 g column of preparative C18 packing (Waters). Bound flavonoids were washed with 100 ml pH 2 H_2O (metaphosphoric acid) and then desorbed with 80% EtOH.

Meridic diets, omitting propyl-gallate and methly-*p*-hydroxy-benzoate (Rutherford et al., 1994), were prepared with flavonoid extracts dried onto the cellulose before diet mixing. For both the resistant and susceptible bulks, flavonoids were added to diet at a 1:1 ratio of fresh mass of budscales to fresh mass of diet. For each treatment, 5 g of diet was prepared and individual first instars larvae were inoculated onto 0.1 g each (five replications of 10 larvae). Feeding initiation and survival were assessed after one week.

RESULTS

Precalibration. Prior to calibration development, one spectral outlier was detected using the CENTER procedure of ISI NIRS 3 software. The outlier (N14) had an H distance of greater than 3 from the mean spectrum of the 61 spectrum set and consequently was excluded from the calibration and validation sets.

Calibration Using Stepwise MLR. Use of the SELECT spectral selection algorithm in constructing calibration and validation sets for the MLR method resulted in all clones with a resistance rating of 1 and two of three clones with

a resistance rating of 9 being included in the calibration set. According to Fearn (1997), spectral selection algorithms ensure that the spectra retained for the calibration set are as widely variable as possible. Those excluded will be spectrally inside the calibration samples and consequently cannot be representative of a true future set, i.e., a validation set. The retention of all but one clone with resistance ratings of 1 and 9 in the calibration set suggests that these spectra were among the most distant from the mean spectrum. To represent spectral variation properly in the validation set, a stratified random sampling technique was preferred to the ISI spectral selection algorithm.

A preliminary calibration was then developed by stepwise MLR using the CALIBRATE procedure of ISI NIRS 3 software with the aim of identifying possible active components through the use of spectra–structure correlation charts such as that of Goddu and Delker (1960). This calibration was found to use wavelengths in the region of 1800–2492 nm. In order to explore the entire spectrum, a second calibration was developed using the region 1108–1796 nm.

Clone J59/3 was found to be a *T* outlier and was deleted from the calibration set. Final calibrations suggest that chemical differences exist in sugarcane budscale extracts that can be significantly correlated with resistance or susceptibility to *E. saccharina* (Tables 1 and 2). The wavelengths chosen, and occasionally divergent predictions (Table 2), suggest that different components may be implicated in model A compared with model B. Thus, the system may be biochem-

TABLE 1. TWO PRELIMINARY MULTIPLE LINEAR REGRESSION (MLR) NIR CALIBRATIONS ON BUDSCALE EXTRACTS OF 39 SUGARCANE CLONES

Model	λ chosen	λ may be characteristic of:	Coefficient of multiple determination R^2
A[a]	1460	aromatic.OH or $CONH_2$	0.480
	1508	$R.NH_2$	
	1244[c]	chlorogenic acid	
	1764	$.CH_2$	
B[b]	1968	starch/sugars or $.NH_2$	0.527
	2464	protein	
	1944	.OH or $.CO_2R$	
	1936	starch/sugars	

[a] Data 1108–1796, 8 nm gap; data treatment 2nd derivative, 8 nm gap, 6 mn smooth; stepwise MLR.

[b] Data 1800–2492, 8 nm gap; data treatment 2nd derivative, 8 nm gap, 6 nm smooth; stepwise MLR.

[c] 1246 nm is reported as characteristic of chlorogenic acid in coffee (Salgo and Izvekov, 1992).

TABLE 2. INTERNAL CALIBRATION SET PREDICTIONS OF *E. saccharina* RESISTANCE FOR 39 SUGARCANE CLONES MADE BY NIR MODELS A AND B AND PREDICTIONS MADE BY AN HPLC FLAVONOID PROFILE CHARACTERISTICS MODEL C

Clone	Resistance ratings (Y)	Model		
		A[a]	B[b]	C[c]
Co281	1	3.11	3.52	3.41
N21	1	2.60	3.61	4.92
N20	2	3.88	4.41	5.46
75L1463	2	5.25	3.30	3.91
75L1157	2	2.65	1.56	4.38
76F879	3	3.97	3.42	5.82
CP57-614	3	4.04	2.33	3.95
79M955	3	3.42	4.00	4.68
78L960	3	3.84	4.40	4.33
74M659	3	4.82	4.79	4.35
81W133	4	3.80	4.00	5.43
N25	4	5.10	4.34	4.69
77L1720	4	3.11	3.74	5.52
75E247	4	4.21	3.42	3.89
N17	4	3.79	2.79	5.01
80F2412	4	3.53	6.53	5.29
84F3680	5	4.40	4.81	4.90
75E1293	5	5.61	4.98	3.80
77W635	5	2.94	5.68	5.12
81E1253	5	5.20	5.39	4.42
81W447	5	4.10	3.39	4.11
NCo376	5	5.91	4.85	4.76
77W1241	5	6.97	4.13	4.02
N23	5	5.76	4.26	5.42
81L1308	6	4.13	5.38	6.56
N18	6	4.21	5.11	5.61
82F2907	6	4.64	6.62	5.66
N19	6	6.78	6.48	4.97
83F1284	6	5.05	6.78	4.44
77F790	6	6.21	4.38	5.40
76H333	7	6.65	6.95	4.40
82F675	7	4.05	7.60	5.27
78F909	7	7.58	5.26	3.73
79H181	7	6.27	5.73	5.75
80F2147	7	5.06	4.71	4.87
77L1143	8	6.51	6.64	4.20
NCo293	8	8.00	9.01	7.36
83F448	9	8.61	6.88	5.45
N11	9	6.23	6.82	6.73
J59/3[d]	8	6.04	2.29	3.98

TABLE 2. CONTINUED

	Model		
	A[a]	B[b]	C[c]
R^{2e}	0.480	0.527	0.176
Standard error of calibration	1.483	1.414	1.919
F ratio	7.85	9.47	3.83
Level of significance for F	$p < 0.01$	$p < 0.01$	$p = 0.031$

[a]Data 1108–1796, 8 nm gap; data treatment 2nd derivative, 8 nm gap, 6 nm smooth; stepwise MLR.
[b]Data 1800–2492, 8 nm gap; data treatment 2nd derivative, 8 nm gap, 6 nm smooth; stepwise MLR.
[c]HPLC flavonoid profile model (see Table 4, variables 1 and 2)
[d]Model B T outlier J59/3 was removed from all calibrations.
[e]R^2 = coefficient of multiple determination.

ically complex. To test the validity of the MLR calibrations, equations for models A and B were used to predict the resistance ratings of the 20-clone validation set (subset 3) excluded from the calibration. The results and prediction statistics presented in Table 3 show that predicted resistance ratings correlated weakly to moderately with actual ratings. The susceptible clone 80L432 was found to be a T outlier by model B.

Differences in Flavonoid Profile Correlated with Resistance and Susceptibility. Of the wavelengths chosen in MLR calibrations (Table 1), two of those in model A may be characteristic of chlorogenates and other aromatics. Subsequent profiling of the same budscale extracts for chlorogenates and flavonoids revealed two extreme HPLC chromatogram types (Figure 1). Integrated individual peak areas were assessed in stepwise MLR analyses for their ability to predict resistance. Of all of the peaks, those indicated in Figure 1, i.e., A, B, C, and D plus total integrated areas, E, i.e., Σ 0–60 min, were the most significant in the development of predictive models. A summary of results in presented in Table 4. Increasing peak D and the total integrated area, E, are associated with susceptibility. On the other hand, increasing peak A expressed as a proportion of total chlorogenates, A/(A + B + C), and total chlorogenates expressed as a proportion of E, (A + B + C)/E, are associated with resistance (Table 4).

In assessing predictive ability for the validation set (subset 3), the two-variable HPLC model was optimal in terms of minimizing SEP(C) and SEP(C)/SEC while maximising r^2. Similarly the regression intercept and gradient are

TABLE 3. VALIDATION PREDICTIONS OF *E. saccharina* RESISTANCE FOR SUB-SET 3 (EXTERNAL TO THE CALIBRATION SET) MADE BY NIR MODELS A AND B; AND PREDICTIONS MADE BY HPLC FLAVONOID PROFILE CHARACTERISTICS MODEL C

Clone	Resistance ratings (*Y*)	Model		
		A[a]	B[b]	C[c]
N8	1	2.83	0.74	4.99
B42231	2	2.38	3.30	3.30
80W1459	2	7.62	1.86	3.66
77F637	3	5.05	−0.25	3.98
84F3078	3	1.66	2.57	4.60
N12	4	8.86	1.22	5.29
79L1294	4	4.79	3.46	6.19
76M1101	4	4.15	4.03	6.41
78W1156	5	4.31	5.13	3.96
79F1043	5	7.91	3.40	6.06
N24	5	7.12	1.75	5.89
81E313	6	7.74	6.26	5.47
68W1049	6	7.68	6.94	5.09
N22	6	6.08	5.48	6.46
N13	7	7.84	7.89	7.04
79L181	7	6.00	4.96	5.31
84F2753	8	3.87	3.21	6.85
N16	8	9.12	5.38	5.89
N52/219	9	4.22	6.37	5.87
r^{2d}		0.128	0.501	0.398
SEP(C)[e]		2.548	1.711	1.765
SEP(C)/SEC[f]		1.718	1.210	0.920
Regression intercept (a)		3.944	0.322	3.855
Regression gradient (b)		0.361	0.709	0.306
		excluded		
N14[g]	7	5.58	−2.54	4.39
80L432[g]	8	3.62	1.08	4.41

[a] Data 1108–1796, 8 nm gap; data treatment 2nd derivative, 8 nm gap, 6 nm smooth; stepwise MLR.

[b] Data 1800–2492, 8 nm gap; data treatment 2nd derivative, 8 nm gap, 6 nm smooth; stepwise MLR.

[c] HPLC flavonoid profile model (see Table 4, two variables).

[d] r^2 = coefficient of determination.

[e] SEP(C) = standard error of prediction corrected for bias.

[f] SEP(C)/SEC = SEP(C)/standard error of calibration.

[g] *H* outlier N14 and model B *T* outlier 80L432 were removed from the analysis.

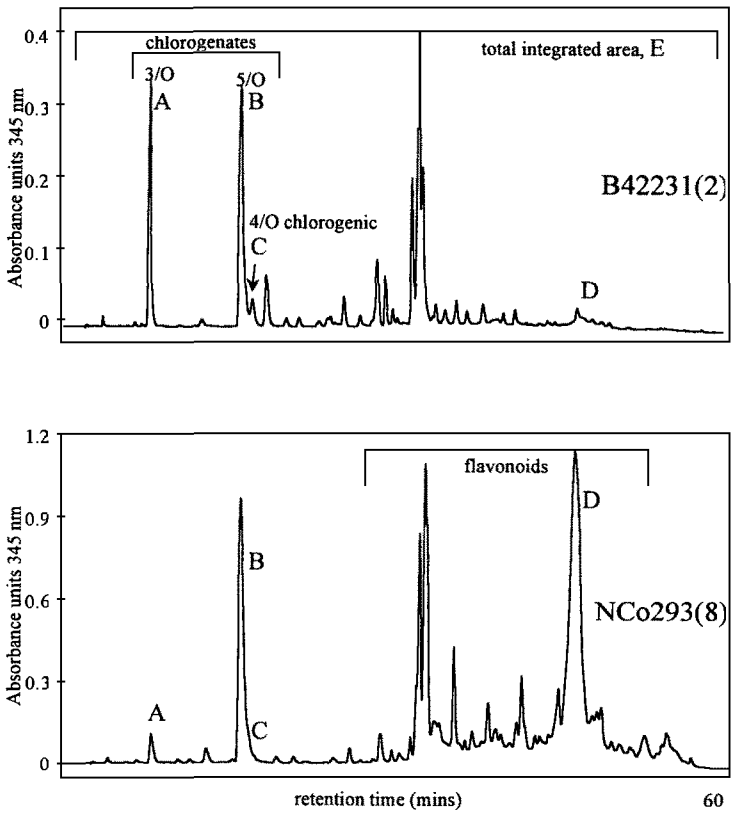

FIG. 1. HPLC chromatogram profiles of resistant (top) and susceptible clones, with resistance ratings in parentheses.

minimized and maximized respectively (ideally these would tend towards zero and one, respectively). The two variable model predictions are presented in Tables 2 and 3 as model C. Clones NCo293 (Table 2) and B42231 (Table 3) have the most extreme predictions.

Effect of Dietary Incorporation of Flavonoid Profiles on E. saccharina *Feeding Initiation and Survival.* Bulked and cleaned flavonoid extracts from the two extreme groups of five clones, as determined by the two-variable flavonoid model (Tables 2 and 3) were fed to *E. saccharina* in meridic diet. These bulks influenced feeding initiation (Figure 2). The diet containing the resistant bulk had noticeably browned by the end of the seven-day assay period, while the susceptible bulk diet retained a straw yellow color. The susceptible bulk appeared

TABLE 4. MODELS BASED ON HPLC FLAVONOID PROFILE CHARACTERISTICS OF 39 CALIBRATION SUGARCANE CLONES AND PREDICTIVE ABILITY ON SUBSET 3 (EXTERNAL TO CALIBRATION SET)

	Variables used for calibration[a]			
	v1	v1 and v2	v1–v3	v1–v4
R^{2b}	0.127	0.176	0.199	0.228
F ratio	5.39	3.83	2.90	2.51
Level of significance for $F(P)$	0.026	0.031	0.049	0.060
SEC[c]	1.947	1.919	1.918	1.910
constant (a)[d]	3.805	5.603	4.995	6.302
(b_1)[d]	+7.790[g]	+5.510	+4.201	+1.889
	(b_2)	−4.562	−4.456	−4.353
		(b_3)	+1.603	+1.967
			(b_4)	−4.711
Prediction results				
r^{2e}	0.315	0.398	0.345	0.276
SEP(C)[f]	1.897	1.765	1.819	1.917
SEP(C)/SEC	0.974	0.920	0.948	1.004
regression intercept (a)	4.200	3.855	3.858	4.114
regression gradient (b)	0.210	0.306	0.296	0.220

[a]Variables: v1, D/E_{clone}: peak D/total integrated area E of the clone; v2, A/(A + B + C): peak A/(peak A + peak B + peak C); v3, E_{clone}/E_{NCo293}: total integrated area E of the clone/total integrated area E of NCo293; and v4, (A + B + C)/E_{clone}: (peak A + peak B + peak C)/total integrated area E of the clone.
[b]R^2 = coefficient of multiple determination.
[c]SEC = standard error of calibration.
[d]Predictive equations take the form $\hat{Y} = a + b_1 \cdot v_1 + \ldots b_n \cdot v_n$.
[e]r^2 = coefficient of determination.
[f]SEP(C) = standard error of prediction corrected for bias.
[g]Positive coefficients denote association with susceptibility; negative coefficients denote association with resistance.

to promote feeding initiation and subsequent survival when compared with the resistant bulk and control. For the susceptible treatment, 25 larvae survived to seven days from the 29 that had initiated feeding. This compared to 5 of 16 and 4 of 10 for the resistant and control diets, respectively. Poor survival in the resistant diet may have been due to progressive autooxidative darkening of this diet.

Calibration Using MPLS Regression. The number of MPLS factors selected is of critical importance. To determine the optimal number, the calibration set was first split into cross-validation segments corresponding to subsets 1, 2, and 3. The SECV was plotted against the number of MPLS factors used. The optimal

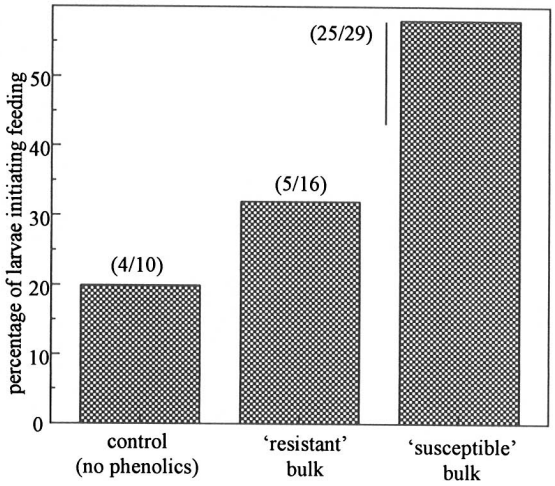

FIG. 2. Effect of sugarcane flavonoid extracts on percentage of larvae initiating feeding and survival of these (survivors/total initiated, in parentheses) at seven days. Bar indicates least significant difference$_{(0.01)} = t_{(0.01)}\sqrt{(2\sigma^2/r)}$ for feeding initiation.

number of factors when three cross-validation segments were employed was found to be four, as indicated where the SECV reaches a plateau (Figure 3).

The results shown in Figure 4 are the predicted values from cross-validation using four MPLS factors and are not the values of the final calibration process. An R^2 of 0.662 (Figure 3) and a SEC of 1.219 were achieved in final calibration with four MPLS factors. Since cross-validation segmentation was into the same subsets as those constructed for the MLR methods, the prediction results for subset 3 (Table 3 and Figure 4) can be compared between methods. Models are ranked for predictive ability using the five measures given in Table 5. The four-factor MPLS calibration method had the best overall ranking for predictive ability on subset 3. However, as a measure of likely performance on future validation sets, the coefficient of determination in cross-validation (r^2 CV) was maximized at 0.53 when cross-validating with six segments rather than three (five rather than four MPLS factors giving the SECV minimum).

DISCUSSION

Results shown in Tables 1–3 for MLR models A and B suggest that predictions of resistance are based on biochemically complex differences. The MPLS calibration method proved to be more effective than MLR for calibrating the

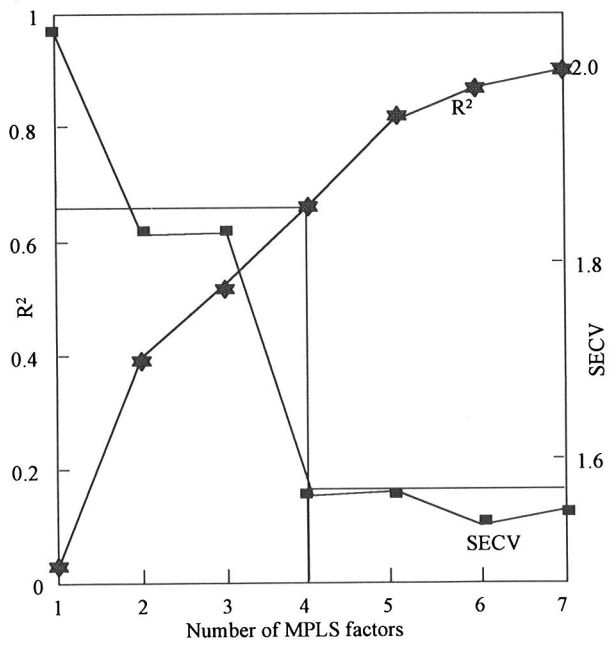

FIG. 3. Calibration statistics (coefficient of multiple determination, R^2, and standard error of cross-validation, SECV) for modified partial least squares (MPLS) regression models based on NIR spectra from bud extracts of 58 sugarcane clones varying in resistance to *E. saccharina*. (J59/3 and 80L432 were *T* outliers omitted from the calibration.

NIR data. MPLS is known to be more suited than MLR for the calibration of complex chemical characteristics, particularly when the data are significantly intercorrelated (Meuret, et al., 1993).

Based on the implicated involvement of aromatics by NIR spectral data and correlated flavonoid profile differences, chlorogenates and flavonoids would appear to be involved with resistance and/or susceptibility to *E. saccharina* in sugarcane. A calibration, optimized for predicting the validation set based on HPLC flavonoid profile characteristics, could explain no more than 17.6% (calibration set) and 39.8% (validation set) of the total variation (Tables 2–4). On the other hand, the MPLS calibration might be expected to explain approximately 53% of total variation in an external validation set. These data, combined with wavelength-component characteristics from Table 1, strongly suggest that other budscale components, in addition to flavonoids, are involved in the NIR predictions. Inclusion of the results from meridic diet incorporation of flavonoids

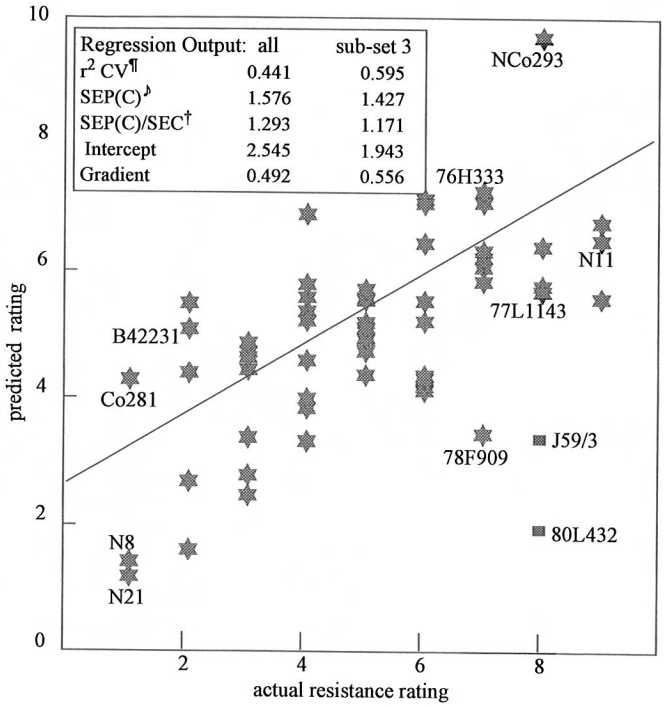

FIG. 4. Four-factor modified partial least squares (MPLS) regression predictions of resistance for samples in cross-validation using three segments corresponding to subsets 1–3. J59/3 and 80L432 were *T* outliers omitted from the calibration. r^2CV = coefficient of determination in cross-validation; SEP(C) = standard error of prediction corrected for bias; and SEP(C)/SEC = SEP(C)/standard error of calibration.

in support of their involvement is limited by the fact that the bulked extracts described represent a single sampling. To confirm these results, similar bulks from subsequent sets will also be tested by meridic diet incorporation.

In South Africa some 22 species of three plant families (Cyperaceae, Gramineae, and Juncaceae) are the only known natural host plants of *E. saccharina* (Conlong, personal communication). These families are shown to be closely related phytochemically, based on flavonoid components (Clifford and Harborne 1969; Williams and Harborne 1977; Harborne et al., 1982, 1985). Flavonoids are involved in many plant–insect interactions and can be active in host-plant recognition, feeding stimulation or deterrency, as well as having effects on insect physiology and nutrition (Schoonhoven, 1972; Slansky, 1992). The initial invasion of sugarcane by *E. saccharina* from neighboring *Cyperus papyrus* L.

TABLE 5. RANKING OF RELATIVE PREDICTIVE ABILITIES ON SUBSET 3 FOR THREE NIR CALIBRATION MODELS AND ONE HPLC CALIBRATION MODEL

	Measures of predictive ability					
Model	r^2 (maximize)[a]	SEP(C) (minimize)[b]	SEP(C)/ SEC (minimize)[c]	(a) (minimize)[d]	(b) (maximize)[e]	Overall ranking
NIR-MLR A)	4	4	4	4	3	4
NIR-MLR B)	2	2	3	1	1	2
HPLC C)	3	3	1	3	4	3
NIR-MPLS	1[f]	1	2	2	2	1

[a] r^2 = coefficient of determination.
[b] SEP(C) = standard error of prediction corrected for bias.
[c] SEC = standard error of calibration.
[d] (a) = regression intercept.
[e] (b) = regression gradient.
[f] Ranking of models, 1 = best; 4 = worst.

swamps in northern KwaZulu-Natal (Atkinson et al., 1981) may have been facilitated by flavonoid similarities between these plant families.

Of interest is the correlation between increasing flavonoid content, particularly peak D, and susceptibility to *E. saccharina*. In contrast, maize lines accumulating greater amounts of the flavonoid maysin are more resistant to the corn earworm, *Helicoverpa zea* (Boddie) (Wiseman et al., 1992). However, peak A in particular and total chlorogenates correlate with resistance to *E. saccharina* (Figure 1). The quinone products of the enzymic or autooxidation of chlorogenates are strong electrophiles that can covalently modify plant or insect proteins through reaction with the amino groups of basic amino acids (Mathew and Parpia, 1971). In the case of plant proteins, this can result in decreased nutritive quality. Following damage to plant tissues, plastidic diphenol oxidases (EC 1.10.3.1) come into contact with vacuolar chlorogenates, suggesting that diphenol oxidases might have a role in defense (Felton et al., 1989).

Clones exhibiting a resistant-type flavonoid profile, but that are in fact susceptible (76H333, 78F909, J59/3, 80L432, and 77L1143; Tables 2 and 3), may lack sufficient expression of diphenol oxidase activity. A preliminary comparison of bud diphenol oxidase activity in J59/3 with that of one of its parents, B44231 (resistant flavonoid profile and is resistant), suggests that J59/3 may have less than one third of the activity of B42231 on both per unit protein and per unit fresh mass bases (Rutherford, unpublished results). Both 80L432 and 77L1143 are progeny of J59/3.

The expression of diphenol oxidase and consequent production of quinones may be necessary for the expression of resistance associated with a resistant type flavonoid profile. In support of this unoxidized diphenols and their quinones have been shown to have opposite effects on insects (Norris, 1970; Kato, 1978). The possible involvement of diphenol oxidases in resistance to *E. saccharina* is under further investigation.

While more clones are desirable for calibration, the NIR models produced do appear to have predictive value, in that partial validations have been achieved. According to calculations made by Schenk and Westerhaus (1993), clones predicted as susceptible (of the three groupings; resistant, intermediate, and susceptible) would include only 4% of actual resistant clones, based on a coefficient of determination during cross-validation in the region of 0.53. However, bud-scale extracts used in this study were sampled from the same site and at the same time. Three subsequent sets of extracts from the same clones, but from different sites, growing seasons, and environmental conditions have produced calibrations that are not able accurately to predict ratings on other sets of samples (Rutherford, unpublished results). This means that a global library of spectra, from which a local calibration set can be drawn, will require a number of growing seasons and environmental conditions for further development. (Dardenne, 1996). Achieving this end and further validation of the NIR technique are the subjects of continuing investigation.

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VARIABILITY IN FLAVONOID COMPOUNDS OF FOUR *Tribulus* SPECIES: DOES IT PLAY A ROLE IN THEIR IDENTIFICATION BY DESERT LOCUST *Schistocerca gregaria?*

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Abstract—We examined how variability in phenolic metabolism of four closely related *Tribulus* taxa might affect palatability to *Schistocerca gregaria*. Plants were collected in the Mauritanian breeding areas of the locust. Eighteen flavonoids (caffeoyl derivatives, quercetin glycosides, including rutin, and kaempferol glycosides) were detected with high-performance liquid chromatography in leaf extracts. Principal component analysis (PCA) differentiated relative abundance of flavonoids of *Tribulus terrestris* from *T. longipetalus*, *T. ochroleucus*, and *T. bimucronatus*. Flavonoid content of *T. terrestris* leaves varied between northern and southern Mauritanian sites. Dual choice tests with plants whose flavonoid profiles had been determined previously were analyzed by multiple regression. The data suggest that the desert locust prefers *T. terrestris* plants rich in quercetin glycosides.

Key Words—*Tribulus* div. sp., *Schistocerca gregaria*, flavonoids, rutin, quercetin glycosides, caffeoyl derivatives, kaempferol glycosides, behavioral responses, host recognition, sympatry.

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INTRODUCTION

Many feeding behavior studies of herbivorous insects have investigated the effect of one chemical, generally the major compound in the host plant. Insect behavioral responses are, however, more likely to be induced by a blend of chemicals rather than by a single compound (Soldaat et al., 1996). These secondary metabolites serve as recognition signals. Dual-choice tests designed to study food preference or insect learning have generally been performed with host plants that differed substantially from each other. Bernays and Weiss (1996) have criticized such experiments, and Marquis (1992) has stressed that herbivores do not accept or reject "Latin binomials" on the basis of taxonomic distinctions, but respond to the food diversity in their environment.

In most plant populations, concentrations of individual natural products are log-normally distributed. In some cases, these intraspecific variations have a genetic basis (Vrieling et al., 1993) and are stable within populations (Till-Bottraud et al., 1988).

In this study, we focused on four *Tribulus* species (Zygophyllaceae) that are closely related on the basis of their phenolic metabolism and that are potentially edible by the desert locust (*Schistocerca gregaria* Forsk.). Field observations in the Saharan breeding areas of the locust indicate that *Tribulus* are attractive to this insect (Ghaout et al., 1991). In Mauritania, the desert locust breeds during the summer in pastures of *Tribulus terrestris*. Bands of hoppers (i.e., juveniles) settle, and later in the year, between October and December, emerging adults move to the northern regions to breed in vegetation patches where *Tribulus* species also occur. *Tribulus* is thought to play a role in the reproductive success of these insects. When rains are abundant, *Tribulus* may represent a large food source. However, the ecology of *Tribulus* and the feeding behavior of the locust are poorly documented (Popov et al., 1978). This study was designed to test whether the locust can discriminate between closely related species, based on complex chemical signatures.

METHODS AND MATERIALS

Insects

Fifth instars of *Schistocerca gregaria* (Forskål) were used in all experiments. The stock culture originated from individuals collected in Mauritania in 1992. They were acclimatized to laboratory conditions and reared according to Louveaux et al. (1994). Mass rearings were conducted in 100- to 220-liter cages that could accomodate 200–400 individuals. Food consisted of wheat seedlings and bran, with occasional additions of cabbage leaves. Rearing temperatures were 33°C ± 2°C (12-hr day) and 20°C ± 2°C (12-hr night). Newly molted, fifth instars were removed each day from the stock cage, dated, and placed in

separate cages under conditions similar to those for mass rearing. At 3–5 days (standard age), nymphs were deprived of food overnight, but supplied with drinking water.

Field Collections of Tribulus *Plants*

Mature specimens of *Tribulus* with fruits were collected between September and December in 1994 and 1995 at five sites. Sites were located in the Inchiri (three sites: 19°54′N, 14°51′W; 19°49′N, 13°55′W; and 19°39′N, 13°59′W), Trarza (17°44′N, 14°14′W), and Assaba (17°06′N, 10°59′W) regions of Mauritania. Whole plants were dried in the shade, brought back to the laboratory, packed in plastic bags, and stored at −40°C. Collected specimens were identified following descriptions and identification keys of Ozenda and Quezel (1956), El Hadidi (1978), and Barry and Celles (1991). In both years, mean vegetation cover was estimated visually by sampling 10 quadrats of 25 m^2 each at all sites. Samples were collected at random within patches of vegetation. Patches ranged from less than one to more than 30 hectares. In Assaba, they reached 100 ha when the rainy season was favorable. Additional observations and collections were done during October 1997 in Trarza to document the distribution of the species. Four patches with fully developed *Tribulus* in Trarza comprised a wadi between sand dunes (100 × 70 m), live dunes (120 × 85 and 70 × 45 m), and a slope (3000 × 1000 m). In each patch, we identified the *Tribulus* species and measured distances between the plants within a single 20 × 20-m quadrat.

Analysis of Flavonoids

Leaf phenolic contents of 90 individual plants of *Tribulus longipetalus* Viv., *Tribulus terrestris* L., *Tribulus bimucronatus* Viv., and *Tribulus ochroleucus* Maire were studied. Dry leaves (200–500 mg) were extracted with EtOH–H_2O (7 : 3) under reflux for 45 min. Crude extract was analyzed by high-performance liquid chromatography (HPLC) on Ultrabase C18 reverse phase columns (5 mm), with a water–acetonitrile gradient with 2% acetic acid. Detection was carried out at 320 nm. A diode array was used to check the UV spectra of compounds associated with each peak (Jay et al., 1975). We detected 18 peaks belonging to compounds in three classes of phenolics comprised of caffeoyl derivatives, quercetin glycosides, and kaempferol glycosides. Principal component analysis (PCA) was performed on the relative areas of the 18 peaks from the chromatographic profiles. The sum of the areas of the 18 peaks represents the 100% value.

Dual-Choice Test

Behavioral Experiments with Chemicals. Nymphs of standard age were offered a choice of filter paper discs (45 mm diameter, Prolabo) impregnated

with sucrose (0.3 M/g) (Cook, 1976) or various concentrations of phenolic compounds. Rutin (quercetin-3-β-D rutinoside, Sigma) and its aglycone (quercetin, Sigma) were dissolved in ammonia solution (20%). Chlorogenic acid (Acros), a caffeoyl derivative, was dissolved in water. Solutions prepared at various concentrations were added to the experimental discs. Control discs (sucrose 0.3 M/g) were impregnated with solvent only. All quantities were calculated in moles per gram dry weight of the paper. The range of concentrations covered natural levels occurring in the collected plants (1–10 μmol/g) up to 150 μmol/g (10% of the dry weight). Test and control discs were impregnated 1 hr before the choice test, and dried at 50°C in two different ovens.

One control and one test disc, each impaled on a pin, were placed in a jar (1 liter) along with cotton wool impregnated with drinking water. Nymphs were discarded at the end of each test to avoid complications related to learning behavior. Ten to 20 replicates were performed at each dose of the tested chemicals. Tests ran until at least 20%, but less than 50%, of one of the two discs was consumed (usually 3–4 hr). The eaten areas of the control and test discs were determined by using an electronic area-measuring device coupled to a video camera (Escoubas et al., 1993).

Results, expressed as a percentage, were calculated as [test disc/(test disc + control disc)] × 100 (Hagerup et al., 1990; Mole and Joern, 1994). Values ranged from 0 (no feeding on the test disc) to 100% (no feeding on the control disc). The eaten areas of the discs were compared by the Wilcoxon U test for matched pairs.

Behavioral Experiments with Plants. Insects were offered dried leaves of individual plants whose flavonoid patterns had been determined previously. Leaves were tightly packed in pills weighing 250 mg to diminish any differences in physical appearance (hairiness, toughness) and to mix leaves thoroughly from different ramets of a single individual. Fifty-seven experimental plants were individually tested against two reference plants, *T. terrestris* and *T. longipetalus*. Twenty-six plants also were tested against themselves, and these are referred to as blank controls. Individual choice tests were conducted in six plastic boxes (12 × 9 × 6 cm) framed together and illuminated with four 100-W electric bulbs. In order to ensure that the design was neutral, the behavior of individuals was checked against blank controls. We observed that in one of the six boxes, the insect had chosen 14 of 18 times to feed on the pill placed on the left side. This box was removed from the analysis since the behavior of the insects was probably influenced by reflected light. Temperature inside the boxes was set to 30–33°C. We observed the feeding behavior of 83 locusts in detail by using a video camera. Each test insect was monitored for 3 hr with a video camera programmed to record events every 2 min. Contact with a plant during two consecutive recordings was considered a feeding bout. Meal length was computed as the total feeding bouts, and meals made of less than 10 bouts were

rejected. The feeding response is expressed as the percentage of contacts with the test plant over the total number of contacts with the test plant and the reference plant.

Additional statistical analyses were performed on the choice tests involving the *T. terrestris* reference plant ($N = 42$) because there was a significant preference shown by the locusts for the *T. terrestris* reference plant. Eighteen chemical variables of the flavonoid pattern were entered in a multiple regression analysis to determine which compounds might explain feeding preference. Linear regressions were performed with StatView (Abacus Concept) software. Data were arc-sine transformed prior to analysis to improve the normality of the variance.

RESULTS

Ecology and Chemotaxonomy of Tribulus *div. sp.*

All Mauritanian *Tribulus* are morphologically and ecologically similar. Four taxa have been described on the basis of their fruit morphology. *T. terrestris* is recognizable by its large spines and *T. longipetalus* by two wings on the mericarp. According to Ozenda and Quezel (1956), *T. ochroleucus* is spineless and *T. bimucronatus* has two spines. Some of the plants collected, however, could not be identified from their morphology and could only be separated on the basis of flavonoid analyses.

In the southeast (Assaba), *T. terrestris* was almost the only *Tribulus* species found. It spreads in large dense monospecific pastures whose cover ranges from 10 to 90% (mean of 41.7% cover, $N = 15$), and can extend over more than 100 ha. These pastures occur in savannah communities dominated by grasses (*Andropogon gayanus*, *Vetiveria nigritaria*, *Cenchrus biflorus*) and trees (*Acacia senegal*, *A. raddiana*, *Combretum glutinosum*). During the summer, these communities constitute potential outbreak areas for the desert locust.

All four species were collected in the winter breeding area of the desert locust, north of the 18° parallel. *T. longipetalus* was dominant (present at 80% of the sites), while *T. terrestris* was rare. *Tribulus* grows as sparse populations of individuals mixed mostly with annuals, such as *Fagonia olivieri*, *Schouwia purpurea*, *Boerhavia diffusa*, *Seetzenia lanata*, and *Psoralea plicata*. This mixed vegetation occurs in patches that are often less than a hectare with plant cover ranging from 3 to 60% (mean of 19% cover, $N = 81$).

Observations on plants collected at 59 sites in Inchiri and Trarza showed that two or more *Tribulus* taxons cooccurred at 28 sites. The association hypothesis was tested on the sample, checking 2×2 frequencies of species in a contingency table (Southwood, 1978). Fisher's test (Table 1) suggests that *T. ochroleucus* is significantly associated with *T. bimucronatus* ($P = 0.045$).

TABLE 1. CONTINGENCY TABLE OF PRESENCE OR ABSENCE OF *Tribulus* SPECIES IN 59 SAMPLES COLLECTED IN NORTHERN MAURITANIA: COMPARISON OF SPECIES PAIRED[a]

Pairs: sp. 1 × sp. 2	Frequencies				Association (cd-ab)[b]	Fisher test *P*
	sp. 1(a)	sp. 2(b)	sp. 1 and sp. 2(c)	no sp. 1, no. sp. 2(d)		
TT × TB	10	6	6	37	+	0.069
TT × TL	3	34	13	9	neutral	0.99
TT × TO	12	19	4	24	–	0.24
TB × TL	5	39	7	8	–	0.11
TB × TO	4	15	8	32	+	0.045
TO × TL	9	33	14	3	–	0.007

[a]TT = *T. terrestris*; TO = *T. ochroleucus*; TB = *T. bimucronatus*; TL = *T. longipetalus*.
[b]Positive association indicates affinity between two species and negative association indicates repulsion.

No significant association was observed between *T. terrestris* and the three other species. Affinity between *T. terrestris* and *T. bimucronatus* was not significant ($P = 0.069$). Distances between 33 individual plants were mapped on four quadrats (20 × 20 m). The nearest distances observed were 15 and 40 cm for two couples of *T. terrestris*/*T. longipetalus* growing in a wadi. Vegetation cover was 15%, and *Tribulus* plants did not exceed 5%. The mean distance between the 12 nearest neighbors of different *Tribulus* species was 5.4 m. This relatively close spacing means that the desert locust may have real choice within these communities. An accurate chemical pattern of the plant community would be helpful in understanding the relationships between the locust and its food plants.

With respect to phenolics, we found that the four species studied accumulated 18 phenolic compounds in their leaves, including five caffeoyl derivatives, six quercetin glycosides, and seven kaempferol glycosides (Table 2).

Qualitative distribution of compounds was studied on a sample of 90 mature plants (33 *T. longipetalus*, 32 *T. terrestris*, 11 *T. ochroleucus*, and 14 *T. bimucronatus*). Of these, 81 were identified on the basis of their fruit morphology. The remaining nine were classified on the basis of the results of multidimensional analysis. Projection of the 18 variables over the first two axes of the PCA, accounting for 45.6% of the total matrix inertia, distinguished *T. terrestris* individuals from all others. This suggests that *T. terrestris* is a valid taxon characterized by a caffeoyl derivative (peak 1) and two quercetin glycosides (peak 6, peak 7) (Figure 1a,b). The relationships among the three other morphotypes (O, L, B) are less clear. Two overlapping domains were observed (Figure 1b), one of which is specific to *T. longipetalus*, while the other includes

TABLE 2. FLAVONOIDS DETECTED IN *Tribulus* DRY LEAVES AFTER ALCOHOLIC EXTRACTION[a]

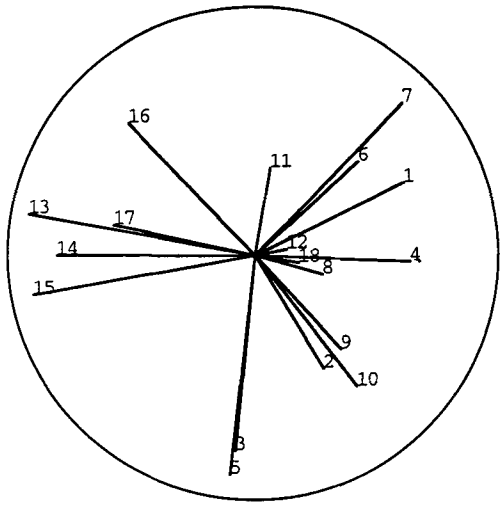
Peak	TR nm	Identification
1	28.50	caffeoyl derivative
2	30.50	caffeoyl derivative
3	31.00	quercetine glycoside
4	32.50	caffeoyl derivative
5	33.00	quercetin glycoside
6	35.00	quercetin glycoside
7	35.75	quercetin-3-*O*-rhamnoglucoside (rutin)
8	36.00	quercetin glycoside
9	37.25	quercetin glycoside
10	38.50	caffeoyl derivative
11	40.00	kaempferol glycoside
12	45.50	caffeoyl derivative
13	49.00	kaempferol-3-*O*-rhamnoglucoside
14	49.50	kaempferol glycoside
15	50.00	kaempferol glycoside
16	62.50	kaempferol glycoside
17	63.50	kaempferol glycoside
18	64.50	keampferol glycoside

[a]Crude extract was analyzed by HPLC.

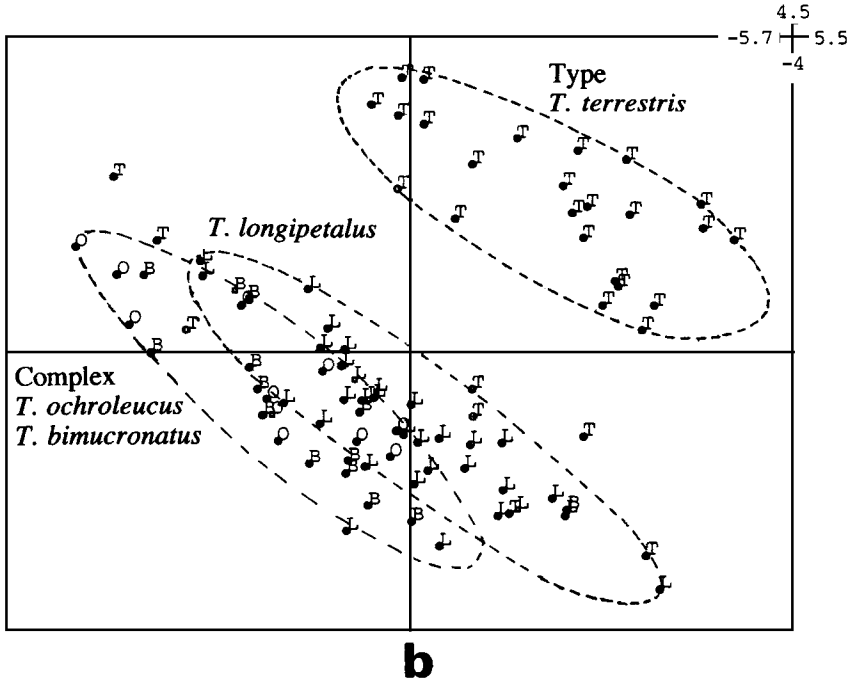
T. ochroleucus and *T. bimucronatus*, which appear more or less randomly distributed over this domain. This complex of species share quercetin glycosides (peaks 3 and 5) and kaempferol glycosides (peaks 13–15).

The relative amounts of the different leaf flavonoids appeared to vary within and among species (Figure 2). A high level of quercetin glycosides was characteristic of *T. terrestris*, especially in some individuals for which rutin constituted the primary compound. *T. ochroleucus* and *T. bimucronatus* synthesize about twice as much kaempferol derivatives as the other species. The kaempferol-3-*O*-rhamnoglucoside (peak 13) in *T. terrestris* and *T. longipetalus* was small, compared to *T. ochroleucus* and *T. bimucronatus*. In contrast, caffeoyl derivatives (except peak 1) could not discriminate *T. terrestris* from the complex of species. Qualitative and quantitative analysis suggests that the phenolic pattern is a good taxonomic marker for *Tribulus terrestris* species differentiating potential food sources.

PCA was also performed at the subspecies level on the pool of 32 *T. terrestris* plants collected in two successive years in the north (Inchiri) and south (Assaba) of Mauritania. PCA showed (Figure 3a,b, Table 3) that *T. terrestris* plants in different locations do not have the same secondary metabo-



a



b

FIG. 1. Ordination 1/2 of 90 *Tribulus* plants. Flavonoid results were submitted to principal component analysis. (a) Correlation circle among the chemical variables. Numbers refer to flavonoids checked in Table 2. (b) Organization of individual plants on both axes 1 and 2 of the PCA ordination. T = *T. terrestris*, L = *T. longipetalus*, O = *T. ochroleucus*, B = *T. bimucronatus*.

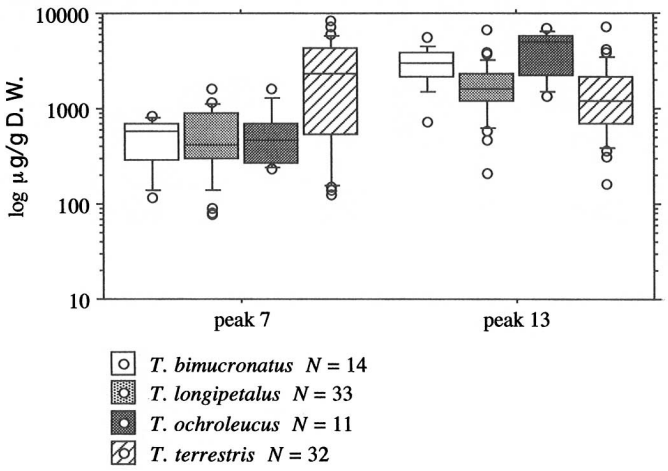


FIG. 2. Box and whisker plots of quantities of flavonoids. Peak 7 is rutin, peak 13 is a kaempferol-3-*O*-rhamnoglucoside. Horizontal bars are the median values of the distribution. Boxes are Q1–Q3 quartiles, small horizontal bars are limits of 90% of the distribution, and dots represent the value of the distribution outliers. Individual values of the four species collected in the north of Mauritania are compared.

lites. Analysis of variance for a two-factor design showed that plants collected in the northern breeding sites ($N = 13$) accumulated significantly less rutin than individuals grown in the southern region ($N = 19$) ($F_{1,28} = 19.80$, $P < 0.001$). Variations between years were not significantly different ($F_{1,28} = 0.28$, $P = 0.59$). On the other hand, the measured quantities of kaempferol glycosides did not differ between the two regions ($F_{1,28} = 2.01$, $P = 0.17$) but seemed to vary from year to year ($F_{1,28} = 5.67$, $P = 0.024$). In all cases, interaction between years and regions were not significant ($0.13 < P < 0.35$).

Behavioral Observations

Experiment with Chemicals. Rutin mixed with 10% sucrose on filter papers increased feeding at doses varying from 10 to 150 µmol/g (Figure 4). A dose of 10 µmol/g is equivalent to 6646 µg/g dry weight, which is close to the highest content observed in *T. terrestris* leaves. Rutin had a stimulating effect up to its solubility point in NH_4^+ (99.7 mg/g). On the other hand, the aglycone moiety (quercetin) was not a phagostimulant at any of the concentrations tested (Figure 4). Chlorogenic acid, a caffeoyl derivative, was neither stimulatory nor deterrent at doses ranging from 1 to 50 µmol/g.

Experiment with Foliar Material. Blank controls (comparing each plant

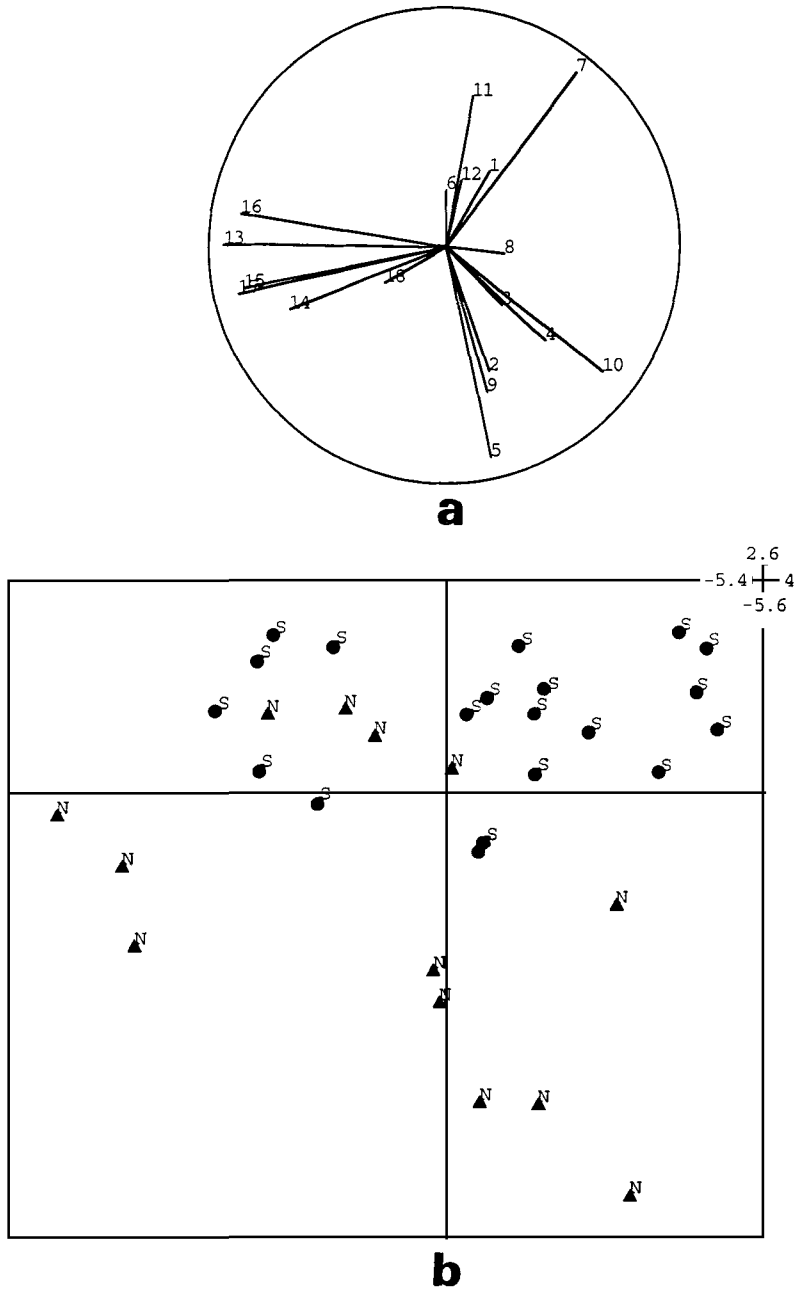


FIG. 3. Ordination 1/2 of *Tribulus terrestris* plants from northern (N) and southern (S) localities of Mauritania. Flavonoid results were submitted to principal component analysis. (a) Correlation circle among chemical variables. Numbers refer to flavonoids checked in Table 2. (b) Organization of individual plants on both axes 1 and 2.

TABLE 3. HPLC ANALYSIS OF FLAVONOID GLYCOSIDES IN *Tribulus terrestris* LEAVES COLLECTED IN NORTHERN AND SOUTHERN MAURITANIA IN NOVEMBER 1994 AND DECEMBER 1995[a]

Origin	*N*	Rutin equivalent				Chlorogenic acid equivalent, total caffeoyl derivatives
		Peak 7	Total quercetin	Peak 13	Total kaempferol	
North 1994	5	1032 (620)	4686 (738)	2925 (1122)	7414 (2546)	2189 (393)
South 1994	11	3473 (674)	5326 (788)	1735 (377)	4629 (862)	1721 (268)
South 1995	8	4441 (754)	6464 (1016)	1056 (334)	3023 (677)	1384 (148)
North 1995	8	791 (366)	2925 (856)	1263 (329)	3518 (839)	942 (359)

[a]Mean weight (±SE) in micrograms per gram dry weight. Peak 7 = quercetin-3-*O*-rhamnoglucoside, peak 13 = kaempferol-3-*O*-rhamnoglucoside.

against itself) showed no effect ($P = 0.53$) (Table 4), and results did not depend on the position of the plants in the experimental design.

In the choice tests, two individuals were used as reference plants: *T. longipetalus* and *T. terrestris*. *T. longipetalus* had 283 μg/g dry weight of rutin and 2773 μg/g dry weight of kaempferol glycosides. *T. terrestris* had a high

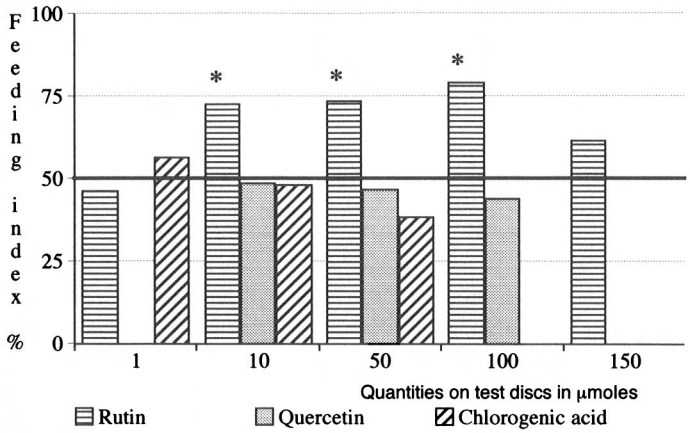


FIG. 4. Choice tests on sucrose-impregnated filter paper discs of rutin, quercetin, and chlorogenic acid. Control discs correspond to sucrose only. There were 10–20 replicates for each dose. The feeding index is the area of test discs eaten as a percentage of the total area eaten (tests discs + control discs). The horizontal line at the 50% level indicates zero level of phagostimulation. Wilcoxon *U* test for matched pairs, $*P < 0.05$.

TABLE 4. DUAL CHOICE TESTS OF *Tribulus* LEAVES AGAINST REFERENCE PLANT *T. longipetalus* OR *T. terrestris*[a]

Reference plant/test plant	*N*	Meal length (mean ± min (SE))	
		Reference plant	Test plant
T. longipetalus/(TO, TB)	15	6.7 (10.2)	7.9 (9.2)
T. terrestris/(TO, TB)	15	19.7 (21.5)	3.1 (4.6)**
T. terrestris/*T. longipetalus*	18	14.4 (15.9)	5.9 (12.0)*
T. terrestris/*T. terrestris*	9	17.6 (21.5)	2.1 (1.1)*
Blank controls	26	8.3 (11.3)	8.3 (8.9)

[a] Plants tested were individuals of the complex *T. ochroleucus*, *T. bimucronatus* (TO, TB) and individuals of *T. longipetalus*. Meal lengths was the total of successive bouts during at least 2 mins. Blank controls are plants tested against themselves. *N* = number of plants tested. Wilcoxon's signed-rank test, $*P < 0.05$, $**P < 0.01$.

content of rutin (7219 μg/g dry weight) and 3780 μg/g dry weight of kaempferol glycosides.

Choice tests failed to reveal any discrimination between the leaves of *T. ochroleucus* or *T. bimucronatus* and those of *T. longipetalus*. The mean times spent feeding on each plant were not different ($P = 0.61$). In contrast, *T. terrestris* was preferred when tested against the other species and even against *T. terrestris* plants with lower quercetin glycosides and a higher in kaempferol-3-*O*-rhamnoglucoside content. Examination of the sequence bouts showed that, in general, the locust preferred the *T. terrestris* from the beginning of the tests (Table 4).

A striking difference was observed in rutin content between *T. terrestris* and the 42 plants tested. A simple regression of the feeding response against plant rutin content failed to display any relationship ($F = 0.09$, $P = 0.77$). A multiple regression analysis of the same feeding responses over the 18 phenolic compounds accounted, however, for 53% of the variation around the mean ($F = 1.46$, $P = 0.20$).

This trend led us to look for independent variables that could contribute to the insect's choice. A backward elimination regression procedure at the $\alpha = 0.05$ level retained 12 variables accounting for 52% of the variation ($F = 2.59$, $P = 0.02$) (Table 5). The null hypothesis stating that the variables chosen agree with a linear model was accepted at the 2% level. This indicates that the 12 flavonoid compounds selected were important in explaining the differential feeding response of the locusts. Residuals plotted against the adjusted feeding response showed no trend, suggesting there was no significant bias in the analysis, such as partial correlations due to variables not taken into account. Five

TABLE 5. CHOICE TEST BETWEEN *T. terrestris* REFERENCE PLANT AND INDIVIDUAL PLANTS OF FOUR *Tribulus* SPECIES ($N = 42$)[a]

	Peak	r	Standard error	Standardized coefficient	Partial F	P
Caffeoyl	1	−0.009	0.005	−0.416	2.845	0.102
derivatives	10	−0.014	0.006	−0.492	5.421	0.027
	3	0.044	0.025	0.542	3.044	0.092
Quercetin	5	0.099	0.055	0.572	3.186	0.085
glycosides	6	−0.133	0.060	−0.584	4.989	0.033
	7	0.021	0.0056	1.278	13.970	0.0008
	8	0.022	0.008	0.462	6.962	0.0133
	11	−0.054	0.034	−0.352	2.611	0.117
Kaempferol	13	−0.016	0.006	−1.221	5.774	0.023
glycosides	14	0.015	0.007	0.741	4.049	0.054
	16	0.032	0.015	0.663	4.349	0.046
	18	−0.045	0.036	−0.214	1.589	0.217

[a] Multiple regression analysis on the feeding response over the 18 flavonoid glycosides. Backward elimination procedure. Result for the best subset with adjusted R^2 optimal. ($R^2 = 52\%$; $P = 0.02$).

of the variables retained in the analysis were significant at the $\alpha = 0.05$ level: two quercetin glycosides (peaks 6 and 8), rutin (peak 7), a kaempferol glycoside (peak 13), and a caffeoyl derivative (peak 10) (Table 5). Rutin (peak 7) and peak 13 were negatively correlated. The standardized correlation coefficient of these two variables indicated that they were equally important in explaining the feeding response.

DISCUSSION

Tribulus terrestris is a cosmopolitan poisonous weed whose ecology has been studied in some detail (El Ghareeb, 1991). It has a fast germination and a short life cycle that is completed within a few weeks. Individuals are productive and can cover relatively large areas with shoots as long as a meter. Little is known about the ecology of the other three species in Mauritania (Mohamed El Hadi, 1996). The taxonomy of the genus remains unclear and has been revised several times (Ozenda and Quezel, 1956; El Hadidi, 1972, 1978; Saleh and El Hadidi, 1977). Twenty-seven flavonoid glycosides have been associated with *Tribulus* species (Harborne and Grayer, 1993). On the basis of a flavonoid analysis, Saleh et al. (1982) inferred that *T. terrestris* forms a separate section, characterized by its high rutin content. This result is supported by our PCA

analysis, demonstrating that rutin and a caffeic derivative are characteristic of *T. terrestris*.

Examination of chromatographic profiles reveals that *T. terrestris* is rather easily identified based on its specific phenolic patterns as compared to *T. ochroleucus* and *T. longipetalus*. The existence of one species, *T. bimucronatus*, seems questionable (Ozenda and Quezel, 1956). Chemically, this species is close to *T. ochroleucus* but its morphological features and, more specifically, the occurrence of spines on its fruits relate it to *T. terrestris*. We hypothesize that *T. bimucronatus* is an introgressed form between *T. terrestris* and *T. ochroleucus*.

Heritability for rutin content has been studied in two varieties of *Fagopyrum esculentum* (Kitabayashi et al., 1995). Rutin content was found to be related to the flowering period of each variety (Ohsawa and Tsutsumi, 1995). Wilkins et al. (1996) identified two chemical populations exhibiting different saponins among *T. terrestris* plants collected in South Africa. These workers could not decide whether the chemotypes were genetically or environmentally determined. Our results confirm the chemical variability of *T. terrestris*.

Many of the phenolics encountered are widely distributed among plants and act as allelochemicals, reducing the growth of nonadapted specialist feeders (Harborne, 1979; McFarlane and Distler, 1982; Bloem and Duffey, 1990). Some generalist species, such as *Spodoptera frugiperda*, are able to detoxify flavones (Wheeler et al., 1993) or are phagostimulated when fed on a diet containing rutin, as is the case for *Schistocerca americana* (Bernays et al., 1991). Our experiments confirm that *Schistocerca gregaria* is stimulated by rutin and not by its aglycone derivative.

Is rutin an effective phagostimulant? We found it was not possible to relate the rutin content of individual plants to the feeding response, but insects selected a blend of five flavonoids. Three of them were positively correlated to the feeding response and two others were negatively correlated. The negative correlation might be the result of competition between two metabolic pathways from the same precursor, the dihydrokaempferol. All flavonoid compounds studied belong to the same phenolic pathway. While compound 10 is derived from an early precursor, compounds 6, 7, 8, and 13 are end products. The enzymes involved are flavonol synthase, leading to kaempferol glycosides, and dihydroflavonol-3′-hydroxylase, which opens the path to quercetin derivatives (Heller and Forkmann, 1988; Jay et al., 1994). It is probable that insect food choice is mainly determined by a flavonoid signature rather than by a single compound. The trend of the feeding response in the choice tests was clear enough to support this conclusion even though we ignored many other secondary compounds present in the plant. For example, it is known that defensive saponin glycosides occur in the aerial parts of *Tribulus terrestris* (Wu et al., 1996; Yan et al., 1996).

In the south of Mauritania, hoppers encounter only *Tribulus terrestris*. The same adults arriving in the northern regions feed on different *Tribulus* species. Thus, they may be in a position to choose on the basis of their previous experiences. Many experiments on locusts suggest the possibility of learning and keeping in memory initial preferences for plants (Kaufmann, 1972; Lee et al., 1987; Muralirangan et al., 1997). We did not study learning behavior in the present work, but learning could reinforce the preference of locusts for plants with a high flavonoid content.

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INHIBITION OF WATER FERN *Salvinia minima* BY CATTAIL (*Typha domingensis*) EXTRACTS AND BY 2-CHLOROPHENOL AND SALICYLALDEHYDE

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Abstract—The allelopathic potential of a cattail species (*Typha domingensis*) towards the common water fern (*Salvinia minima*) was studied in a short-term bioassay. Aqueous extracts of cattail plants (roots, stems, and leaves) as well as two of its phenolic compounds (2-chlorophenol and salicylaldehyde) were found to inhibit the growth of *Salvinia* when incorporated in the growth medium. After a period of seven days, chlorophyll concentration, fresh weight, dry weight, and number of fronds of *Salvinia* were compared and correlations were found among the different parameters. The results suggest an inhibitory effect by components of *Typha*, especially of the root fractions. Results from pure chemicals are in qualitative and quantitative agreement with those obtained from the cattail extracts.

Key Words—Aquatic plant, bioassay, biological control, phenolic compounds, phytotoxicity.

INTRODUCTION

Salvinia minima Willd. is a small, rapidly growing, free-floating fern widely distributed in many aquatic ecosystems worldwide (DeBusk and Reddy, 1987). The weed is found in Florida waters, usually associated with *Lemna* and other small floating species, and can become a nuisance due to its explosive growth rates and ability to shade underwater life (Oliver, 1993). Related species (especially *Salvinia molesta* Mitchell) are considered a major problem in Africa, Southeast Asia, and Australia. They are known to be invasive, choking canals,

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rivers, ponds, and even rice fields, thereby causing agricultural losses and habitat disturbance (Pandey, 1994a,b). *S. minima* is known to increase the pH of its growth media and has been proposed as a natural buffering agent in the control of acidification of aquatic ecosystems, as well as a moderator of toxic effects of certain metals such as aluminum (Gardner and Al-Hamdani, 1997) and cadmium (Singh et al., 1996).

Salvinia poses a challenge because it must be managed in a way that it is allowed to participate in the aquatic community without taking over, so that its beneficial effects can be felt without detrimental impact on overall balance. It has been shown that the growth of *S. molesta* can be inhibited by extracts of the tropical weed parthenium (*Parthenium hysterophorus*) and its purified toxin, parthenin (Pandey, 1994a,b, 1996).

Cattails, *Typha* spp., may be candidates for biological control. They are aggressive aquatic plants, and their ability to expand over areas that were previously occupied by other species has been the subject of study (Gallardo et al., 1998). The species can be troublesome in wetland environments. In South Florida, *T. domingensis* is believed to be a natural component of the Everglades ecosystem (Davis, 1994), but in many cases it has become the dominant marsh species, outcompeting native plants (Toth, 1988). In Florida public waters, cattails are the dominant emergent species of aquatic plants (Schardt, 1997). There are several factors responsible for cattails' opportunistic expansion, including size, growth habit, adaptability to changes in the surroundings, and the release of compounds that prevent the growth of other species.

Previous research (Prindle and Martin, 1996) dealing with aqueous extracts of *T. domingensis* noted its phytotoxic properties. It was found to inhibit the germination of lettuce and radish seeds and to reduce the oxygen production rates of the filamentous alga *Lyngbya majuscula*. Extracts were active after autoclaving. Gas chromatography–mass spectrometry analysis revealed two of the major components of the extracts as 2-chlorophenol and salicylaldehyde (Prindle et al., 1997). Relative concentrations of these compounds in the extracts have been found to be on the order of milligrams per kilogram of fresh material (Gallardo and Martin, 1997). The compounds are present in sediments in the *T. domingensis* growth front but not in sediments 2 m away from the cattail infestation (Albalat et al., 1997).

This investigation deals with inhibitory effects of *T. domingensis* extracts and the above-mentioned compounds towards the growth and propagation of *S. minima*.

METHODS AND MATERIALS

Salvinia *Culture*. *Salvinia minima* Willd. was collected at Lettuce Lake (Lettuce Lake Park, Hillsborough County, Florida), where it grows mixed with

Lemna spp. and covers most of the lake shore and adjacent swamp areas. After collection, the material was cleaned by rinsing three times under running tap water. After this, plants were dipped in a 5% solution of commercial bleach for 1 min and thoroughly rinsed with deionized water. Plants were divided into clumps with approximately 10 leaves and kept in sterile foam-stoppered 125-ml Erlenmeyer flasks containing 50 ml of E medium, prepared at a pH of 4.6 and sterilized by autoclaving for 1 hr (Cleland, 1979). Stock cultures were maintained for up to a week prior to use and kept at 28°C under constant cool white light (150 $\mu E/m^2/sec$).

Cattail Extracts. Fully mature samples were taken from a storm water ditch near the University of South Florida campus in Tampa, Florida. Samples were identified and, after collection, were rinsed with deionized water and divided into three sections: the root system, the stem (taken from just above the root to the part where the leaves start separating), and the leaves. A portion (usually about 150 g) of each section was weighed and extracted in a blender with deionized water (5 ml/g material) at room temperature. The resulting crude extracts were filtered through Whatman No. 1 filter paper, and the remaining liquid was autoclaved for 1 hr, and placed in a refrigerator at 0–4°C. Extracts were disposed of after two weeks.

Determination of Organic Carbon. Organic carbon analysis of cattail extracts was done with a Dohrmann (model DC-180) automated carbon analyzer. Samples of root, stem, and leaf extracts were analyzed. Calibration was done with potassium hydrogen phthalate in deionized water, serially diluted from 2000 ppm to give a 10 ppm working standard. The concentration of organic carbon was then calculated.

Chemicals. Known phytotoxic substances were studied. Concentrations of 10, 20, 50, and 100 ppm of 2-chlorophenol (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) and salicylaldehyde (Mallinckrodt, Inc., St. Louis, Missouri) in distilled water were prepared and added to the growth medium. Because of the dilution factor, the effective concentrations of the chemicals in the bioassay were 2.5, 5.0, 12.5, and 25.0 ppm.

Determination of Inhibitory Effects. Bioassays were performed according to the methods described by Einhellig et al. (1985). Aseptic conditions were maintained and fresh medium was prepared and autoclaved for each bioassay. Diluted E medium (1.5 ml) was dispensed into each well of a 24-well tissue culture plate with a sterile pipet; then 0.5 ml of the substance being tested (either a cattail extract or a solution of the chemical) was added to the E medium. Each test substance was replicated twice in a randomized design. Control wells received 0.5 ml of distilled water, and there were three control wells on each plate. *Salvinia* was divided into 2-frond units of comparable size, and a unit was placed on each well by using a flamed culture loop. Plates were covered and placed in a constant-temperature bath under lights as described. Plates were arranged randomly, and their position in the bath was changed daily to minimize

effects of light, temperature, and relative humidity differences. In order to ensure appropriate gas exchange, the lids of the culture plates were lifted for 5 min every day. At this time, any observed evaporation of the medium was corrected by replenishing the wells with distilled water. The experiment was conducted in a controlled temperature bath at 25°C, 150 $\mu E/m^2/sec$ light intensity, and a 12-hr photoperiod.

Bioassays were repeated twice, always randomizing the position of the test solutions in the wells and the position of the wells in the bath. Frond number and condition were recorded daily for seven days. After this period, *Salvinia* was removed and weighed. Representative samples were air dried at room temperature for two days to obtain the dry weight. Other samples were ground in a glass tissue grinder and steeped in 3 ml of 90% acetone in dark containers for 2 hr to determine chlorophyll *a* content (Standard Method 10200 H; Eaton et al., 1995; Beckman model 25 spectrophotometer). Calculations of chlorophyll *a* content were done with a Chlorophyll Calculator program (WindowChem Software, Fairfield, California). All data presented were evaluated by using Student's *t* test.

RESULTS

Salvinia minima is sensitive to phenolic compounds in its growth medium. Even low concentrations (2.5 ppm) have an impact on the fern's capability to produce new fronds, its chlorophyll production, and its weight. After a seven-day exposure, the number of new fronds was significantly reduced by all cattail fractions and 2-chlorophenol and salicylaldehyde solutions, as compared to controls. Loss of the bristle hairs on the surface of the fronds (trichomes) and intense darkening were also observed. In the most concentrated solutions (12.5 and 25 ppm) and the cattail root fraction, the detrimental effect was visible after two to three days.

With the cattail fractions, all observed parameters were decreased, and the root fraction was the most inhibitory (Table 1). Results from fresh and dry weight determinations are in agreement with measured chlorophyll *a* for all the bioassays performed. When the values obtained for the control are normalized to 100%, it can be seen that cattail root extracts reduce dry weight to 41.9% $\pm$ 6.0% of control; this weight reduction is a consequence of the combined loss of the tips of the trichomes from the leaf and the inhibition of new growth. Chlorophyll *a* concentration was reduced by the cattail root fraction to 4.1% $\pm$ 3.0% of control. At the early stages of exposure, the leaves become dull and the initial bright green color is subdued. After three days (for the root fraction, later for other fractions), the leaves change to a brownish green and finally turn dark brown. The color change starts at the base of the leaves and spreads to

TABLE 1. EFFECT OF CATTAIL EXTRACTS (5:1 WATER–FRESH MATERIAL) ADDED TO GROWTH MEDIUM OF *Salvinia*[a]

Fraction	Fresh weight (%)	Dry weight (%)	Chlorophyll *a*, (%)
Root	45.0 ± 5.8	41.9 ± 6.0	4.1 ± 3.0
Stem	55.1 ± 7.6	52.6 ± 5.3	15.3 ± 3.3
Leaves	81.0 ± 6.7	80.6 ± 6.2	19.5 ± 2.9
Control (deionized water)	100	100	100

[a] All values are normalized by considering the control value to be 100%. Values are mean ± standard deviation. $N = 4$. Values are different from control at the 95% confidence level according to Student's t test ($P = 0.05$).

cover the surface. Usually, in the less concentrated fractions, one of the leaves changes colors while the other remains visibly healthy through the duration of the experiment. In the most concentrated fractions, both leaves undergo the color change and eventually turn completely brown.

Inhibitory activity decreases in extracts from stem and leaves, but the effect is somewhat noticeable even in the less active fractions. Organic carbon determinations indicate a larger concentration of organic carbon in the root fraction (616.5 ppm) compared to stems (28.9 ppm) and leaves (129.4 ppm). Previous determination of 2-chlorophenol and salicylaldehyde concentrations showed that these are more concentrated in the roots and that their concentration decreases in the stem and leaves. Both 2-chlorophenol and salicylaldehyde are present on the order of milligrams per kilogram of fresh cattail material (Gallardo and Martin, 1997). In root fractions, we have found 76.4 ± 7.5 mg/kg fresh material of 2-chlorophenol and 21.0 ± 3.0 mg/kg fresh material of salicylaldehyde. This corresponds to an effective concentration (under the conditions of the bioassay) of approximately 4 ppm 2-chlorophenol, and 1 ppm salicylaldehyde. We used the concentrations of both chemicals in stem and leaf extracts in order to estimate a working range for testing.

When pure 2-chlorophenol and salicylaldehyde were tested for biological activity (Tables 2 and 3), a similiar inhibitory pattern was found. Changes in color and leaf surface appearance were evident in the short term, and the overall effect was close to the changes induced by concentrated cattail fractions. The results suggest that the phenolic fraction may play a role in the phytotoxic action of the cattail extracts.

Effects observed for the cattail root fraction are comparable to effects of the 25 ppm 2-chlorophenol fraction or the 12.5 ppm salicylaldehyde fraction; these concentrations, however, correspond to higher amounts of the chemicals than what we have found in cattail extracts. The two phenolic compounds are

TABLE 2. EFFECT OF 2-CHLOROPHENOL SOLUTIONS ADDED TO GROWTH MEDIUM OF *Salvinia*[a]

Effective concentration (ppm)	Fresh weight (%)	Dry weight (%)	Chlorophyll *a* (%)
2.5	96.7 ± 5.1[b]	90.6 ± 5.6[b]	20.9 ± 3.3
5.0	84.9 ± 4.7	85.8 ± 6.8	9.5 ± 4.6
12.5	70.5 ± 2.3	76.7 ± 4.5	6.8 ± 3.8
25.0	39.1 ± 7.8	35.1 ± 4.8	4.6 ± 2.2
Control (deionized water)	100	100	100

[a] All values are normalized by considering the control value to be 100%. Values are mean ± standard deviation. $N = 4$. Values are different from control at the 95% confidence level according to Student's t test ($P = 0.05$) unless indicated otherwise.
[b] Not statistically different from control at the 95% confidence level.

TABLE 3. EFFECT OF SALICYLALDEHYDE SOLUTIONS ADDED TO GROWTH MEDIUM OF *Salvinia*[a]

Effective concentration (ppm)	Fresh weight (%)	Dry weight (%)	Chlorophyll *a* (%)
2.5	89.9 ± 3.3	90.9 ± 5.7[b]	18.1 ± 4.1
5.0	81.6 ± 5.1	80.8 ± 3.5	20.9 ± 3.4
12.5	49.3 ± 4.0	48.5 ± 2.9	14.4 ± 4.8
25.0	25.7 ± 5.5	28.6 ± 5.1	9.8 ± 2.9
Control (deionized water)	100	100	100

[a] All values are normalized by considering the control to be 100%. Values are mean ± standard deviation. $N = 4$. Values are different from control at the 95% confidence level according to Student's t test ($P = 0.05$) unless indicated otherwise.
[b] Not statistically different from control at the 95% confidence level.

not the only components of the cattail extracts, and the existence of other toxic substances has not been ruled out.

Propagation of *Salvinia* was inhibited. Due to the impact of the compounds studied on vegetative growth, growth rates could not be used as a working parameter. Over a period of seven days, no new fronds were detected in the presence of the cattail root and stem extracts or the 12.5 and 25 ppm solution

of the chemicals (in comparison to the control, which normally went from two to eight leaves over the same period of time). Detrimental effects were not reversible. Even if the well plates were left uncovered to promote evaporation of phenolic substances, the plants did not recover and eventually died. Removal of the plants from the well plates and placement in fresh E medium also proved unsuccessful in reversing the observed darkening of the leaves and loss of trichomes. However, by using less concentrated chemicals (2.5 ppm) and the cattail leaves fraction, damaged *Salvinia* plants were able to produce new growth after a period of seven days if placed in fresh E medium. The potential of *Salvinia* plants to overcome detrimental effects appears to be related to time of exposure and to concentration of the chemicals in the growing medium.

During the bioassay it was noticed that, in the presence of cattail extracts or chemicals, plants consumed less water than controls. This observation, coupled with the fact that roots and margins of the fronds showed rotting after exposure to the inhibitory substances, indicates that a root dysfunction was induced that probably contributed to the desiccation and death of plants. It has been suggested (Pandey, 1994a) that loss of membrane integrity and disruption of enzymatic pathways are possible actions for phenolic-type allelochemicals. Our results, including the massive weight loss and the reduction in chlorophyll production, are in agreement with such a proposed action mode.

There is a possibility that *Salvinia* and other aquatic weeds could be managed by allelochemicals. Chemicals derived from wetland species would not be likely to build up in waterbodies and would represent an economically and ecologically sound alternative to traditional herbicide treatments.

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FIELD ATTRACTION OF *Hoplia communis* TO 2-PHENYLETHANOL, A MAJOR VOLATILE COMPONENT FROM HOST FLOWERS, *Rosa* spp.

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Abstract—The attractiveness of volatile compounds from the floral scent of *Rosa*, one of the most preferred plants for adult *Hoplia communis*, was evaluated under field conditions. The beetles were attracted to most compounds tested, but 2-phenylethanol exhibited the highest capture rate. Catches increased with increasing emissions of between 9.1 and 287.2 mg/day. Catches in white traps were significantly larger (17.4-fold) than those in green traps when both were baited with anethole, an already known attractant; however, the trap color was not significant when a more attractive lure, 2-phenylethanol, was used. The use of a single funnel trap baited with 2 g of 2-phenylethanol at a heavily infested nursery exhibited promising results for mass trapping. Approximately 90,000 beetles of both sexes, which nearly corresponds to the estimated maximum population per 1000 m^2, were captured within six days.

Key Words—*Hoplia communis*, Scarabaeidae, Coleoptera, attractant, kairomone, 2-phenylethanol, geranyl acetate, floral scent, *Rosa* spp.

INTRODUCTION

The long-legged chafer *Hoplia communis* Waterhouse (Coleoptera: Scarabaeidae) is one of the major scarabaeid turf pests and is distributed on the Japanese mainlands of Honshu, Shikoku, and Kyushu, and the islands of Sado, Izu, and Yakushima (Kobayashi, 1985). The grubs feed on the roots of turfgrasses and damage turf when population density is high (Ikka, 1996). Adults emerge intensively in May, fly and forage during the daytime in fine weather, and aggregate

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and feed on the petals of whitish flowers, especially *Rosa* and *Pyracantha* spp. (Rosaceae). They are strongly attracted even to artificial objects with whitish surfaces such as clothes and cars, making this chafer notorious as a nuisance pest for golfers, the residential population, and agriculture (Ikka, 1996; Hatsukade, 1995).

Because the grub lives underground and the adult disperses, most of the pests cannot be contacted with chemical pesticides at an effective dosage through ordinary application, and chemical control cannot be achieved economically. Efforts have been made to look for alternative methods of control, and the development of a mass trapping device has been suggested (N. Izuhara, personal communication). The attractiveness of anethole and of a mixture of eugenol, geraniol, and phenethyl propionate for the adult beetles has been reported, and these compounds, formulated as commercial lures in a white trap, have been used for monitoring and also for mass trapping (Yokomizo and Nagata, 1984; Hatsukade, 1995; Hirai, 1993). However, the performance of the present commercial trap is not good enough to control the pest population.

We have tested the attractiveness of floral volatiles of *Rosa* that have been described (Knudsen et al., 1993), and other related compounds, in order to obtain a more effective chemical lure, as well as to evaluate chemical and physical contributions in the attraction of adults to whitish flowers.

METHODS AND MATERIALS

Preparation of Chemical Lures. All test chemicals were obtained from commercial sources. Each compound (1 ml) was applied to glass wool (500 mg), sealed with a polyethylene film (0.03 mm thick) and attached to a JT Windspack funnel trap (green traps were used except when evaluating the attractiveness of white traps). For dose–response evaluation and practical application, lures were prepared by pouring 2 g of 2-phenylethanol over Tosoh Ultrasen polyethylene-vinyl acetate copolymer pellets (4 g) in a glass bottle, and allowing them to stand for a day, with occasional shaking by hand, to ensure uniform absorption. For the dose–response evaluation, 2, 5, 15, 30, and 100 pellets were each weighed and placed in traps just before a test. After a test, the pellets were weighed again, and the difference in the weights before and after the test was calculated to estimate the amount of 2-phenylethanol evaporated during a test.

Field Tests. Field tests were conducted in turf nurseries in Tsukuba, Ibaraki, in May 1995 and 1996. Traps were arranged around the nurseries in a randomized linear design, approximately 10 m apart at a height of 1 m. Trap positions were rerandomized when the test was replicated. Capture data (except data from the initial screening of candidate attractants) were transformed to log $(x + 1)$ to normalize variances. Paired means and multiple means were separated

with Student's t test and with ANOVA followed by Tukey-Kramer test, respectively ($P < 0.05$). Untransformed means and standard errors are presented in the tables.

RESULTS AND DISCUSSION

Our observations of the foraging behavior of adult *Hoplia communis* revealed that they are accustomed to feeding on whitish flowers, including species of *Rhododendron* (Ericaceae), *Anthemis* (Compositae), and *Quercus* (Fagaceae), but the most preferred were species of *Rosa* and *Pyracantha* (Rosaceae). Thirteen volatile compounds that comprise 91.2% of the volatile compounds of *R. rugosa* (Dobson et al., 1990) and two related compounds were used for the initial screening of attractants. Because the test was conducted early in the season when the field population was low, the data obtained were insufficient for statistical analysis. Five of 15 compounds, especially 2-phenylethanol and geranyl acetate, exhibited larger catches than anethole, the attractiveness of which already had been demonstrated (Yokomizo and Nagata, 1984) (Table 1). 2-Phenylethanol is the most abundant component (36.2%) of the floral scent of *R. rugosa* (Dobson et al., 1990) and other *Rosa* cultivars (Mookherjee et al., 1990) and is one of the most common volatile compounds in diverse taxa. It has been identified in flowers of 34 of 174 genera (Knudsen et al., 1993). The adult chafer's wide preference of flowers for feeding could be explained on a chemical basis by the wide distribution of 2-phenylethanol among plants, in addition to the chafer's response to diverse compounds. 2-Phenylethanol also attracts other phytophagous insects, including onion and seed-corn flies, *Hylemya antiqua* and *H. platura* (Ishikawa et al., 1983), rootworm beetles, *Diabrotica* spp., and the scarab beetles, *Popillia japonica* (cited in Metcalf and Metcalf, 1992) and *Anomala octiescostata* (Leal et al., 1994). However, only a few chafers, *Oxycetonia juncunda*, were mixed with catches on rare occasions during the tests.

2-Phenylethanol attracted a larger catch than geranyl acetate when the compounds were applied separately. No synergistic effect was observed in these two compounds, although a mixture attracted larger catches at all mixing ratios tested than geranyl acetate alone (Table 2).

The attractiveness of 2-phenylethanol and some related compounds, three phenyl alkyl alcohols with C_1 to C_3 chains, the propyl ester, and amino-substitution for the hydroxyl of 2-phenylethanol, were investigated. The lower attractiveness of the phenethyl acetate was revealed in the previous test (Table 1). Among these compounds, only phenethyl amine and 2-phenylethanol exhibited significantly higher attractiveness than a standard compound, anethole (Table 3). The attractiveness of phenethyl amine was almost identical to 2-phenylethanol, although it smells quite different to the human nose. These results

TABLE 1. CATCHES OF *Hoplia communis* IN TRAPS BAITED WITH VOLATILE COMPOUNDS FROM FLORAL SCENT OF *Rosa* SPP. AND SOME RELATED COMPOUNDS[a]

Compound	Total catches (relative attractiveness)[b]		
	May 7–10	May 11–14	May 15–17
Anethole (standard)	4 (1.00)	13 (1.00)	8 (1.00)
Citronellal[c]	0 (0)		
Citronellol[c,d]		1 (0.08)	
Citronellyl acetate[c,d]			6 (0.75)
Nerol[c,d]			5 (0.63)
Linalool[c]		12 (0.92)	
Geraniol[c,d]	5 (1.20)		
Geranyl acetate[c,d]		59 (4.54)	
Limonene[c,e]		13 (1.00)	
2-Phenylethanol[c,d]	21 (5.25)		
Phenethyl acetate[c,d]			12 (1.50)
Eugenol[c,d]	3 (0.75)		
Methyl eugenol[c,d]			7 (0.88)
Isoeugenol		18 (1.38)	
3 (*Z*)-hexenol	0 (0)		
3 (*Z*)-hexenyl acetate[c]			14 (1.75)

[a]Test conducted May 1995.
[b]Total of four traps. Relative attractiveness is based on anethole catch, which is arbitrarily set to equal 1.
[c]Listed in Knudsen et al. (1993).
[d]Listed in Dobson et al. (1990).
[e]D-Limonene is listed in Knudsen et al. (1993), however, DL-limonene was used in this test.

TABLE 2. CATCHES OF *Hoplia communis* IN TRAPS BAITED WITH 2-PHENYLETHANOL (PE) AND GERANYL ACETATE (GA)[a]

Bait PE/GA	Mean catch ± SE
10/0	7097 ± 1176 b
8/2	11829 ± 582 b
5/5	11824 ± 1197 b
2/8	10580 ± 1530 b
0/10	1797 ± 396 a

[a]Test conducted May 20–21, 1995. $N = 6$ (3 traps × 2 replications). Means followed by the same letters are not significantly different according to Tukey-Kramer test ($P < 0.05$).

TABLE 3. CATCHES OF *Hoplia communis* IN TRAPS BAITED WITH 2-PHENYLETHANOL AND RELATED COMPOUNDS[a]

Compound		Mean catch ± SE
2-Phenylethanol	(C_6H_5)-C_2H_4OH	132.10 ± 79.67 bc
3-Phenylmethanol	(C_6H_5)-CH_2OH	9.93 ± 4.97 abc
3-Phenylpropanol	(C_6H_5)-C_3H_6OH	5.75 ± 2.87 ab
Phenethyl propionate	(C_6H_5)-$C_2H_4OCOC_2H_5$	5.50 ± 1.32 ab
Phenethyl amine	(C_6H_5)-$C_2H_4NH_2$	96.00 ± 47.27 c
Anethole		2.25 ± 0.48 a

[a]Test conducted May 17-19, 1995. $N = 4$ (2 traps × 2 replications). Means followed by the same letters are not significantly different according to Tukey-Kramer test ($P < 0.05$).

suggest that $C_6 + C_2$ with a compact electron releasing group is essential for attraction.

Dose-response tests revealed that catches increased with increasing emission of 2-phenylethanol, without apparently reaching a maximum or saturation point, within a range of about 9–287 mg/day (Table 4).

The effect of trap color on the catch was evaluated by using white and green traps baited with anethole and 2-phenylethanol. Catches in white traps were significantly larger than those in green traps when both were baited with anethole. The trap color was not significant, however, when a more attractive lure, 2-phenylethanol, was used (Table 5). Data that quantify the physical attractiveness of trap whiteness have not been obtained; however, the results suggest

TABLE 4. CATCHES OF *Hoplia communis* IN TRAPS BAITED WITH DIFFERENT AMOUNTS OF 2-PHENYETHANOL[a]

Dose of 2-phenylethanol		
No. of pellets	Released amount (mg/day)	Mean catch ± SEM
2	9.1	234.3 ± 72.2
5	18.9	324.2 ± 99.9
15	57.2	401.7 ± 143.3
30	107.9	672.2 ± 292.1
100	287.2	1053.8 ± 534.5

[a]Test conducted May 20-21, 1996. $N = 6$ (3 traps × 2 replications).

TABLE 5. EFFECT OF TRAP COLOR ON CATCHES OF *Hoplia communis* IN TRAPS BAITED WITH ANETHOLE OR 2-PHENYLETHANOL[a]

Bait	Trap color		
	Green	White	
Anethole	2.25 ± 0.48	39.25 ± 11.08	*[b]
2-Phenylethanol	61.75 ± 15.21	140.50 ± 44.75	NS

[a]Test conducted May 17–19 for anethole and May 23–29 for 2-phenylethanol, 1995. Data represent the mean of four trap catches ± standard errors.
[b]Means within a row are significantly different according to Student's *t*-test ($P < 0.05$).

that both physical and chemical cues play a role in the host-searching behavior of the adult beetles.

A funnel trap baited with 2 g of 2-phenylethanol was used at a heavily infested nursery during the peak season, May 19–24, 1995. Although daily catches fluctuated drastically depending on the weather, more than 20,000 beetles, the full capacity of the trap, were captured in a single trap on a single day (Table 6). The total catch during six days, approximately 90,000 beetles, was close to the estimated maximum population per 1000 m^2 (Ikka, 1996). Fifty beetles sampled from the catches on May 19, 23, and 24 were sexed, and the sex ratios were biased toward the female (Table 6). Although it is still uncertain whether the lure is more effective for females, or whether the sex ratio in the catches represented that of the field population, mass trapping with 2-phenylethanol might be promising due to its attractiveness to both sexes as well as its high performance (Lanier, 1990).

TABLE 6. CATCHES OF *Hoplia communis* IN A SINGLE FUNNEL TRAP BAITED WITH 2-PHENYLETHANOL[a]

	Date (May 1995)					
	19	20	21	22	23	24
Catch[b]	22,000	17,900	76	21	23,500	15,800
Male/female[c]	19.31	ND[d]	ND[d]	ND[d]	0:50	4:46

[a]Test conducted May 19–24, 1995.
[b]Ascertained from the weight when exceeding 10,000.
[c]Fifty beetles sampled from each catch were sexed.
[d]Not determined.

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STRUCTURE-PHAGOSTIMULATORY RELATIONSHIPS FOR AMINO ACIDS IN ADULT WESTERN CORN ROOTWORM, *Diabrotica virgifera virgifera*

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Abstract—The effects of 52 amino acids and derivatives on feeding responses of adult western corn rootworm *Diabrotica virgifera virgifera* (WCR) were investigated. L-Alanine was the most phagostimulatory amino acid, followed by L-serine, and β-alanine. All three amino acids showed a dose-response pattern but at the highest dose (500 nmol/disk) feeding by WCR decreased. In structure-activity relationship studies, four structural features for stimulating WCR feeding were found. First, terminal carboxyl and amino acid groups must be intact since all structural modifications in these groups led to a considerable decrease in feeding. Second, a hydrophobic region sterically limited to a two-saturated-carbon insert between the amino and carboxyl groups is associated with stimulatory effects at the L-alanine taste receptor. Third, L-alanine and L-serine are much more potent stimulants than the D enantiomers of these amino acids. Fourth, the hydrogen domain on the α-carbon of L-alanine is sterically constrained. A β-hydroxyl group on the amino acid side chain, such as in L-serine, does not reduce stimulatory taste, which indicates the presence of another binding domain. Therefore, L-alanine and L-serine either have separate binding sites on the taste cells of WCR or an un-ionized, polar grouping extending from the hydrophobic domain of the L-alanine binding site is permitted. The three most phagostimulatory amino acids (L-alanine, L-serine, and β-alanine) are nonessential amino acids, suggesting that amino acid sensitivity in WCR may be tuned to quantitatively relevant signals rather than just nutritionally important amino acids in a food.

Key Words—Feeding stimulants, gustation, insect chemoreception, Chrysomelidae, nonprotein amino acids, QSAR, structure-taste relationships, L-alanine receptor.

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INTRODUCTION

Free amino acids are ubiquitously distributed in both terrestrial and aquatic environments and are inevitable nutrient sources and important chemical stimuli to a variety of organisms. Nutrient chemicals such as amino acids, sugars, sterols, phospholipids, ascorbic acid, and B-vitamins are known phagostimulants to many phytophagous insect species (Thorsteinson, 1960; Beck, 1965; Bernays and Simpson, 1982). Studies of the feeding responses to amino acids have been done with several insects from various orders, with highly variable effects of amino acids between the species (Bernays and Simpson, 1982). For example, L-serine was a feeding stimulant to three species, the migratory locust *Locusta migratoria* L., the pea aphid *Aphis fabae* Scopoli, and the large milkweed bug *Oncopeltus fasciatus* Dallas, but L-methionine only stimulated the taste receptors of the migratory locust among these species. Moreover, for southwestern corn borer *Diatraea grandiosella* Dyar, L-alanine was more phagostimulatory than β-alanine followed by L-serine (Hedin et al., 1993).

Among Coleoptera, the phagostimulatory effects of amino acids have been studied most extensively for the scarab *Sericesthis geminata* Boisduval (Wensler and Dudzinski, 1972) and for the Colorado potato beetle *Leptinotarsa decemlineata* Say (Hsiao and Fraenkel, 1968). In *S. geminata* larvae, amino acids such as L-leucine, L-isoleucine, and L-alanine were stimulatory at lower concentrations but strongly phagodeterrent at higher concentrations. Meanwhile, L-alanine, γ-aminobutyric acid (GABA), and L-serine elicited marked feeding stimulation in Colorado potato beetle larvae, and threshold responses to these amino acids were at lower concentration than for phagostimulation by sucrose.

Adult western corn rootworm *Diabrotica virgifera virgifera* LeConte (WCR) readily consume all pollens tested to date (Mullin et al., 1993), including corn (*Zea mays* L.), winter squash (*Cucurbita maxima* Duchesne), sunflower (*Helianthus annuus* L.), rye (*Secale cereale* L.), orchard grass (*Dactylis glomerata* L.), and Johnson grass [*Sorghum halepeuse* (L.) Pers.]. The highest feeding stimulatory activity was associated with polar extracts of pollen, and amino acids, not sugars, turned out to be the primary phagostimulants for WCR. Pollens contain high amounts (90–130 mmol/g fresh pollen) of free amino acids (Hollister, in preparation; Erhardt and Baker, 1990; Stanley and Linskens, 1974). In cellulose disk bioassays, blends of GABA, alanine, and proline at concentrations similar to those in pollen were strong feeding stimulants for adult WCR (Mullin et al., 1993).

Presented here are feeding responses of adult WCR to individual amino acids and the structure–activity relationships between the amino acids. Most previous works on amino acid feeding responses have been accomplished with limited doses, which may be below or above the phagostimulatory sensitivity range of taste receptors. Therefore, five doses were used, ranging from 10 to

500 nmol/disk, which are within the physiological range of amino acid content in pollen (Hollister and Mullin, 1998). Amino acids were presented to the beetles on cellulose disks in a no-choice consumption bioassay. The goal was to determine structural requirements for phagostimulatory amino acid taste chemoreception in this important herbivorous pest.

METHODS AND MATERIALS

Beetles. A nondiapausing laboratory strain (French Agricultural Research, Inc., Lamberton, Minnesota) of western corn rootworm was received as pupae. Emerged beetles were placed into plastic shoe boxes with sliced store-bought acorn squash fruit and water, and maintained at 25°C and 16L:8D photoperiod. Food and water were changed every two days, and beetles were deprived of food, but not water, for 24 hr prior to bioassay. Beetles were used for a single amino acid consumption bioassay from 5 days until they were 20 days post-emergence, and no significant age-related difference in sensitivity to L-alanine phagostimulation was found during this age period (Kim, unpublished).

Chemicals. 3-Chloro-L-alanine and DL-2-fluoro-3-alanine were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, and PCR Inc., Gainesville, Florida, respectively. Purity of 19 protein L-amino acids plus glycine was >99% by TLC (Sigma LAA-21 kit of standards). Other amino acids and derivatives were obtained from Sigma Chemical Co., St. Louis, Missouri, and were the highest purity commercially available.

Regenerated Cellulose Disk Bioassay. Dose–phagostimulant studies on 52 amino acids and their derivatives were performed using a no-choice regenerated disk bioassay. After 24 hr of starvation, two randomly selected beetles, without sex discrimination, were placed into a 100- × 20-mm Petri dish containing one regenerated cellulose disk (27.4 mm^2, 0.45-μm pore size; Schleicher and Schuell, Keene, New Hampshire) suspended in the middle of the dish on a minuten pin 2 mm above a paraffin layer covered with moistened filter paper. Generally, five different concentrations of an amino acid (2.5, 12.5, 25, 62.5, and 125 nmol/μl) dissolved in water were separately applied with a Hamilton repeating dispenser (Reno, Nevada) in 4 × 1-μl aliquots to the disks to ensure complete coverage. Control disks were treated with only 4 × 1 μl water. Each amino acid treatment was usually replicated five times, but ranged from 4 to 14 times. Dry disk areas after 24 hr of bioassay were determined to the nearest 0.1 mm^2 with an Optomax V image analyzer (Analytical Measuring Systems, Saffron Walden, UK) and then subtracted from the mean disk area (27.4 mm^2) of 10 disks not offered to beetles, to give the area consumed.

Data Analysis. Consumption data are presented as a mean percentage of the disk area consumed ± standard error of the mean. The significance of

differences between the consumption of a control (water-treated, $N = 90$) and amino acid-treated disks by beetles was determined by Student's t test (JMP version 3.1, SAS Institute Inc., Cary, North Carolina). In this bioassay, beetle mortality was not significant until after 72 hr, provided the filter papers were kept moist.

RESULTS

Single Amino Acids. The effects of 20 protein amino acids and their analogs on feeding responses of adult WCR are summarized in Table 1. L-Alanine (**1**) was the most phagostimulatory amino acid. The total amount eaten by beetles at five doses tested was the highest. Compared to solvent control, L-alanine disk consumption at all doses tested was significantly different (Student's t test, $P < 0.01$). L-Serine (**12**) induced significant disk consumption at doses greater than 10 nmol/disk, and β-alanine (**39**) had a stimulatory effect on feeding at higher doses (100, 250, and 500 nmol/disk). L-Serine-treated disks were consumed more than L-alanine-treated disks at 50 and 100 nmol/disk. However, L-alanine consumption increased up to 250 nmol/disk, whereas L-serine consumption leveled off and then decreased with doses greater than 250 nmol/disk. All three amino acids showed increasing phagostimulation with increasing dose and then an antifeedant effect, particularly at 500 nmol/disk.

L-Asparagine (**16**) was a weak, but consistent stimulant, over the dose range tested, especially at greater than 50 nmol/disk ($P < 0.05$), and L-valine (**5**) also showed a weak to moderate effect at 50 and 500 nmol/disk doses ($P < 0.05$). However, other protein amino acids did not elicit significant stimulatory responses compared to the control (water). The D isomers of alanine (**28**) and serine (**13**) did not evoke feeding over the range of doses tested.

Among amino acid analogs and nonprotein amino acids, only DL-γ-amino-β-hydroxybutyric acid (**47**) at 10 nmol/disk (Table 1) had a significant phagostimulatory effect on adult WCR feeding (Student's t test, $P < 0.05$). No other amino acid analogs showed significant differences in amount consumed from the control.

Side-Chain Modifications of L-Alanine or L-Serine. Substitution on the L-alanine β-carbon with aliphatic or aromatic groups dramatically decreased phagostimulatory responses. Aliphatic side-chain substitution with ethyl, *sec*-butyl, and isobutyl groups to give L-α-amino-*n*-butyric acid (**2**), L-isoleucine (**3**), and L-leucine (**4**), respectively, lowered feeding responses. Isopropyl substitution at the α-carbon position (i.e., L-valine **5**) elicited a moderate feeding activity at doses of 50 and 500 nmol/disk. The aromatic amino acids, L-histidine (**6**), L-phenylalanine (**7**), L-tryptophan (**8**), and L-tyrosine (**9**), were inactive or

antifeedant for the beetles. Glycine (**10**), which has the minimal side-chain volume, did not elicit a feeding response, whereas α-aminoisobutyric acid (**11**), with an additional methyl group on the α-carbon, greatly reduced feeding.

Replacement of one hydrogen on the methyl group of L-alanine with polar functional groups had less pronounced effects on phagostimulation. Hydroxyl substitution (L-serine **12**) retained feeding activity, while attachment of an amide group (L-asparagine **16**) led to a weaker feeding response. Inserting an additional methylene group between the α-carbon and polar functional group on the amino acid side chain led to decreased activity [e.g., asparagine (**16**) and aspartic acid (**17**) versus glutamic acid (**18**) and glutamine (**19**)]. One of the side-chain amide amino acids (**16**) was more active than the respective free acid (**17**). However, β-cyano-L-alanine (**20**), with similar polarity and steric and hydrogen bonding capabilities to the amide group, gave a much weaker phagostimulatory response as did the carboxylic acid group (L-aspartic acid **17**), and the chloro group (3-chloro-L-alanine **21**). Other replacement of a hydrogen in the methyl group of L-alanine with polar functional groups similar to hydroxyl of serine such as thiol (L-cysteine **22**), and amino groups (DL-2,3-diaminoproprionic acid **23**) greatly reduced the stimulatory response. Other protein amino acids (**24, 25,** and **26**), which contain a long side chain on the α-carbon, diminished feeding activity. Substitution of a methyl group on the β-carbon of L-valine (**5**) with a hydroxyl group (L-threonine **27**) also lowered feeding responses.

Side-chain modifications of L-serine were similarly restrictive as in L-alanine for maintenance of feeding stimulation. Methylation of the side-chain hydroxyl terminus (*O*-methyl-DL-serine **14**) decreased feeding consumption, and an additional methylene group between the α-carbon and hydroxyl group of the side chain (L-homoserine **15**) did not stimulate feeding. The opposite enantiomer (D-serine **13**) lacked significant activity.

Effects of Amino or Carboxyl Group Modification. Substitution on the amino group of alanine also reduced feeding. Acetylation (*N*-acetyl-L-alanine **29**), substitution of the amino group with a hydroxyl group (L-lactic acid **31**), and methylation (*N*-methyl-L-alanine **30**) resulted in poor feeding stimulation. Similarly, any alterations to the free carboxylic group of alanine greatly reduced phagostimulatory activity. Esterification of a methyl group (L-alanine methyl ester **33**) and replacement of the carboxyl group with a phosphonic acid group [(*R*)-1-aminoethyl phosphonic acid **34** and (*S*)-1-aminoethyl phosphonic acid **35**] markedly attenuated the feeding response. For serine, amidation (L-serine amide **36**), methylation (L-serine methyl ester **37**), and hydroxymethyl substitution at the carboxyl group (DL-serinol **38**) greatly lessened consumption by beetles.

Extended and Cyclic Amino Acids. All changes to the basic β-alanine structure (Table 1) reduced feeding. When functional groups such as $-CH_3$,

TABLE 1. PHAGOSTIMULATORY ACTIVITY OF 52 AMINO ACIDS AND DERIVATIVES IN NO-CHOICE TEST WITH ADULT WESTERN CORN ROOTWORM[a]

Compound	Structure (R)	Mean % disk consumption (± SE) at dose (nmol/disk)				
		10	50	100	250	500
α-amino acids	HOOC(CH-R)NH2					
1. L-alanine	CH_3	17.39 ± 5.60	21.98 ± 5.41	22.15 ± 5.85	44.71 ± 9.06	23.87 ± 6.38
2. L-α-amino-*N*-butyric acid	CH_2CH_3	6.47 ± 1.55	nt (not tested)	9.73 ± 3.76	4.39 ± 0.58	5.50 ± 2.08
3. L-isoleucine	$CH_2(CH_3)CH_2CH_3$	4.87 ± 0.77	4.67 ± 0.60	5.93 ± 0.63	3.84 ± 0.47	4.53 ± 1.43
4. L-leucine	$CH_2CH(CH_3)_2$	2.27 ± 1.21	3.26 ± 0.75	7.81 ± 6.10	1.94 ± 0.60	8.16 ± 4.63
5. L-valine	$CH_2(CH_3)_2$	9.38 ± 5.45	20.06 ± 9.27	6.77 ± 1.80	4.12 ± 1.57	20.10 ± 10.30
6. L-histidine[b]	[c]	10.31 ± 9.82	4.13 ± 2.69	1.83 ± 0.92	0.15 ± 0.15	0
7. L-phenylalanine	$CH_2C_6H_5$	3.02 ± 0.70	0.12 ± 0.08	0.60 ± 0.45	0.77 ± 0.77	0
8. L-tryptophan	[c]	2.94 ± 0.84	1.29 ± 0.65	5.66 ± 3.77	1.58 ± 0.69	1.42 ± 0.48
9. L-tyrosine	$CH_2C_6H_4OH$	6.04 ± 0.79	5.75 ± 0.31	5.10 ± 0.58	4.07 ± 0.45	3.38 ± 0.75
10. glycine	H	1.62 ± 0.49	nt	5.26 ± 1.65	4.79 ± 1.03	2.88 ± 0.52
11. α-aminoisobutyric acid	$HOOCC(CH_3)_2NH_2$[d]	2.36 ± 0.37	2.75 ± 0.58	2.23 ± 0.74	2.46 ± 0.93	2.14 ± 1.41
12. L-serine	CH_2OH	6.97 ± 1.57	28.00 ± 8.38	29.33 ± 9.32	26.21 ± 6.92	11.70 ± 2.96
13. D-serine	CH_2OH	4.68 ± 1.65	6.10 ± 1.08	4.34 ± 0.62	3.73 ± 0.71	5.46 ± 0.66
14. *O*-methyl-DL-serine	CH_2OCH_3	5.26 ± 0.79	4.77 ± 0.61	5.28 ± 0.41	8.37 ± 1.68	3.60 ± 0.83
15. L-homoserine	CH_2CH_2OH	6.75 ± 0.35	6.30 ± 0.67	6.63 ± 0.97	13.18 ± 6.55	13.91 ± 8.91
16. L-asparagine	CH_2CONH_2	14.99 ± 7.83	20.50 ± 12.3	15.31 ± 4.06	15.04 ± 6.72	18.8 ± 10.20
17. L-aspartic acid	CH_2COOH	6.55 ± 0.39	5.16 ± 1.14	6.64 ± 0.90	6.57 ± 0.75	5.57 ± 1.27
18. L-glutamic acid	CH_2CH_2COOH	4.99 ± 1.14	6.23 ± 1.09	6.54 ± 0.99	6.82 ± 0.47	5.13 ± 0.90
19. L-glutamine	$CH_2CH_2CONH_2$	3.00 ± 0.0	4.19 ± 0.36	10.18 ± 6.02	3.73 ± 0.95	5.57 ± 1.27
20. β-cyano-L-alanine	CH_2CN	10.57 ± 2.48	13.11 ± 2.51	6.04 ± 1.61	11.28 ± 4.80	11.45 ± 4.74
21. 3-chloro-L-alanine	CH_2Cl	4.90 ± 0.40	6.75 ± 1.13	4.02 ± 0.42	2.63 ± 0.66	nt
22. L-cysteine[b]	CH_2SH	7.13 ± 0.71	7.02 ± 0.96	8.44 ± 0.56	6.11 ± 1.22	7.89 ± 0.68
23. DL-2,3-diaminoproprionic acid[b]	CH_2NH_2	6.99 ± 1.02	5.74 ± 1.26	7.17 ± 1.39	5.26 ± 0.29	5.03 ± 0.74
24. L-arginine[b]	$(CH_2)_3NHC(NH)NH_2$	5.80 ± 1.24	5.02 ± 0.63	5.80 ± 1.18	4.79 ± 1.01	3.10 ± 0.84
25. L-lysine	$(CH_2)_4NH_2$	5.47 ± 0.62	5.08 ± 1.31	5.77 ± 1.14	5.47 ± 1.05	5.09 ± 1.13
26. L-methionine	$CH_2CH_2SCH_3$	7.11 ± 1.47	4.90 ± 0.30	5.02 ± 0.77	9.25 ± 4.41	3.84 ± 0.92
27. L-threonine	$CH(OH)\text{-}CH_3$	10.94 ± 6.70	5.76 ± 1.02	4.26 ± 1.27	13.19 ± 7.69	5.26 ± 0.87
28. D-alanine	CH_3	4.84 ± 0.22	nt	5.28 ± 0.91	5.14 ± 0.29	4.31 + 0.82

NH_2 modified amino acids	$HOOC(CH\text{-}R)CH_3$					
29. *N*-acetyl-L-alanine	$NHCOCH_3$	5.44 ± 0.43	6.30 ± 0.49	6.12 ± 0.61	4.99 ± 0.50	4.07 ± 0.95
30. *N*-methyl-L-alanine	$NHCH_3$	7.55 ± 0.63	4.92 ± 1.81	6.13 ± 0.53	6.30 ± 1.06	3.32 ± 1.03
31. L-lactic acid	OH	4.47 ± 0.87	6.06 ± 0.52	6.60 ± 1.07	6.35 ± 0.76	4.81 ± 0.75
32. sarcosine	CH_3NHCH_2COOH[d]	4.62 ± 2.20	nt	4.56 ± 1.34	2.40 ± 0.61	0.79 ± 0.29
COOH modified amino acids	$H_2N(CH\text{-}R)CH_3$					
33. L-alanine methyl ester[b]	$COOCH_3$	4.69 ± 0.46	6.25 ± 0.64	6.42 ± 0.94	6.79 ± 0.31	4.07 ± 0.37
34. (*R*)-1-aminoethyl phosphonic acid	$P(OH)_3$	7.46 ± 0.59	7.16 ± 1.56	7.81 ± 0.95	5.15 ± 1.27	8.74 ± 0.71
35. (*S*)-1-aminoethyl phosphonic acid	$P(OH)_3$	3.93 ± 1.04	3.79 ± 0.86	4.52 ± 0.55	5.35 ± 0.69	3.97 ± 0.88
36. L-serine amide[b]	$H_2N(CH\text{-}CONH_2)CH_2OH$[d]	5.76 ± 0.29	12.28 ± 5.49	5.52 ± 1.00	4.32 ± 1.07	3.74 ± 1.06
37. L-serine methyl ester[b]	$H_2N(CH\text{-}COOCH3)CH_2OH$[d]	2.89 ± 0.61	3.55 ± 0.43	3.47 ± 0.88	2.54 ± 0.69	1.17 ± 0.33
38. DL-serinol[b]	H2N(CH-CH2OH)CH2OH[d]	6.56 ± 0.93	6.86 ± 0.96	7.44 ± 0.93	5.98 ± 0.89	4.37 ± 0.51
β- or γ-amino acids	$H_2N\text{-}R\text{-}COOH$					
39. β-alanine	CH_2CH_2	3.83 ± 0.52	6.53 ± 1.80	16.43 ± 5.50	24.26 ± 8.23	17.99 ± 6.41
40. DL-β-aminoisobutyric acid	$CH(CH_3)CH_2$	3.19 ± 0.57	nt	3.12 ± 0.60	1.86 ± 0.65	2.80 ± 0.53
41. DL-β-amino-N-butyric acid	$CH_2CH(CH_3)$	4.42 ± 0.42	nt	7.71 ± 3.06	6.16 ± 1.60	3.55 ± 0.56
42. DL-2-fluoro-3-alanine	$CH_2CH(F)$	4.90 ± 0.42	4.71 ± 0.98	6.20 ± 1.40	3.02 ± 0.60	3.55 ± 0.80
43. DL-isoserine	$CH_2CH(OH)$	4.02 ± 0.48	5.29 ± 1.08	4.82 ± 1.05	7.48 ± 3.69	2.52 ± 0.74
44. aminooxyacetic acid[e]	OCH_2	5.53 ± 3.93	2.96 ± 1.39	4.19 ± 0.64	2.78 ± 1.06	1.38 ± 0.37
45. taurine	$H2NCH_2CH_2SO_2H$[d]	5.67 ± 0.87	7.14 ± 1.50	6.74 ± 0.64	6.58 ± 0.51	8.10 ± 3.15
46. γ-aminobutyric acid (GABA)	$CH_2CH_2CH_2$	5.83 ± 0.54	nt	6.23 ± 0.59	4.40 ± 0.92	5.17 ± 1.21
47. DL-γ-amino-β-hydroxybutyric acid	$CH_2CH(OH)CH_2$	15.80 ± 11.70	12.83 ± 6.35	5.68 ± 1.49	9.01 ± 2.34	3.14 ± 0.61
48. L-carnitine inner salt	$(CH_3)_3NCH_2CH(OH)CH_2COOH$[d]	4.33 ± 0.42	3.70 ± 1.04	4.10 ± 0.80	4.83 ± 0.53	2.41 ± 0.67
Cycloamino acids						
49. L-azetidine-2-carboxylic acid	[c]	5.56 ± 1.91	nt	2.81 ± 0.33	2.28 ± 0.71	1.44 ± 0.50
50. proline	[c]	3.43 ± 1.04	nt	6.05 ± 2.53	5.84 ± 2.34	3.26 ± 1.14
51. *cis*-4-Hydroxy-L-proline	[c]	6.46 ± 0.37	9.29 ± 0.40	5.28 ± 0.46	5.32 ± 0.23	10.03 ± 3.09
52. L-cycloserine	[c]	7.86 ± 0.86	9.99 ± 4.48	8.20 ± 1.11	7.17 ± 0.50	6.99 ± 1.17

[a] The average % disk consumption of control (water) was 6.18 ± 0.70 (N = 90).
[b] Hydrochlorides used.
[c] See Figure 1.
[d] Entire structure shown.
[d] Hemihydrochloride used.

—F, or —OH were attached to either α- or β-carbon such as in DL-β-aminoisobutyric acid **40**; DL-β-amino-*N*-butyric acid **41**; DL-2-fluoro-3-alanine **42**; and DL-isoserine **43**, feeding responses were reduced. Replacement of the methylene adjacent to the amino group with oxygen (aminooxyacetic acid **44**) or the carboxylic acid by a sulfonic acid (taurine **45**) also decreased activity. A single carbon increase [γ-aminobutyric acid (GABA) **46**] between the carboxyl and amino residues of β-alanine diminished phagostimulation. Adding an —OH group to the β-carbon of GABA significantly increased its phagostimulatory activity at low doses (DL-γ-amino-β-hydroxybutyric acid **47**). Further exhaustive methylation of the amino group of **47** to give carnitine (**48**) obliterated feeding enhancement.

None of the low-molecular-weight cyclic amino acids (**49–52**) at the doses tested here had significant phagostimulatory activity in the no-choice bioassay (Table 1, Figure 1).

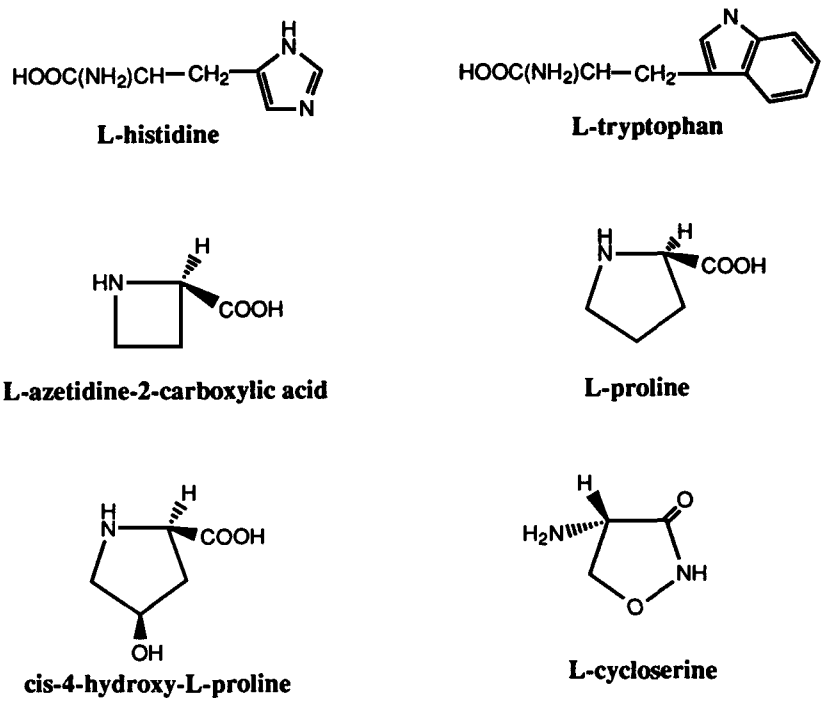

FIG. 1. Chemical structures of two free amino acids and cycloamino acids used in this study.

DISCUSSION

Considerable loss of phagostimulation with even minor structural changes in L-alanine indicates that phagostimulatory amino acid taste receptors in WCR are selective for L-alanine. Effective feeding stimulation by amino acids in WCR requires primarily four structural features (Figure 2). First, terminal carboxyl and amino groups must be intact since all structural modifications in these groups led to a considerable decrease in feeding (compounds **29–38**). It is likely that the carboxyl and amino groups bind to respective positively and negatively charged domains within the receptor since they will be fully ionized at physiological pH near neutrality. Second, a hydrophobic region sterically limited to a two-saturated-carbon insert between the amino and carboxyl groups is needed for optimal stimulatory effects at the L-alanine taste receptor. L-Alanine and β-alanine have only two carbons between the amino and carboxyl groups, and lengthening the chain by even one carbon (e.g., GABA **46**, or L-γ-amino-*n*-butyric acid **2**) drastically reduced feeding responses. Third, L-alanine and L-serine (*S* configuration) are much more potent stimulants than the respective D enantiomers (*R* configuration), verifying the strict stereospecificity of the receptor site. Fourth, the hydrogen domain on the α-carbon of L-alanine is sterically constrained since replacement with a methyl group (α-aminoisobutyric acid **11**) obliterated phagostimulatory activity. To fully characterize the steric

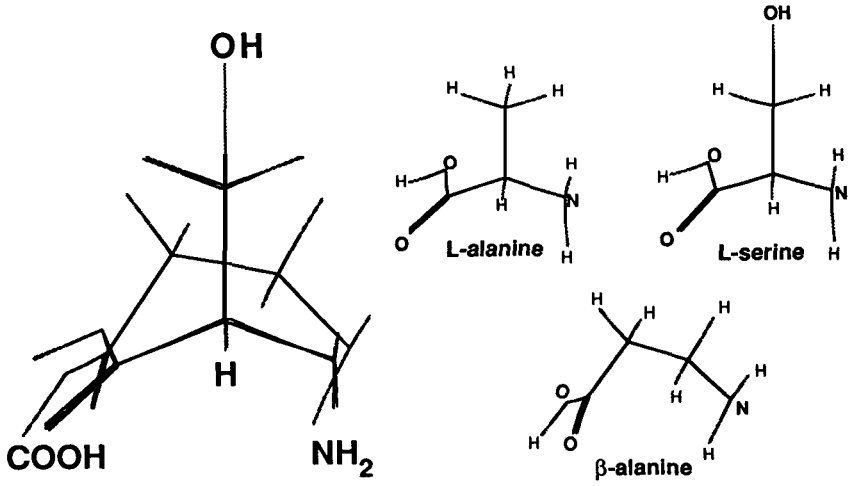

FIG. 2. Superimposition of three phagostimulatory amino acid structures: L-alanine, L-serine, and β-alanine from Alchemy III (Tripos Associates, St. Louis, Missouri).

hindrance on the hydrogen domain will require substitution with smaller groupings such as a fluorine atom.

In Colorado potato beetle, L-alanine, GABA, and L-α-aminobutyric acid were equally potent followed by L-serine, L-proline, and β-alanine in stimulating galeal taste cells with the sensillar tip recording method (Mitchell and Schoonhoven, 1974; Mitchell, 1985). The similar phagostimulatory potency and presumed binding of GABA and L-alanine with Colorado potato beetle taste receptors, due to the flexibility of the GABA molecule (Mitchell, 1985), is not directly applicable in WCR. However, other work with adult WCR with selected dual combinations of L-alanine and other amino acids (Kim, 1997), or more complex combinations of pollen-dominant amino acids including GABA (Hollister, unpublished) demonstrated an additive or synergistic effect on the L-alanine response, which suggests multiple amino acid sites are interacting to produce the net phagostimulatory input to the central nervous system. While GABA only elicits nibbling on regenerated cellulose disks, it does stimulate adult WCR feeding on plant disks, particularly lettuce disks, and evokes dose-dependent action potentials from a galeal chemosensory cell (Mullin et al., 1994; Chyb et al., 1995). Two cooperative binding sites may be hypothesized, one restricted to L-alanine and another less sterically hindered by bulkier short chain neutral amino acids, such as GABA. In β-alanine, the reduced bulk of two saturated carbons between the amino and carboxyl groups could contribute to a weak hydrophobic interaction with both an alanine and a less sterically restricted amino acid receptor site, resulting in a weak feeding response in WCR.

That a β-hydroxyl group on the amino acid side chain, such as in L-serine, does not reduce stimulatory taste indicates the presence of another binding domain. A hydrophilic hydroxyl group on the β-carbon of L-serine would not be expected to share the same side-chain hydrophobic binding domain with L-alanine because of the obvious differences in functions (polar vs. nonpolar). Electronegativity of functional groups on the β-carbon is not a sole determinant for feeding response because of the low significance ($P > 0.1$) of a modest positive correlation (Pearson correlation coefficient, $r = 0.584$, $N = 9$ without alanine) between phagostimulatory activity and sidechain electronegativity (Table 2). However, a much stronger negative correlation occurs with the π hydrophobic parameter (collinear with the octanol/water partition coefficient of the group) provided the substituent is nonionic at physiological pH, which excludes compounds **17** and **23** (Table 2 and Figure 3). Decreasing hydrophobicity (increasing polarity) of the group improves phagostimulatory activity ($r = 0.808$, $P < 0.05$ without alanine, and $r = 0.518$, $P > 0.1$ with alanine). Meanwhile, there was no correlation with the size (steric factor) of the β-carbon functional group (data not shown). Therefore, L-alanine and L-serine may have distinct binding sites on the taste cells in WCR, or an un-ionized and polar grouping extending from the hydrophobic domain of an L-alanine receptor is permitted.

TABLE 2. AMINO ACID SIDE-CHAIN GROUP ELECTRONEGATIVITIES, π VALUES, AND FEEDING RESPONSES

Amino acid	Group	Electronegativity[a]	π[b]	Feeding responses[c]
L-alanine	$-H$	2.176	0	44.71
L-serine	$-OH$	3.494	−0.67	29.33
L-asparagine	$-CONH_2$	2.731	−1.49	20.5
β-cyano-L-alanine	$-CN$	3.208	−0.57	13.11
L-α-amino-*n*-butyric acid	$-CH_3$	2.472	0.56	9.74
L-cysteine	$-SH$	2.616	0.39	8.44
DL-2,3-diaminoproprionic acid	$-NH_2$	2.992	−1.23	7.17
3-chloro-L-alanine	$-Cl$	3.000	0.71	6.75
L-aspartic acid	$-COOH$	2.824	−0.32	6.64
L-phenylalanine	$-C_6H_5$	2.717	1.96	3.02

[a]Inamoto and Masuda (1982).
[b]Hansch and Leo (1979).
[c]Maximal % consumption based on Table 1.

Three phagostimulatory amino acids (L-alanine, L-serine, and β-alanine) showed an increasing dose–response pattern followed by inhibition at a high concentration (i.e., 500 nmol/disk). L-Alanine, L-serine, and β-alanine are dominant free amino acids in pollens and the range of phagostimulation (about

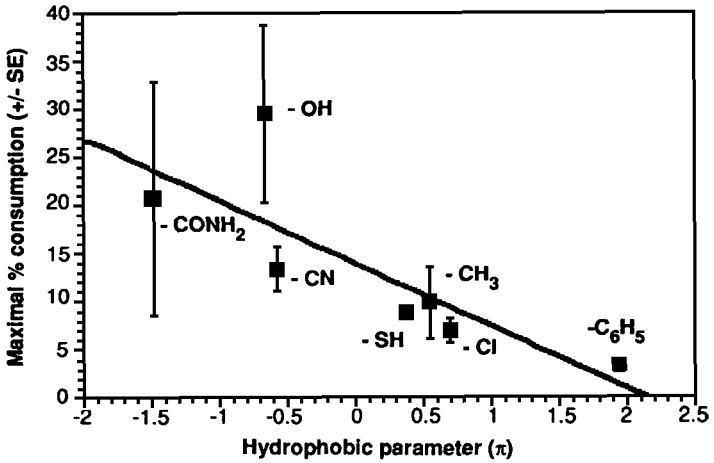

FIG. 3. The relationship between π value of functional groups on the amino acid β-carbon and its maximal phagostimulation (% consumption ± SE) for western corn rootworm.

50–250 nmol/disk) is within the physiological concentration in rootworm food pollens (Hollister and Mullin, 1998; Mullin et al., 1993; Hollister, in preparation) so naturally occurring concentrations of food amino acids overlap with their dynamic phagostimulatory dose range for chemoreception in WCR. Interestingly, these three phagostimulants are nonessential amino acids in insect diets (Dadd, 1985). Although nutritionally essential amino acids were more effective stimulants in several Lepidoptera species (Dethier, 1973; van Loon and van Eeuwijk, 1989), blow flies (Goldrich, 1973), flesh flies (Shimada and Tanimura, 1981), and a termite (Chen and Henderson, 1996), amino acid taste stimulation in insects is predominantly due to short-chain nonessential amino acids including L-alanine, L-serine, β-alanine, and GABA (Bernays and Simpson, 1982; Hedin et al., 1993; Hsiao and Fraenkel, 1968; Mitchell, 1985; Mullin et al., 1994; Thorsteinson, 1960). Only in the latter cases have distinct amino acid structure–phagostimulatory relationships been obtained. Thus, amino acid sensitivity in WCR may be tuned to quantitatively relevant signals rather than just nutritionally important amino acids in a food.

Chapman (1995) reviewed the vast literature establishing that food choice is based on response to a mixture of various chemical stimuli and that feeding behavior may not be explained through correlations relying only on inputs from sensory chemicals tested singly. Future investigations on interactions between mixtures of taste chemicals such as amino acids at the peripheral sensory neurons should better reveal chemoreception mechanisms leading to observed feeding behaviors. This work is essential for providing additional phagostimulatory bait components to that of the cucurbitacins (Metcalf, 1985) for effective Diabroticite pest control.

Acknowledgments—This study was supported, in part, by a USDA-NRICGP 95-37302-1807 grant.

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VERTEBRATE HERBIVORY ON *Eucalyptus*—IDENTIFICATION OF SPECIFIC FEEDING DETERRENTS FOR COMMON RINGTAIL POSSUMS (*Pseudocheirus peregrinus*) BY BIOASSAY-GUIDED FRACTIONATION OF *Eucalyptus ovata* FOLIAGE

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Abstract—Factors determining the acceptance of *Eucalyptus ovata* foliage by common ringtail possums (*Pseudocheirus peregrinus*) were studied. Bioassay-guided fractionation was used with foliage from both browser-susceptible and browser-resistant trees to identify the chemical components underlying the resistance. In foliage from browser-resistant trees, the deterrent principles were contained in the base-soluble fraction of the chloroform extract. Further fractionation of this material yielded polar and nonpolar fractions that contained acylphloroglucinol derivatives, and from the polar fraction we isolated macrocarpal G. Addition of this compound to an artificial diet at a concentration of 2.1% of dry matter resulted in a 90% reduction of voluntary food intake compared with solvent-treated controls. This is the first time that a specific compound in *Eucalyptus* has been shown to inhibit feeding of any marsupial folivore.

Key Words—Plant secondary metabolites, terpenes, feeding, folivory, bioassay, marsupial, *Eucalyptus ovata*, *Pseudocheirus peregrinus*.

INTRODUCTION

Herbivory in *Eucalyptus* forests and woodlands varies widely (Landsberg and Cork, 1997). *Eucalyptus* dominates more than 90% of Australian forests, and

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studies have shown that some of the 600 species suffer little damage (e.g., Pahl, 1987). Even more striking are variations within species, with some trees being killed while an adjacent tree is apparently untouched (Edwards et al., 1993; Pahl, 1987). An inability to identify the factors controlling herbivory in *Eucalyptus* forests impedes our ability to manage these environments or to contribute to testing many recent theories about herbivory. Consequently, there have been many attempts to identify the chemical basis for this difference in palatability. A number of studies have sought correlations between food choice and primary constituents such as leaf nitrogen, leaf sugars, and fibre (Degabriele, 1981; Cork and Pahl, 1984; Geritz, 1987; Kavanagh and Lambert, 1990; Osawa, 1992; Hume and Esson, 1993). Few trends have emerged other than a weak influence of water and total nitrogen on feeding by koalas, but even so, this result explains little of the between-tree variation.

Other studies have attempted to relate the concentration of plant secondary metabolites (PSMs) in the leaves to the level of herbivory. *Eucalyptus* trees appear to be well defended plants because terpenoids, tannins, and associated phenolics can comprise up to 40% of the leaf dry matter (Fox and Macauley, 1977; Morrow and Fox, 1980; Foley, 1992), but again no consistent patterns of food choice or feeding behavior in relation to PSMs have been found. For example, in koalas (*Phascolarctos cinereus*), Southwell (1978) found no association between browsing and cineole concentrations in foliage, Betts (1978) suggested that the ratio of cineole to sesquiterpenes best explained the use of *E. globulus* foliage by koalas, and Hume and Esson (1993) concluded that koalas use foliar terpenoids as a positive feeding cue, preferring those with a relatively high concentration of monoterpenes. Taken together, these studies suggest either that an important component has been omitted from the analyses or that we have little understanding of what actually constitutes high nutritional quality in *Eucalyptus*.

The difficulty of this approach, which correlates food choice or feeding pressure with the chemical composition of the foliage, is that the analyses are not necessarily those that are relevant to the animal. Bryant et al. (1983) and Reichardt et al. (1984, 1990) showed the value of bioassay-guided fractionation in their studies of food choice of snowshoe hares in boreal ecosystems. This approach allowed specific compounds to be identified and tested for their repellent properties. Recognizing that correlative approaches had been exhausted, we adopted a bioassay-guided fractionation protocol to identify the specific constituents of *Eucalyptus ovata* foliage that determine its acceptability for a small marsupial browser, the common ringtail possum (*Pseudocheirus peregrinus*).

METHODS AND MATERIALS

Animals and Basal Diet. This research was approved by the Animal Experimentation Ethics Committee of James Cook University and conforms with the

Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Common ringtail possums (*Pseudocheirus peregrinus*) were used in all bioassays. They were caught by hand in *Eucalyptus* woodland near Townsville and Melbourne and maintained individually in large outdoor cages (preliminary experiments) or in metabolism cages as described by Foley (1992). A period of at least four weeks was allowed for acclimation to captivity, during which time the animals were fed mixed *Eucalyptus* foliage, fruit, and bread. In preliminary experiments (described below) they were offered only *Eucalyptus* foliage, but in all bioassay experiments, they were maintained on a palatable artificial diet made from cereals and fruit (Foley, 1992). The diet was made consistently to be 32% dry matter, and all animals maintained mass when offered this diet. Drinking water was supplied ad libitum.

Preliminary Identification of Resistant Trees and Susceptible Trees. Foliage was collected from 24 individual *E. ovata* trees and offered to captive ringtail possums. Initially, branches from six individual trees were weighed and placed in buckets of water in the cages at 18:00 hr. The weight of control branches did not change by more than 0.2%, and thus no correction for water uptake or loss was necessary. All branches were reweighed at 08:00 hr the next day, and any plants that were uneaten were noted. This procedure was repeated until we had preliminary identifications of four resistant and four susceptible trees. We then provided each foliage individually to ringtail possums and measured wet matter intake (Foley, 1992). Resistant trees were taken to be those of which animals ate less than 15 $g/kg^{0.75}$/day, whereas susceptible trees were defined as those of which animals ate at least 90 $g/kg^{0.75}$/day.

Extraction and Purification of Plant Secondary Metabolites. Foliage was collected from four resistant trees, mixed thoroughly, immediately frozen in liquid nitrogen, and stored at −20°C. A similar collection was made from four susceptible trees. The dry matter content of this foliage was 32–41% dry matter. Volatile constituents were extracted by steam distillation in an all-glass apparatus for 12 hr. The product of this distillation was extracted with diethyl ether and the organic layer dried over Na_2SO_4 and evaporated in vacuo to yield the steam-distillate fraction.

Crude methanol extracts of both resistant and susceptible foliages were prepared, and these extracts were partitioned until specific feeding deterrents were identified. *E. ovata* foliage was extracted in a Soxhlet apparatus with methanol for 24 hr. The methanol was filtered and reduced under vacuum to near dryness. This was designated as the methanol extract. A methanol extract was then partitioned between water (water extract) and chloroform. The organic layers were combined and evaporated to yield the chloroform extract. Extraction of the chloroform-soluble fraction with base (1.0 M NaOH, 3 × 200 ml) followed by reacidification and back-extraction of the caustic layer [5.0 M to pH 2.0, 3 × 200 ml dichloromethane (DCM)] afforded a mixture of low-molecular-weight (< 1000 daltons) phenolic compounds and fatty acids. This was designated the crude phenolic extract.

Further fractionation of the crude phenolic extract by repeated vacuum-assisted silica gel chromatography (90:10 DCM–methanol) (Coll and Bowden, 1986) resulted in a polar and a nonpolar fraction. Examination of these fractions by ^{1}H NMR suggested that the nonpolar fraction comprised compounds similar to the euglobals isolated from *E. globulus* by Kozuka et al. (1982a,b) and some fatty acids, whereas the polar fraction contained compounds similar to the macrocarpals isolated by Nishizawa et al. (1992) together with fatty acid.

Individual compounds were purified from these fractions by a combination of column chromatography on silica and Sephadex LH-20 and reversed phase HPLC. Structures of the known compounds reported here were confirmed by comparison of ^{1}H, ^{13}C, and high-resolution mass spectra with published data.

Bioassay-Guided Fractionation. Two types of bioassay were used to identify which fractions acted as antifeedants. The first was a two-choice protocol used by past workers (e.g., Bryant et al., 1983; Reichardt et al., 1984). Each bioassay used an extract from an original 600-g wet mass of foliage and was applied to 600 g wet mass of the basal diet described above (see bioassay conditions below). Extracts were dissolved in a minimum volume of hot methanol, then thoroughly mixed with the dry components of the basal diet. The solvent was removed under vacuum and the diet then made up as normal to produce the test food. Controls were prepared in exactly the same manner using methanol alone, and the position of the control and treated food in the cage was randomized each day. Each experiment was performed over four days with six common ringtail possums. We followed Sinclair et al. (1988) and calculated the proportion of the total intake resulting from the test and control diet each day and tested whether this was significantly different using a Wilcoxon matched pairs, signed rank test. When there was a statistically significant difference between treated food and controls in this protocol, we conducted a no-choice experiment, in which the animals were offered treated food alone. Comparisons were then made between the amount of food eaten when animals had no choice and the amount of food consumed on the previous day when control diets alone were offered.

Analytical. ^{1}H NMR spectra were recorded at 300.133 MHz and ^{13}C NMR at 75.47 MHz using a Bruker AM-300 spectrometer. Chemical shifts are quoted as parts per million relative to $CHCl_3$ set to δ7.26 (^{1}H NMR) and 77.0 (^{13}C NMR), unless otherwise stated. Capillary gas–liquid chromatography (GLC) was performed on a Hewlett-Packard 5890 gas chromatograph with flame ionization detection and on bonded-phase silica columns (BP 1.0.25). Waters Radial Pac 25×10 C18 reversed-phase cartridges were used for all high-performance liquid chromatography (HPLC).

Cyanogenic glycosides were isolated and characterized as described by Brimer and Dalgaard (1984) and Cardona et al. (1992) and quantified using the procedure of Lambert et al. (1975). Feigl-Anger papers were used as a quali-

tative test for cyanogenic glycosides in both whole foliage and foliar extracts prior to purifications of the cyanogenic principle.

RESULTS

Summary figures showing the path taken during the bioassay procedures and the intake of each fraction by common ringtail possums are shown in Figures 1 and 2.

Intake of Foliage from Resistant and Susceptible Trees. When provided a choice between foliage cut from resistant or susceptible trees, common ringtails strongly preferred that from the susceptible trees ($P < 0.001$). Intake of foliage from susceptible trees was 105 $\pm$ 7 g wet matter (32.1 $\pm$ 2.3 g dry matter), whereas only 5 $\pm$ 4 g (wet mass) of foliage from the resistant trees was consumed. In a no-choice test with resistant foliage, animals consumed 10 $\pm$ 6 g wet matter.

Methanol Extracts from Resistant and Susceptible Foliage. In a two-choice test there was no significant difference between controls and food treated with a methanol extract of susceptible foliage ($P = 0.14$). In contrast, controls were preferred over food treated with a methanol extract from resistant foliage ($P = 0.04$).

Steam Distillate from Resistant and Susceptible Foliage. The yield of steam distillate from susceptible foliage was 0.1% wet matter and that from resistant foliage was 1.0%. Gas–liquid chromatography showed that 1,8-cineole was the dominant component of the oil from mature foliage from resistant trees, comprising about 80% of the total extract. Although cineole was also the dominant fraction of the mature foliage from the extract of the susceptible foliage, linalool was the principal component in young leaf from the same trees again comprising about 80% of the extract. No young leaves were produced on the resistant trees during the study for comparison.

Animals preferred ($P = 0.05$) controls over the food treated with the steam distillate of resistant trees applied to the basal diet at a concentration of 1.0% (wet mass). Animals showed no preference for controls over food treated either with the steam-distillate fraction of the susceptible foliage or with a 0.1% concentration of steam distillate from resistant foliage ($P = 0.29$).

Pure cineole (>99% by GLC) added to the basal diet at a concentration of 0.8% was a strong deterrent ($P = 0.01$) to feeding in a two-choice test. However, when the same concentration was offered in a no-choice protocol, there was no significant difference ($P = 0.16$) between food intake during the test days and in the days both before and after the test when only control food was offered. Pure linalool at a concentration of 0.1% and 0.8% (equal to the range of cineole concentrations tested) did not have a significant effect on food intake in a two-choice test ($P = 0.40$ and $P = 0.15$, respectively).

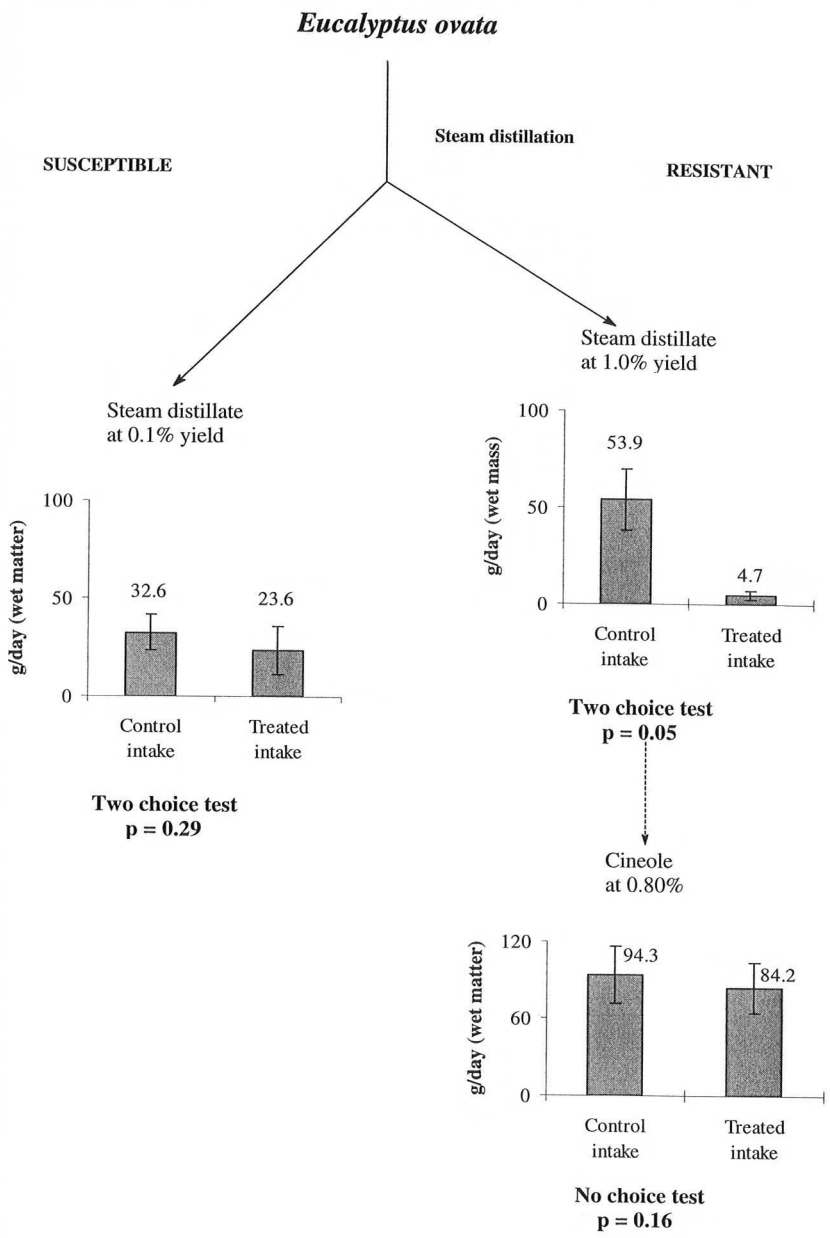


FIG. 1. Bioassay-guided fractionation of volatile constituents of browsing-resistant and susceptible foliage of *Eucalyptus ovata*. Each graph shows the mean ± SE of wet matter intake for six common ringtails. All results are from two choice tests except where indicated. *P* values are the significance of the difference in intake between control and treated food.

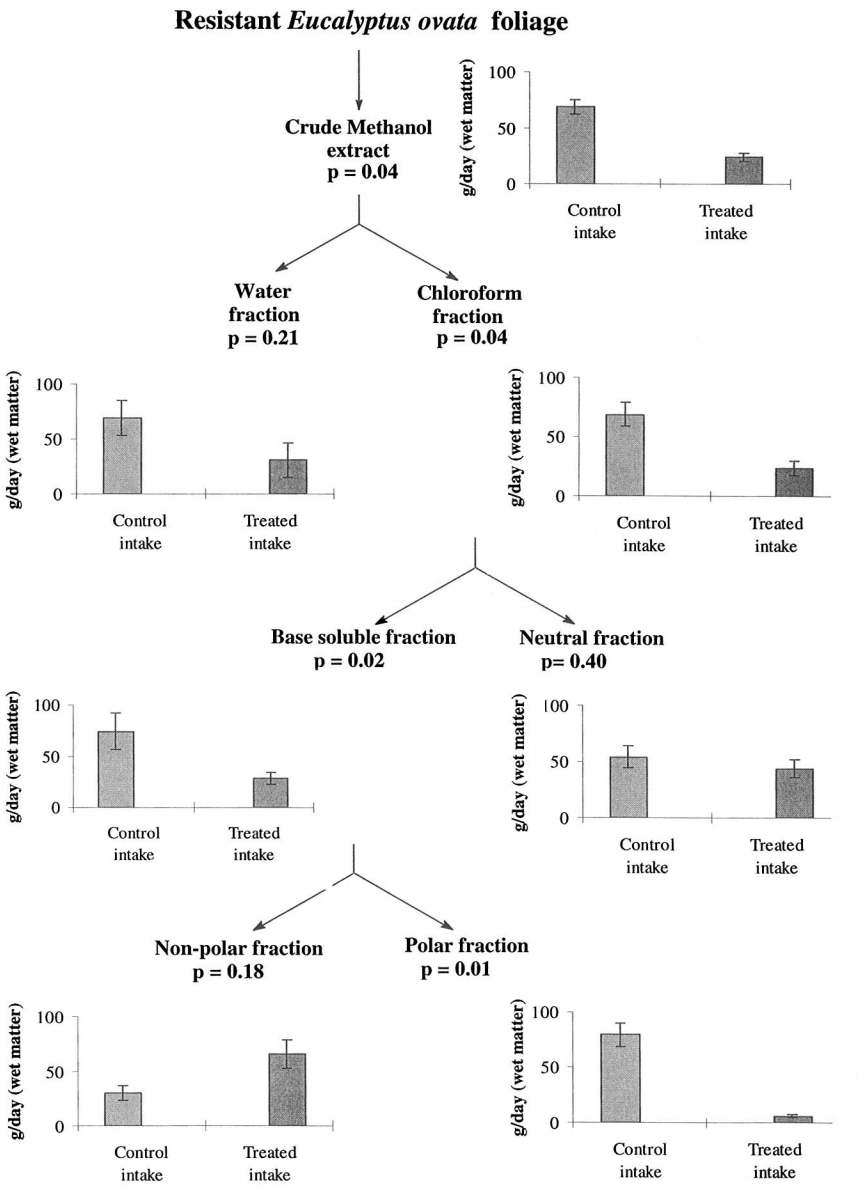


FIG. 2. Bioassay-guided fractionation of nonvolatile constituents of browsing-resistant foliage of *Eucalyptus ovata*. Each graph shows the mean ± SE of wet matter intake for six common ringtails and results from two choice tests. *P* values are the significance of the difference in intake between control and treated food.

Water-Soluble Fraction of Methanol Extract of Resistant Foliage. The presence of a cyanogenic compound was detected by the use of Feigl-Anger papers in the water-soluble fraction of resistant foliage. This cyanogenic activity was isolated and shown by ^{1}H NMR to be prunasin by comparison with literature values (Cardona et al., 1992). Prunasin occurred at a concentration 44 $\pm$ 5 μg cyanide/g fresh leaf in resistant foliage but no cyanide was detected in the foliage from the susceptible trees. Despite this, the water-soluble extract containing these glycosides was not significantly deterrent to the animals, and there was no significant difference between the intake of food treated with the water-soluble extract or controls ($P = 0.21$) in a two-choice test.

Chloroform-Soluble Fraction of Methanol Extract of Resistant Foliage. Controls were preferred over the chloroform-soluble material from the methanol extract ($P = 0.04$) in a two choice test.

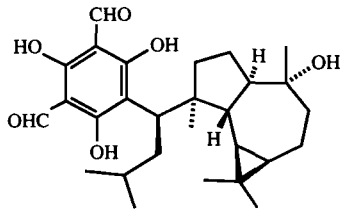
Base-Soluble and Neutral Extracts of Chloroform Solubles from Resistant Foliage. Animals strongly preferred controls ($P = 0.02$) over food treated with the base soluble extract of chloroform solubles from resistant foliage in a two-choice test. In contrast, there was no significant difference in food intake between controls and food treated with the acid-soluble fraction. This confirmed that the deterrent fractions were likely to be phenolic in nature.

Polar and Nonpolar Fractions of Base-Soluble Extract. The base-soluble extract was chromatographed (see conditions above) to yield two fractions of differing polarity. Animals showed no significant preference ($P = 0.18$) between controls and food treated with the nonpolar fraction in two-choice tests. In contrast, controls were strongly preferred ($P = 0.01$) over food treated with the polar fraction of the base-soluble extract of resistant foliage.

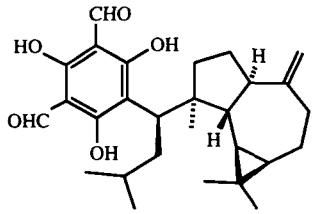
Identification of PSMs. Examination of both the polar and nonpolar fractions of the base solubles by ^{1}H NMR suggested the presence of hydrogen-bonded hydroxyls on a fully substituted aromatic chromophore attached to a hydrocarbon/terpene moiety. These features closely matched the structures of a group of acylphloroglucinol–terpene adducts previously identified in *Eucalyptus*, namely the euglobals (Kozuka et al., 1982a,b) and the macrocarpals (Nishizawa et al., 1992; Yamakoshi et al., 1992).

Chromatography on Sephadex LH-20 and reverse-phase HPLC (95:4.9:0.1 acetonitrile–water–acetic acid) of the nonpolar fraction afforded a number of euglobals (euglobal Ib, Ic, IIa, Bl$_1$, III, IVb, and V) and another simpler phloroglucinol derivative [compound 1 of Qi and Snyder (1991)]. Euglobal III (Figure 3) was the dominant component of this fraction.

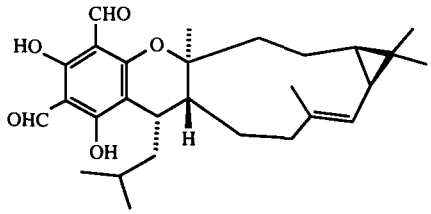
The dominant component of the polar mixture was isolated by chromatography on Sephadex LH-20 and reverse-phase HPLC (97:2.5:0.5 acetonitrile–dichloromethane–acetic acid). Comparison of the ^{13}C NMR data (in d_4-methanol) with published values (Yamakoshi et al., 1992) allowed this compound to be assigned as macrocarpal G (Figure 3).



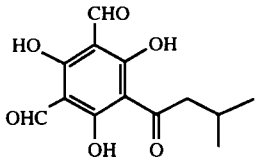
Macrocarpal A



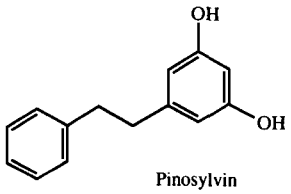
Macrocarpal G



Euglobal III



Jensenone



Pinosylvin

FIG. 3. Planar structures of acylphloroglucinol–terpene adducts of *Eucalyptus* foliage and of pinosylvin.

Macrocarpal G. The macrocarpal fraction was refined by repeated chromatography over silica (0.5:99.5 acetic acid–light petroleum) to yield macrocarpal G (Figure 3) in sufficient purity (>95% by HPLC) for bioassay. The impurities were long-chain fatty acids that proved very difficult to separate. We added this material to the diet at a concentration of 0.7% of wet matter (2.1% dry matter) and used a no-choice protocol. Animals ate too little of the treated food to maintain themselves, and the intake was significantly different from controls ($P = 0.03$), demonstrating that macrocarpal G is at least partially responsible for the selective feeding behavior of common ringtail possums on *E. ovata* foliage.

DISCUSSION

The striking differences in food intake of different *E. ovata* trees quantifies the field observations of many biologists (e.g., Hindell and Lee, 1987; Pahl, 1987; Geritz, 1987) that *Eucalyptus* displays interspecific variation in its susceptibility to vertebrate herbivores, but this study is the first to explain how this is mediated. Although the trees that we subsequently examined in detail were collected 20 km apart, resistant and susceptible *E. ovata* trees can be found side by side (Geritz, 1987; W. Foley, personal observation).

Bioassay-guided fractionation provided clear evidence that common ringtail possums fed little on resistant *E. ovata* foliage because of the presence of the phloroglucinol-terpene adduct known as macrocarpal G. This is the first time that a specific molecule in *Eucalyptus* has been identified as a feeding deterrent for either insects or mammals. Macrocarpal G was first isolated in 1992 (Yamakoshi et al., 1992) and is one of several related bioactive phloroglucinol derivatives with widespread biological activity (Ghisalberti, 1996).

There remains some confusion in published work regarding the name macrocarpal G since the same planar structure has been assigned to both macrocarpal G (Yamakoshi et al., 1992) and macrocarpal C (Nishizawa et al., 1992). Since the NMR experiments described in these two reports were carried out in different solvents, direct comparisons are not possible. Our NMR spectra were obtained in methanol as were those of Yamakoshi et al. (1992), and so we have followed those workers and used the name macrocarpal G.

The structure of both euglobals and macrocarpals suggests that they are derived from two separate biosynthetic pathways. Both contain a terpene moiety coupled to a fully substituted aromatic ring. How these pieces are coupled has been the subject of some speculation. Kozuka et al. (1982a,b) argued for a Diels-Alder-based mechanism for the biosynthesis of the euglobals, whereas the addition of the appropriate benzylic cation to a sesquiterpene has been proposed to generate macrocarpals. Bicyclogermacrene is the terpene involved in the biosynthesis of macrocarpals A and G. Macrocarpal G is presumed to be the

dehydrated product of macrocarpal A or one of its isomers. Both euglobal III and macrocarpal G can, however, be envisaged as being derived from addition of bicyclogermacrene to the same benzylic carbocation. Euglobal IVa and IVb could also arise by similar condensations (see Figure 4).

We presume that the terpene moiety confers a high degree of lipid solubility on the macrocarpals and so serves to carry the reactive aldehyde and phenol groups across membranes. If this is true, then we would expect that the level of deterrence of these compounds is a function of both the number of reactive groups on the molecule and the degree of lipid solubility conferred by the terpene moiety. Evidence for this suggestion comes from the relative antifeedant activity of the polar (principally macrocarpal G) and nonpolar fractions (principally euglobal III) in our experiments. Both compounds have the same acylphloroglucinol skeleton but the macrocarpals have a free hydroxyl where the euglobals have an ether linkage connecting the aromatic portion with the terpene. It is possible that the free hydroxyl of the macrocarpal species contributes significantly to the antifeedant activity. Preliminary studies (D. M. Pass and W. Foley, unpublished) using jensenone (Boland et al., 1992) (Figure 3), a related acylphloroglucinol compound from *Eucalyptus jensenii* support this hypothesis, although a directed structure–activity study is in progress.

Lipophilic phenols have been implicated as feeding deterrents for other mammalian browsers (e.g., snowshoe hares, *Lepus americanus*). For example, Clausen et al. (1986) showed that foliar pinosylvin was a significant factor in the choice of snowshoe hares for green alder, and the relative activities observed between pinosylvin and its mono- and dimethylated derivatives parallel the differences in the antifeedant activities demonstrated here for euglobal III and macrocarpal G. Interestingly, there are structural and biosynthetic similarities between compounds such as pinosylvin and dihydrochalcone [which is also deterrent to snowshoe hares; (Clausen et al., 1986)] and macrocarpal G. This raises the possibility that aspects of the chemical structures of these compounds provides some deterrent properties against a wide range of mammalian browsers.

We do not know at present what effects the macrocarpals have on common ringtail possums and thus why they are so strongly avoided. Preliminary evidence (Lawler, Foley and G. J. Pass unpublished data) is that acylphloroglucinols cause nausea in marsupials and so lead to the development of a conditioned food aversion but whether this occurs as a result of the native compound or its metabolites is not yet known.

Previous correlative studies of diet choice in both vertebrate and insect browsers of *Eucalyptus* have implicated the terpenoid 1,8-cineole as the deterrent molecule (Betts, 1978, Edwards et al., 1993) but our study is the first to use bioassays to test that hypothesis. It is therefore significant that we found that cineole was not deterrent when the animals were given no choice at a concentration of cineole similar to that in resistant foliage.

High concentrations of cineole were measured in the unpalatable *E. ovata*

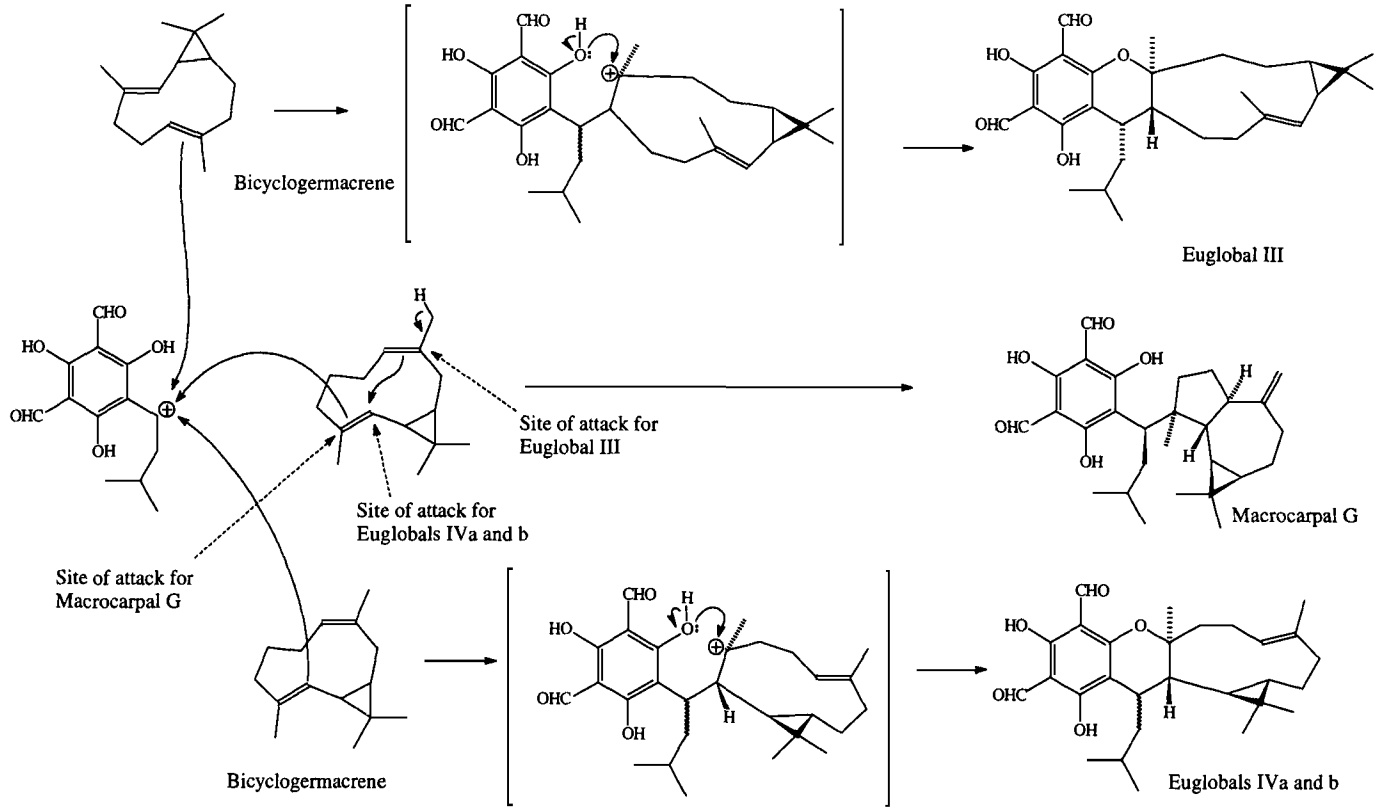


FIG. 4. Proposed addition of bicyclogermacrene to the appropriate carbocation for generation of euglobals III, IVa, IVb, and macrocarpal G.

as well as in a number of other unpalatable trees within other species of *Eucalyptus* [e.g., in *E. polyanthemos* (D. M. Pass unpublished data) and in various other *Eucalyptus* species (Edwards et al., 1993)]. This suggests the possibility that cineole may be used as a cue by some animals to the presence of high levels of macrocarpal G and related compounds. Both the euglobals and the macrocarpals are composed of a (poly)acylated phloroglucinol moiety with either a monoterpene or sesquiterpene attached through a C-5 unit. In most cases the monoterpene functionality appears to derive from β-pinene (or a related monoterpene), whereas sesquiterpene functionalities are derived from bicyclogermacrene (see Figure 4). Cineole has never been recorded as part of the acylphloroglucinol–terpene adduct, and we suggests that any involvement of cineole as an adjunct to deterrent macrocarpals must be only indirect. It is possible that high levels of cineole signal to animals that macrocarpals are present, but this idea requires testing.

In these studies it was found that animals chose to eat little cineole when offered a choice between food containing cineole and food that did not. However, when no choice was offered, animals consumed food with 0.8% (wet mass) cineole readily. This implies that ingestion of cineole involves a cost for the animals but one that they are willing to pay when no other food is available. However, the nature of this cost is unclear. For example, Krockenberger (1988) found no difference in the digestibility or metabolizability of food when common ringtails were fed cineole for a prolonged period of time at a concentration of 1.4% dry matter. Lawler (unpublished) found that at concentrations of 12% dry matter common ringtails could maintain body mass on high intakes of artificial diets. Costs may be related to general disturbances to acid–base metabolism as described by Foley (1992) and Foley et al. (1995), in which biotransformation of ingested PSMs leads to production of a proton that must in turn be excreted. However, Bull et al. (1993) have shown that urinary metabolites of cineole may act as pheromones in common brushtail possums, allowing discrimination between the scent marks of female and male possums, and so in this instance the animal is deriving some value from the PSM.

The acylphloroglucinol backbone of the macrocarpals is present in a variety of related compounds found within different species of *Eucalyptus* (Ghisalberti, 1996; D. M. Pass, unpublished). Given the demonstration of antifeedant activities and other wide-ranging biological actions [e.g., tumor suppressant, and antibacterial and antifouling activities (Kozuka et al., 1982a,b; Nishizawa et al., 1992; Yamakoshi et al., 1992)], we postulate that this group of compounds are significant herbivore defenses within the whole genus. Given the dominance of *Eucalyptus* in Australian forests, these compounds are likely to significantly modulate other plant–animal interactions within the continent.

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BIOLOGICAL ACTIVITY OF *Datura wrightii* GLANDULAR TRICHOME EXUDATE AGAINST *Manduca sexta* LARVAE

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Abstract—Natural populations of *Datura wrightii* in southern California consist of two distinctly different phenotypes. The leaves of one phenotype are densely covered with nonglandular trichomes and feel velvety. The other phenotype is covered with larger type IV glandular trichomes that excrete a sticky exudate. Neonate larvae of *M. sexta* reared on velvety leaves developed significantly faster than larvae on sticky leaves. Larvae on sticky leaves took 28% longer to reach the prepupal stage. Survival and pupal weight were not significantly different between the two groups. First instars of *M. sexta* had a significantly higher consumption rate on velvety leaves than on sticky leaves. Removal of the exudate from stickly leaves significantly increased larval consumption rates compared to unwashed controls. Female moths did not show an oviposition preference; both in the lab and in the field the two trichome phenotypes of *D. wrightii* received similar egg loads. Because there were no significant differences in other nutritional factors between the two plant phenotypes, we concluded that the exudate was responsible for the effect. We isolated a complex mixture of sugar esters (SE) as the biologically active compounds in the exudate of *D. wrightii*. The SE mixture was composed of glucose esterified with several combinations of straight chain C_6–C_9 acids. By comparing GC-MS spectra of synthetic SE with the SE extracted from *D. wrightii*, we identified one of the SE as 3′-*O*-hexanoyl glucose.

Key Words—Tobacco hornworm, Solanaceae, insect–plant interactions, glandular trichomes, exudate, resistance polymorphism, oviposition choice, acyl sugar esters, Lepidoptera, Sphingidae.

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INTRODUCTION

Glandular trichomes and their exudates confer insect resistance in several solanaceous plant species. They may form a physical barrier hampering feeding or movement of herbivores on the leaves, or a chemical defense that increases insect mortality (Tingey, 1981; Duffey, 1986). These mechanisms are active against a wide range of insect herbivores, such as aphids (Goffreda et al., 1989; Neal et al., 1990; Severson et al., 1994), whiteflies (Buta et al., 1993; Liedl et al., 1995), beetles (Neal et al., 1989; Yencho et al., 1994), and caterpillars (Thurston, 1970; Kennedy and Yamamoto, 1979; Dimock and Kennedy, 1983). Several chemical compounds involved in insect resistance have been isolated from glandular trichomes of solanaceous plants. The relatively small globular type VI (Luckwill, 1943) trichomes on tomato leaves produce a volatile compound, 2-tridecanone, that increases mortality of *Manduca sexta* and *Helicoverpa zea* larvae (Williams et al., 1980; Dimock and Kennedy, 1983). Type VI trichomes on tomato also contain phenolics and polyphenol oxidases (PPO) (Duffey, 1986; Steffens et al., 1990). Phenolics and PPO are stored in different compartments of the tetracellular tips of these trichomes. Rupture of the head by herbivores leads to enzymatic polymerization of the phenolics, resulting in entrapment of the insect (Duffey and Isman, 1981; Duffey, 1986). Moreover, several Solanaceae also possess type IV trichomes, which are longer than type VI trichomes and have ovoid glands on their tips. These glands excrete a sticky exudate that contains sugar esters (King and Calhoun, 1988; King et al., 1988; Goffreda et al., 1990; Liedl et al., 1995; Liu et al., 1996). Sugar esters either isolated from plants, or produced synthetically, significantly increase whitefly mortality (Buta et al., 1993; Chortyk et al., 1996) and reduce settling by aphids (Goffreda et al., 1989). Moreover, sugar esters have antagonistic effects on growth and survival of *H. zea* and *Spodoptera exigua* (Juvik et al., 1994).

Exudate-producing type IV trichomes are also found on leaves of *Datura wrightii* L. (Solanaceae) plants. However, not all individuals of this species possess type IV glandular trichomes; some plants are densely covered with nonglandular trichomes (van Dam et al., in press). All plants possess equally low numbers ($\sim 5\%$ of total trichomes) of the tetralobed type VI trichomes. The appearances of plants with and without type IV glandular trichomes are distinctly different and the two phenotypes can be easily discriminated by eye and touch; plants with nonglandular trichomes feel velvety, while plants with glandular trichomes feel sticky. Only a few (less than 5%) intermediate types are found in the field. All southern Californian populations censused to date contained both velvety and sticky phenotypes. Trichome type in *D. wrightii* is determined by a single gene, with glandular trichomes being dominant (van Dam et al., in press).

Because trichomes are known to be involved in insect resistance, we hypothesize that insect herbivores play a role in the maintenance of this trichome polymorphism. Both nonglandular and glandular trichomes are involved in insect resistance (Tingey, 1981), but the production of exudate by sticky types may be an additional physical or chemical barrier for herbivores. Therefore, it is not unreasonable to expect that sticky types, overall, are more resistant to herbivores than velvety types.

One of the naturally occurring herbivores on *D. wrightii* in southern California and Arizona is *Manduca sexta* (Lepidoptera: Sphingidae), the tobacco hornworm (Casey, 1976). The larvae consume up to 3400 cm^2 of leaf area to reach the prepupal stage (Madden and Chamberlin, 1945), so grazing by *M. sexta* may constitute a significant selection pressure. Previous studies on Solanaceae, such as *Petunia*, tobacco, and tomato, indicated that *M. sexta* larvae are negatively affected by trichomes and their various exudates (Thurston, 1970; Kennedy and Yamamoto, 1979).

We have studied the biological activity of trichomes and exudate of *D. wrightii* on *M. sexta* performance. First, we assessed differences in development, feeding rates, and oviposition of *M. sexta* on velvety and sticky leaves. We evaluated the significance of the exudate indirectly by washing it off sticky leaves and comparing the feeding rate of neonate *M. sexta* larvae on these leaves with untreated leaves. Then, we assessed the biological activity of the exudate in isolation by adding leaf surface extracts to artificial diets and comparing the developmental rates of the larvae. Thus, we eliminated other factors, such as trichome morphology and nutritional factors as being responsible for the differences in growth rates. Finally, we analyzed the chemical composition of the active fraction and compared it with biologically active exudate compounds in other Solanaceae.

METHODS AND MATERIALS

Description of Species and Populations

Datura wrightii Regel (Solanaceae), is a self-compatible perennial species whose geographical distribution is western Texas to California and Mexico (Avery et al., 1959; Munz, 1973). *D. wrightii* is commonly found in sandy or gravely dry places, such as river washes and slopes (Munz, 1973). One population in this study (UCR) is in an undisturbed site on the hills in and around the botanical garden at the University of California, Riverside campus. The Moreno Valley (MV) population is located 10 km southeast of the UCR population, near a residential area in a dry creek that is periodically tilled for weed control. Based on their appearance and differences in percentages of glandular

trichomes, plants with less than 15% glandular trichomes were designated as velvety, while plants with more than 85% glandular trichomes were designated as sticky (van Dam et al., in press).

Manduca sexta (L.) (Lepidoptera: Sphingidae) is a naturally occurring herbivore on *D. wrightii* plants throughout southern California and Arizona (Hodges, 1971; Casey, 1976). The geographic range of *M. sexta* in North America stretches from southern California to Florida, with northern limits in southeastern New York (Hodges, 1971). The moths lay single eggs on the abaxial side of the leaves of *D. wrightii* and other solanaceous plant species (Yamamoto et al., 1969; Hodges, 1971). The larvae go through five larval instars. In southern California, *M. sexta* typically goes through two generations per season: the first lasts from June until August, the second, numerically larger generation, is from September until December (Oatman and Platner, 1978). Pupae of the second generation overwinter in the soil (Gilmore, 1938).

M. sexta *Rearing*

In 1994 a colony was started with *M. sexta* larvae and eggs collected from *D. wrightii* from several natural populations in and around Riverside. To prevent inbreeding, the colony was supplemented annually with new *M. sexta* eggs and larvae, collected from natural populations between June and December. The larvae were reared individually on artificial diet (Hoffman et al., 1966) in a growth room (28°C const., 16L:8D). After the moths had emerged, they were transferred to a flight cage (1 × 1 × 1 m) for mating and oviposition. From October until April, the cage was placed in the greenhouse (20–30°C, ambient light conditions), while for the remainder of the year the cage was placed outside under a tree to protect it from excessive solar heat and radiation. The moths were provided with a 25% sucrose solution. The eggs were collected daily from leaves of sticky and velvety *D. wrightii* plants placed in the cage and brought to the growth room. There they hatched within three to five days after collection.

Performance of M. sexta *Larvae on Velvety and Sticky Plants*

Toxicity and Effect on Early Development. Groups of 15 neonate *M. sexta* larvae were placed in plastic boxes (16 × 30 × 8.5 cm) that contained either a sticky or a velvety branch, cut from plants grown at the Agricultural Operations experimental station at UCR (AgOps UCR). Plant type was initially determined by eye and touch and later confirmed by observation under a dissection microscope (25×). The branches were put in a Aqua-pic (Syndicate Sales Inc., Kokomo, Indiana) vial filled with water to prevent wilting. The boxes had ventilation holes in the sides, covered with brass screen, and were lined with a wet paper towel. Three boxes per trichome type were placed in the growth room

(conditions as above). After four days, the larvae were transferred to fresh branches cut from the same plants as previously. After six days the experiment was ended and the head capsule width and weight of each larva were measured. The numbers of larvae in each of the first three instars were noted. Differences in survival were analyzed with a *t* test, larval weight with a nested ANOVA (box nested in plant type; SAS, version 6.02, SAS Institute Inc., Cary, North Carolina), and the distribution of larvae over instars was analyzed with a G-test (BIOM statistical package, F. J. Rohlf, 1982).

Long-Term Effect on Development. To determine the effect of trichome type on larval development of *M. sexta*, branches were cut from neighboring sticky and velvety plants growing in the MV (three plants per type) and UCR (two plants per type) *D. wrightii* populations and placed in boxes as described above. Ten (MV plants) or 12 (UCR plants) neonate *M. sexta* larvae were placed on the leaves in each box. The boxes were placed in the colony room (conditions as above). The larvae fed ad libitum and the branches were replaced with freshly collected branches from the same plants twice a week or more frequently when necessary. When a larva reached prepupal (wandering) stage, the date was noted and the larva was transferred to a labeled cup filled with sterile vermiculite. Seven days after transfer, when the pupal case had hardened, the pupae were sexed and weighed. Differences in developmental time and pupal weights were analyzed with a mixed model ANOVA. Plant type was tested against the box within type error; sex and type × sex interaction were both tested against the sex × box within type interaction. Nonsignificant interactions were pooled with the error.

Consumption Rates. Neonate larvae of *M. sexta* were placed singly on a *D. wrightii* leaf disc (18.5 mm diameter) that was placed with the abaxial side up on wet filter paper in a Petri dish (10 cm diameter). The plants used for this experiment were grown in the greenhouse from seeds obtained by selfing of plants grown at AgOps UCR. During the experiment, the survival of the larvae was monitored every 24 hr. After 72 hr, when the leaf discs started to show signs of decay, the larvae were removed and the leaf area consumed was measured on 1-mm graph paper. In the first experiment, the consumption by larvae on discs punched from four velvety plants was compared to that on discs from four sticky plants (10 larvae per treatment). These data were analyzed with a *t* test. In the second experiment, the discs were punched from four different sticky plants. A pair of discs were punched from the same leaf: one was washed with demineralized water to remove the exudate, while the other served as a control (10 larvae per treatment). The washed discs were carefully dried with a low-lint tissue (Kimberley-Clark, Atlanta, Georgia) and examined under a dissection microscope (25×) to confirm that the exudate droplets were indeed removed. The differences in consumption rates on washed and control discs of the same

leaf were analyzed with a paired *t* test. In both cases, differences in the distribution of survival over time (one, two, or three days) were analyzed with a *G* test.

Oviposition Choice. Oviposition activity by the moths peaks in the fall in southern California (Oatman and Platner, 1978). On September 13, 14, and 19, 1995, a total of 99 mature *D. wrightii* plants in the UCR population were censused for the presence of *M. sexta* eggs. Trichome type of the plants was assessed by sight and touch. Differences in egg distribution were analyzed with a *G* test.

To study the oviposition choice of the moths under controlled conditions, we cut branches from adjacent velvety and sticky plants either in the MV population (November 15–December 3, 1995; 15 choice tests) or in the UCR population (June 12–24, 1996; 10 choice tests). Flowers, seed capsules, and the occasional wild *M. sexta* egg were removed, and the branches were pruned to equal size and appearance. The branches were placed in individual 500-ml flasks filled with tap water. The phenotype of the plant was confirmed by observation under a dissection microscope. Two branches of a sticky plant and two branches of a velvety plant were placed 46 cm apart in the four corners of the *M. sexta* cage. Sticky and velvety branches alternated, and every day the positions of the sticky and the velvety branches were exchanged. After one night in the cage, the branches were taken out and the number of eggs and the number of leaves on each branch were counted. The moths were supplied with fresh branches from a different set of plants. To correct for differences in plant size, we divided the number of eggs by the number of leaves on a branch. Overall differences in numbers of eggs per leaf on the two types were analyzed on log transformed data with a mixed model ANOVA (type × experiment, with day nested into experiment). Nonsignificant interactions were pooled with the error term.

Identification of Active Compound

To determine the cause of the difference in performance of *M. sexta* on velvety and sticky plants, we carried out several analyses. First, we measured five general nutritional variables that may differ between the two plant phenotypes and are known to influence insect growth in general. Then we focused on the active compounds in the trichome exudates.

Differences in Nutritional Parameters. All solvents used for extraction and analysis were of certified ACS quality or better and were purchased from Fisher Scientific (Fair Lawn, New York) unless indicated otherwise. In November 1995, 100 leaves of seven sticky and seven velvety *D. wrightii* plants grown at AgOps UCR were collected. The leaves were weighed, dried to constant weight at 70°C for 48 hr, and weighed again to assess percentage of water. The dried leaves were ground with a Wiley mill equipped with a 20-mesh sieve. The

samples were analyzed for total nitrogen by a Leco gas analyzer (Sweeney, 1989) at the DANR Analytical Laboratories, University of California, Davis. Soluble sugars and phenolic contents were determined with colorimetric methods: soluble sugars with anthrone reagent (Allen, 1974) and total phenolics with Folin-Denis reagent (Allen, 1974). Alkaloids were extracted from 125 mg leaf powder under reflux (2× 1 hr) with 25 ml $CHCl_3$–NH_3OH (24 : 1) (Fliniaux et al., 1993). Before extraction, 0.375 mg anisodine in MeOH (Sigma, St. Louis, Missouri) was added to the leaf powder as an internal standard. The two extracts were combined and evaporated under reduced pressure. The alkaloids were recovered from the crude extract with 10.0 ml mobile HPLC phase. After filtration through a 0.45-μm Acrodisc PTFE filter (Gelman Sciences, Ann Arbor, Michigan), 20 μl of extract was injected on the HPLC. The method used for HPLC analysis was adapted from Fliniaux et al. (1993) with the following changes: flow rate: 1.2 ml/min, column: Beckman ODS 250 × 4.6 mm, 5-μm particle size (Beckman, Fullerton, California), mobile phase: 15% CH_3CN and 0.3% phosphoric acid in water, pH adjusted to 2.2 with triethylamine. Retention times and UV spectra of peaks in the leaf extracts were compared with reference solutions of scopolamine and hyoscyamine (Sigma) which have been described as the major alkaloids in related *Datura* species (Cheeke and Shull, 1985). Scopolamine and hyoscyamine also were the main alkaloids found in *D. wrightii* leaf extract; in addition we found a minor unidentified alkaloid with a UV spectrum similar to that of hyoscyamine. The quantities of these alkaloids were added to obtain total alkaloid contents.

Test for Volatile Toxicity of Sticky Leaf Surface Extract. A crude chloroform extract was prepared from sticky leaves (1120 g) collected in a population close to the UCR campus (CC population, see van Dam and Hare, 1998) and bioassayed as described in Kennedy et al. (1981). A $CHCl_3$ solution of the extract was applied to five Whatman filter paper circles (4.5 cm diam.), so that each contained 2.5 mg/cm^2 of the extract, and placed in individual glass Petri dishes (5 cm diam.). Another five filter paper circles were treated with solvent only. The $CHCl_3$ was evaporated at room temperature prior to placing three neonate larvae on the filter paper. The dishes were sealed with parafilm. Surviving larvae were counted after 24 hr.

Extraction and Purification of Type VI Trichome Exudate. The first part of the extraction and purification procedure is a modified version of the sugar ester isolation procedure described in Severson et al. (1994). Young branches were cut from 25 sticky plants growing at AgOps UCR on October 10, 1996. The leaves [652.2 g fresh weight (FW)] were soaked for 1 hr in 3 liters $CHCl_3$ (Figure 1). The extract was filtered over Na_2SO_4 to remove water and the $CHCl_3$ was removed by rotary evaporation and an airstream. The yield of this brown residue of the crude extract (coded CS) was 5.794 g or 0.89% of the leaf FW. A sample of 25 velvety plants (240.7 g FW) was extracted likewise in 1.2 liters

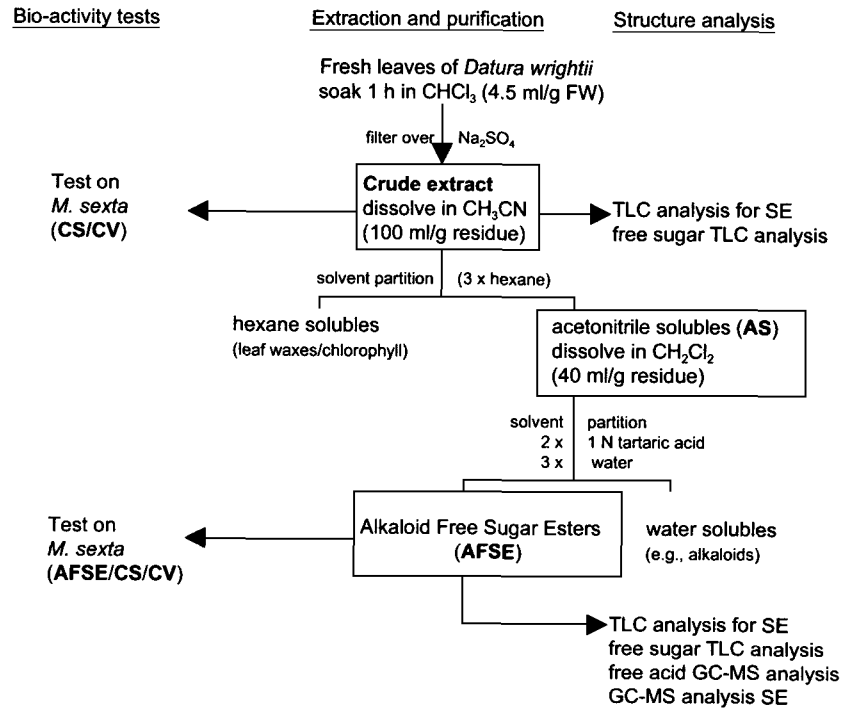

FIG. 1. Outline of the extraction and purification procedure for sugar esters of *Datura wrightii*. Left side: extracts that have been tested for biological activity on *M. sexta* larvae; right side: methods used for structure elucidation. Abbreviations: SE = sugar esters, CS = crude extract of sticky leaves, CV = crude extract of velvety leaves.

$CHCl_3$, yielding 0.388 g of a brown-green gum (coded CV, 0.16% FW). Both extracts were analyzed for the presence of sugar esters (SE) on TLC (see below). Because the CV extract appeared to be inactive against *M. sexta* larvae and to contain no compounds consistent with R_f values for SE, further purification and analysis was carried out with the CS extract only.

The CS residue was suspended in 100 ml CH_3CN and placed in an ultrasonic bath for 10 min to dissolve all material. A portion (25 ml) of the CH_3CN solution was kept for analysis, and 75 ml was partitioned three times against hexane in a separatory funnel (140 ml and 2× 100 ml hexane, respectively). In the last separation, 70 ml CH_3CN was added. The CH_3CN fraction was removed in vacuo, which resulted in 1.238 g of a light brown gum (code AS for acetonitrile solubles). The AS residue then was dissolved in 50 ml dichloromethane (CH_2Cl_2), 40 ml of which was partitioned two times against 20 ml of 1 N tartaric acid solution, and three times against 15 ml water. The tartaric acid and

water layers with alkaloids and other water-soluble compounds were combined and kept at −20°C. Evaporation of the CH_2Cl_2 resulted in a light brown residue with the alkaloid-free SE (AFSE) (Severson et al., 1994), weighing 0.792 g (0.202% FW, corrected for aliquots taken).

To obtain sufficient material for testing, an additional 700.2 g of sticky branches were collected on October 17, 1996. They were extracted as described above and yielded 5.250 g CS (0.75% of leaf FW) and eventually 0.663 g (0.095% FW) of AFSE residue. The two AFSE fractions were combined before testing.

Procedures Used for SE Analysis

TLC Procedure. Between 1 and 10 μl of test solution was applied with a glass microcapillary to 2.5 × 7.5 cm TLC plates (MK6F Si-gel, 60 Å, 205 μm layer, Whatman, Clifton, New York). The presence of SE on the plates after elution with $CHCl_3$–MeOH (9:1) was tentatively detected as described in King and Calhoun (1988).

Hydrolysis of SE and Identification of Sugar Moiety. Dried samples of purified plant extracts were dissolved in 2 ml MeOH, and 25 μl was vortexed with 25 μl 0.1 M NaOH in 1 ml microcentrifuge tubes (Goffreda et al., 1990). After 10 min at room temperature, 2 μl of the hydrolyzed sample were applied to a cellulose TLC plate (13245 Cellulose, Kodak, Rochester, New York) and developed twice in the top layer of a BuOH–acetic acid–H_2O (4:1:5) mixture (Harborne, 1973). After air drying, the plates were sprayed with 2.5 g aniline hydrogen phthalate in 100 ml BuOH–acetone–H_2O (49:49:2) and heated for 10 min at 105°C (Harborne, 1973). The sugars were identified by comparison with reference solutions of glucose and fructose. Glucose and fructose could clearly be discriminated based on their R_f values (0.77 and 0.81 in this system, respectively) and a distinct difference in color (brown for glucose vs. yellow for fructose) (Harborne, 1973).

Derivatization and GC-MS Analysis of SE. The SE in the AFSE fraction were characterized by GC and GC-MS analysis of their trimethylsilyl (TMS) derivatives. Between 1 and 10 μg of oven dried (50°C) sample were combined with 1.0 ml dimethylformamide (DMF) and 100 μl *N*, *O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) in 4 ml Reacti-Vials (Pierce, Rockford, Illinois). The vials were closed, vortexed, and heated to 60°C for 15 min (Pierce Silylation Sample Reagent Kit Instruction Booklet; Pierce). Samples of 1 μl were injected (splitless) on a 30-m × 0.32-mm-ID DB-5 column, film thickness 0.25 μm (J&W Scientific, Folsom, California) in an HP 5890 GC connected to an HP 3392A integrator (Hewlett Packard). Injector temperature: 250°C; FID detection; detector temperature: 300°C, column pressure: 50 kPa. The initial temperature of 150°C was maintained for 1 min, after which the temperature was increased to 300°C at 10°/min. The

final temperature was maintained for 10 min. GS-MS (EI, 70 eV) analysis was performed on an HP 5890 GC coupled to an HP 5989 A mass spectrometer, with a scan range of 40–650 Da at 2 scans/sec. Apart from the temperature program rate (6°C/min), the conditions and column were the same as for GC analysis.

Saponification of SE and Identification of Free Acids. A 30-mg sample of AFSE was saponified following the exact procedure described in Buta et al. (1993). The free acids were identified by comparison with reference samples on GC and GC-MS. We used the same GC conditions as for the TMS derivatives of the SE, except for the temperature program. Initial column temperature was set to 50°C for 3 min, followed by a 10°C/min increase until 250°C, which was maintained for 10 min. The identity of the acids was confirmed with GC-MS analysis (EI 70 eV, 40–400 Da, 1.7 scans/sec) on an HP 5890 GC coupled to a 5970 B Mass Selective Detector equipped with a 25-m × 0.2-mm Ultra 2 column (Hewlett Packard). The temperature program was 40°C for 1 min, increasing by 10°/min to 250°C. Identities were confirmed by comparison of retention times and mass spectra with those of authentic standards.

Synthesis of Reference Sugar Esters. To synthesize hexanoyl and heptanoyl reference SE, we followed the exact procedure described by Chortyk et al. (1996), with β-D-glucose (ICN, Cleveland, Ohio) and either caproyl (hexanoyl) chloride or heptanoyl chloride (Sigma) as reagents. The synthetic SE were derivatized as described above and analyzed on GC and GC-MS. This procedure resulted in a mixture of mono-, di-, and triacyl sugar esters, which can be detected as three separate groups on GC (Chortyk et al., 1996). Their retention times and fragmentation patterns were used as references for the AFSE extract of sticky *D. wrightii* plants.

Biological Activity of Extracts

General Procedures. Figure 1 summarizes the extracts tested on *M. sexta* larvae. In all cases, the extracts were first weighed to calculate the original leaf concentration, based on the yield of residue per gram fresh weight. Corrections were made for aliquots taken for chemical analysis during the purification process. The residues were dissolved in 4 ml CH_2Cl_2 before they were added to a weighed amount of warm (50°C) standard *M. sexta* rearing diet. The diets with the extracts were heated and stirred on a hot plate until the CH_2Cl_2 had evaporated. To adjust for losses during the purification and diet preparation, the test concentrations in the diets were prepared at concentrations four to eight times the original leaf content. Small cubes (ca. 125 mm^3) of diet were placed in 28-ml clear polystyrene cups with a lid. Neonate larvae were individually placed on the diet, and the lids were sealed with parafilm. The diet was replaced every

other day, so the larvae could eat ad libitum. The number of surviving larvae in each group was counted daily, and at the end of the experiment, the head capsule width of the larvae was measured under a dissection microscope.

Experiment 1: CV and CS. We mixed 0.158 g CV with 12 g diet (eight times more concentrated than original), and 0.779 g CS with 15 g diet (5.8 times concentrated). The experiment started with 15 larvae per group and was ended after eight days.

Experiment 2. CV, CS, and AFSE. We mixed 0.329 g CV with 40 g diet (5.1 times more concentrated), and 0.824 g CS extract with 20 g diet (4.6 times more concentrated). An 0.174-g aliquot of the AFSE fraction was taken and mixed with 13.5 g diet (4.7 times more concentrated than on the plant, corrected for aliquots taken). The experiment started with 15 neonate larvae in each group and was ended after seven days.

RESULTS

Performance of M. sexta *Larvae on Velvety and Sticky Plants*

Toxicity and Effect on Early Development. The development of *M. sexta* on sticky leaves was significantly delayed: after six days, the majority of larvae in this group was still in their second instar, while most larvae reared on velvety leaves were third instars (Table 1; G test, $G_2 = 54.171$, $P < 0.001$). The larvae feeding on sticky leaves also had a considerably lower body weight (Table 1, nested ANOVA, $F_{1,68} = 42.70$, $P < 0.001$, with no significant effect of box within plant type). However, survival rates of neonate larvae feeding on sticky leaves were not significantly different from those on velvety leaves (Table 1; $t_4 = 1.99$, $P = 0.12$).

Long-Term Effects on Development. Larvae of both sexes developed significantly slower when reared on sticky leaves [mixed ANOVA, sex effect $F_{1,42}$

TABLE 1. AVERAGE SURVIVAL, WEIGHTS (SE), AND NUMBER OF *Manduca sexta* LARVAE PER INSTAR AFTER SIX DAYS ON NONGLANDULAR OR GLANDULAR *Datura wrightii* LEAVES

Plant type	Survival (%)	Larval weight (g)	First instars	Second instars	Third instars
Nonglandular	84.5 (2.3)	47.0 (2.61)	0	6	32
Glandular	80.0 (0.0)	24.6 (2.12)	2	32	2

= 2.93, $P = 0.0942$ (NS); type effect $F_{1,8} = 8.61$, $P = 0.0189$]. Female *M. sexta* reached greater pupal weights than males, but the absence of a significant sex × type interaction indicated that both sexes responded similarly to plant type. On average, larvae on sticky leaves took 22.2 ± 0.4 days to reach the prepupal stage, which is 28% longer than larvae on velvety leaves (17.3 ± 0.5 days). Plant type, however, did not have a significant effect on pupal weight. As before, larvae reared on velvety and sticky leaves had comparable survival rates (48.7 ± 5.6% on sticky vs. 49.3 ± 9.1% on velvety; $t_8 = 0.145$, $P = 0.89$). For both developmental time and pupal weight there was a significant effect of box within type (mixed ANOVA, $F_{8,42} > 4.0$, $P < 0.001$ for both variables). Because the boxes each contained leaves of a different plant, this may indicate variation in the nutritional value of individual plants within type.

Consumption Rates. In the first experiment, neonate larvae on velvety leaf discs consumed significantly more leaf area (63.6 ± 18.2 mm^2) than those on sticky leaf discs (4.7 ± 1.5 mm^2; $t_{18} = 3.066$, $P = 0.0067$). In the second experiment, the larvae fed significantly more on those leaf discs whose exudate was removed (16.2 ± 2.3 mm^2 on washed discs vs. 6.0 mm^2 on unwashed discs; paired t test, $t_8 = 3.38$, $P = 0.008$), indicating that the exudate is at least partly responsible for the lower feeding rates on sticky plants. In neither experiment were survival rates of neonate larvae on leaves with and without exudate significantly different (G test on survival classes, $G_2 < 3$ and $P > 0.05$ in both cases).

Oviposition Choice. There was no significant difference in egg distribution over velvety (24 with eggs, 43 without) and sticky (15 with, 17 without) plants in the UCR population (G test: $G_1 = 1.09$, $P = 0.29$). In the cage experiments, there was also no significant difference in the numbers of eggs per leaf between the two plant types (0.17 ± 0.06 eggs per leaf on velvety vs. 0.16 ± 0.2 on sticky in 1995, and 0.85 ± 0.18 on velvety and 0.64 ± 0.8 on sticky in 1996, respectively; $F_{1,23} = 0.07$, $P = 0.80$). We found a significant difference in numbers of eggs between the two experiments ($F_{1,48} = 312.27$, $P < 0.001$) and between test days within the experiments ($F_{22,48} = 11.29$, $P < 0.001$), which is probably due to variable numbers of female moths available in the colony. The absence of a significant interaction between plant type and experiment, however, indicates that the moths were consistent in their lack of preference.

Identification of Active Compounds

Differences in Nutritional Value of Plants. None of the internal leaf characteristics differed significantly between the two types of *D. wrightii* (Table 2). This reduces the probability that differences in growth rates of the larvae are caused by differences between the phenotypes other than trichome morphology.

TABLE 2. CONSTITUENTS OF LEAVES OF GLANDULAR AND NONGLANDULAR *Datura wrightii* PLANTS GROWN AT UCR IN 1995[a]

	Nonglandular plants	Glandular plants
Water (%)	86.9 (0.1)	85.7 (1.5)
Nitrogen (% DW)	4.02 (0.11)	4.31 (0.14)
Soluble sugars (% DW)	6.58 (0.37)	6.97 (0.66)
Total alkaloids (mg/g DW)	4.79 (1.11)	6.12 (0.88)
Total phenolics (mg/g DW)	27.01 (1.49)	26.18 (1.83)

[a]None of the plant constituents was significantly different between types (t test, $P > 0.1$ for all tests). Values in parentheses are standard errors.

These results, together with the lower consumption rates on sticky leaves, indicate that the glandular trichomes, and more specifically the exudate, are responsible for the adverse effects on the developmental rate of *M. sexta* larvae reared on sticky leaves. Therefore, we isolated exudate from sticky leaves to test its biological activity in *M. sexta* larvae and to identify the chemical compounds responsible for the activity.

Volatile Toxicity of Sticky Exudate Extract. Larvae confined to Petri dishes with surface extract had exactly the same survival rates as larvae in the control treatment (93.5 ± 6.5%), although the concentration of the *D. wrightii* extract was over 50 times the LD_{50} reported for crude extract from tomato leaves (44.2 $\mu g/cm^2$) (Williams et al., 1980). This indicates that trichomes of *D. wrightii* do not produce volatiles, e.g., 2-tridecanone, in sufficient quantities to be lethal to first-instar *M. sexta* larvae. Therefore, we pursued extraction and detection procedures for biologically active, nonvolatile compounds of type VI trichomes. We focused on sugar esters, because they are known for their antiherbivore activity (Buta et al., 1993; Liedl et al., 1995) and have been extracted from a closely related plant species, *D. metel* (King and Calhoun, 1988).

Biological Activity and Chemical Composition of Type IV Trichome Exudate. After eight days, most larvae on the diet with crude extract of sticky *D. wrightii* plants (CS) were still in their first instar and thus developed significantly slower than larvae on the diet with velvety leaf (CV) extract, which were mostly second and third instars ($G_2 = 8.89$, $P = 0.0117$). TLC analysis of the crude extracts revealed that the CS extract showed two major charred spots ($R_f = 0.48$ and 0.69) in the R_f region typical of SE (King and Calhoun, 1988), while the CV extract showed no charred spots at all. Analysis of hydrolyzed aliquots of the extracts indicated that the sugar moiety of the SE in the CS extract consisted of glucose. In the hydrolyzed CV extract, no monosaccharides

were detected on TLC, which is consistent with the above finding that there are no SE present on the leaf surfaces of velvety plants. These results suggested that the presence of SE in the CS extract plays a role in the difference in development rates of *M. sexta* larvae on diet with CS vs. diet with CV. Therefore, only the CS extract was subjected to further analysis.

The purified extract (AFSE) had a significant deleterious effect on the development of *M. sexta* larvae ($G_2 = 6.84$, $P = 0.0327$). After seven days, the instar distribution of larvae on AFSE diet (six second and eight third instars) was not significantly different from that on CS extract (six second and six third instars; $G_1 = 0.133$, $P = 0.715$), while larvae on CV (one second and 12 third instars) developed significantly faster than on either CS ($G_1 = 5.96$, $P = 0.014$) or AFSE diet ($G_1 = 4.73$, $P = 0.029$). The differences in larval survival (87–93%) were small and did not indicate a toxic effect of SE.

To identify the acyl side chains of the SE, the AFSE extract was saponified. GC-MS analysis showed that hexanoic acid was the most abundant residue (57%), followed by heptanoic (19%), nonanoic (13%), and octanoic (9%), acid residues. Pentanoic acid was only a minor compound (2%). GC-MS analysis of the derivatized AFSE fraction showed that the extract contained at least 13 peaks that contained SE (Figure 2). Although mass spectra of sugar esters cannot

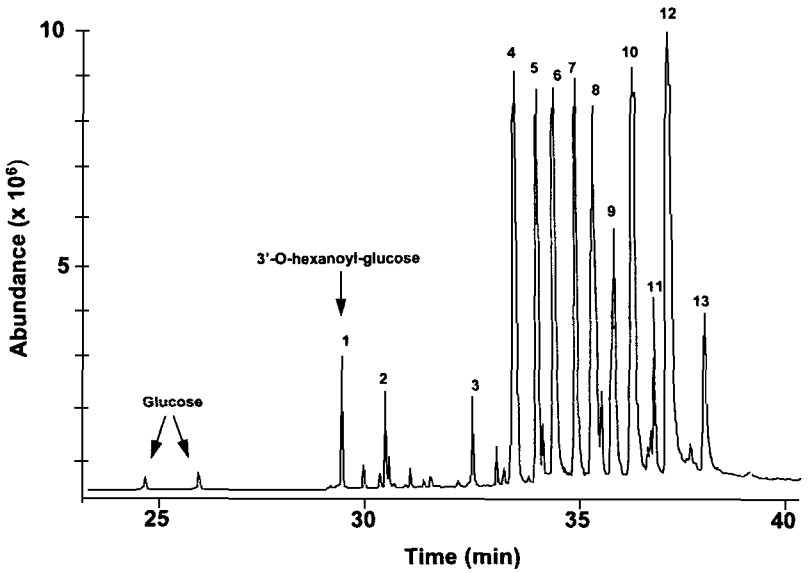

FIG. 2. GC trace of trimethylsylil-derivatized alkaloid-free sugar ester (AFSE) extract from glandular *Datura wrightii* plants.

reveal the exact stereochemistry, MS data can adequately determine the position and the size of the acyl groups (Patouraux-Promé and Promé, 1984; Severson et al., 1994). Detailed analysis of the mass spectra revealed that peaks 1 and 2 were monohexanoyl esters (MW = 566), while the mass spectra of peaks 3–7 all showed signals for hexanoyl (*m/z* 99) or heptanoyl (*m/z* 113) side chains. Peaks 8 and 9 were the only two peaks whose mass spectra contained strong signals for octanoyl (*m/z* 127) side chains in addition to hexanoyl signals. Peaks 10–13 all showed a strong *m/z* 141, indicating the presence of nonanoyl side chains, in addition to hexanoyl or heptanoyl signals. The presence of a strong *m/z* 273 ion (ion 14 in Patouraux-Promé and Promé, 1984) indicated that the nonanoyl side chain in peaks 10–13 probably was substituted at the 6′ position. Unfortunately, the parent ion for TMS derivatives of SE is generally not observed (Severson et al., 1994).

Additional evidence for the presence of SE in the AFSE extract of *D. wrightii* was provided by comparison with synthetic SE. Comparison of retention times and mass spectra with the GC-MS data of synthetic hexanoyl and heptanoyl glucose esters revealed that peak 1 in the AFSE extract had exactly the same retention time (29.548 min), molecular weight (MW = 566), and mass spectrum (Figure 3) as one of the monoacyl esters in the hexanoyl–glucose ester mixture. The presence of a strong *m/z* 243 signal (Figure 3) (F1 ion, Patouraux-Promé and Promé, 1984) indicated that the glucose was acylated at either the 2′ or 3′ position. However, in the case of a 2′ acylated monohexanoyl SE, we would have expected a strong *m/z* 316 signal (ion 13, Patouraux-Promé and Promé, 1984). Because the *m/z* 316 signal was completely absent from the MS, we identified peak 1 as 3′-*O*-hexanoyl glucose, derivatized with four trimethylsilyl groups (Figure 3). The position of the hexanoyl group was further confirmed by the low *m/z* 218/217 rate, which excludes 4′-acylation (Patouraux-Promé and Promé, 1984), and the presence of a strong *m/z* 132 signal (Figure 3), which is absent in MS from 6′-acylated SE (Patouraux-Promé and Promé, 1984).

We conclude that the complex mixture of SE in the exudate produced by type IV glandular trichomes on *D. wrightii* is responsible for the differences in performance of *M. sexta* on sticky and velvety leaves. The mixture is composed of glucose esterified with several combinations of C_6–C_9 acids.

DISCUSSION

Exudate of sticky *D. wrightii* phenotypes contains a mixture of sugar esters that significantly reduces the developmental rate of *M. sexta* larvae, one of its naturally occurring herbivores. The chemical structures of the SE from *D. wrightii* are similar to those of SE in the exudate of the closely related species

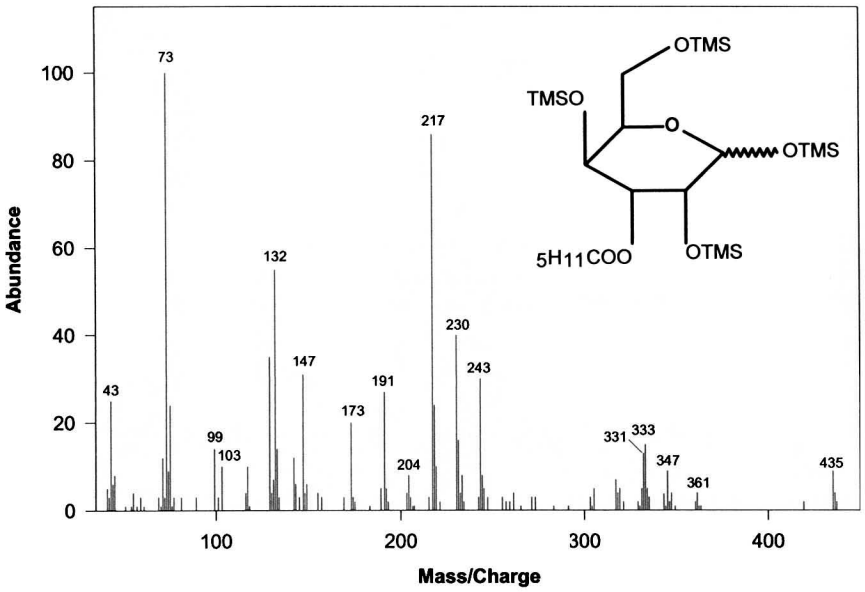

FIG. 3. Mass spectrum and molecular formula of 3′-*O*-hexanoyl glucose derivatized with TMS.

D. metel (L.) (King and Calhoun, 1988). In both species, the SE consist of a glucose, substituted with straight chain acid groups. This is unique in comparison with SE from other Solanaceae, which generally produce SE with branched acid side chains (King et al., 1988; Walters and Steffens, 1990; Severson et al., 1994).

The effects of SE in *D. wrightii* on *M. sexta* are similar to those described for SE from wild tomato species. Both *H. zea* and *S. exigua* larvae developed significantly slower on diets with SE, while their survival was not affected. Unlike *M. sexta*, their pupal weights were lower when fed on diets with SE (Juvik et al., 1994). Although we showed that trichome IV exudate alone significantly affects *M. sexta* growth, we cannot exclude that other factors, such as size differences between glandular and nonglandular trichomes, play an additional role. The presence of type VI glandular trichomes with PPOs and phenolics, which are present in low densities on both phenotypes of *D. wrightii*, may have a synergistic effect on type IV trichome-based resistance on sticky plants (Duffey, 1986; Neal et al., 1990).

Exudates with SE can act both as a physical and a chemical barrier to insect herbivores (Duffey, 1986). The exudate may reduce feeding activity by entrapping the insects or gumming the mouth parts, or it may act as an antifeedant

(Juvik et al., 1994; Duffey, 1986). When the insects do feed, the SE in the exudate may also negatively affect the metabolism of the insect, resulting in lower growth rates (Appel and Martin, 1992). Physical entrapment of *M. sexta* larvae does not seem to be the main mode of action of *D. wrightii* exudate, because the effects of SE on growth rates were preserved after incorporation in artificial diet. This, together with the reduced feeding rates on leaves with exudate, suggest that the SE in *D. wrightii* act as antifeedants and possibly also as metabolic toxins. This combination of biological activities has also been proposed as the mechanism behind the effects for orally ingested nicotine and other secondary metabolites on third instars of *M. sexta* (Appel and Martin, 1992).

Female moths did not choose to oviposit more frequently on the phenotype that maximizes the developmental rate of their offspring. It is not uncommon to find a poor relationship between oviposition preference and offspring performance in Lepidoptera (Thompson, 1988). For example, *H. zea* oviposition was even positively correlated with SE presence, despite a significantly lower larval performance on diets and leaves with SE (Juvik et al., 1994). As for many other moths (Juvik et al., 1994), oviposition is a crucial step that greatly determines the fitness of *M. sexta*, because the larvae normally do not leave the plant on which they hatch (McFadden, 1968). This raises the question why *M. sexta* moths do not oviposit more frequently on velvety plants than on sticky plants.

It has been argued that moths oviposit on suboptimal hosts because they provide enemy-free space (Thompson, 1988; Juvik et al., 1994; Berdegue et al., 1996). Glandular trichomes and their exudate have indeed been reported to reduce parasitization rates of *M. sexta* eggs on tobacco (Rabb and Bradley, 1968). However, the overall parasitization rates for *M. sexta* eggs and larvae in southern California are low (0–14.1%) (Oatman et al., 1983; N. M. van Dam and J. D. Hare, personal observations for seasons 1994–1997), which makes it unlikely that the search for enemy-free space motivates oviposition choices of *M. sexta* on *D. wrightii* phenotypes.

The results of our experiments indicate that, overall, sticky phenotypes are more resistant to *M. sexta*. The larvae of *M. sexta* are voracious herbivores that consume large amounts of leaves to complete their life cycle (Madden and Chamberlin, 1945) and occasionally completely defoliate *D. wrightii* plants (J. D. Hare, personal observation). The production of exudate by glandular trichomes significantly reduces the consumption and developmental rates of neonate larvae on sticky leaves. However, larvae on both phenotypes eventually attain similar pupal weights, which suggests that they ingest similar amounts of leaf biomass. Total herbivory on sticky plants may still be reduced if the longer developmental time on these phenotypes leads to a reduction of *M. sexta* numbers over time. The slow developmental rate may not only lead to a slower population growth within one season, but also increase the probability that the

larvae are exposed to poor climactic conditions at the end of their growth season (Juvik et al., 1994). Because *D. wrightii* is a perennial plant, long-term effects on herbivore numbers can indeed result in lifetime benefit gains for sticky phenotypes.

The net fitness costs or benefits for sticky and velvety phenotypes will not only depend on *M. sexta* resistance, but also on other factors influencing selection for trichome type. Glandular trichome production and exudate may be costly compared to the production of nonglandular trichomes (N. M. van Dam and J. D. Hare, unpublished data). Allocation costs (sensu Simms, 1992) may occur when limited resources are shunted into the production of defenses rather than into the primary metabolism. The production of SE in tomato not only requires glucose molecules, but also amino acids for the synthesis of the acid side branches (Walters and Steffens, 1990). Moreover, the production of SE solution by type IV glandular trichomes may be costly if it diverts a portion of the water resources of sticky plants, especially in the arid natural environment of *D. wrightii* (Lauter and Munns, 1986).

Additionally, there may be ecological costs (Simms, 1992) involved with glandular trichome production when, for example, resistance against one herbivore leads to susceptibility for another. In the wild, *D. wrightii* is attacked by a suite of herbivores of different orders, among which are whiteflies (Homoptera: Aleyrodidae) and *Tupiocoris notatus* (Heteroptera: Miridae). The same exudate that makes sticky plants less suitable for *M. sexta* and whitefly also renders them more attractive to *T. notatus* (van Dam and Hare, 1998). Hence, it is not yet possible to predict, on the basis of the interaction with one herbivore alone, which of the two phenotypes is the most fit. The quantification of the costs and benefits involved with glandular trichome and exudate production are the subject of our current studies.

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THE EFFECT OF LIGNIN AND BARK WOUNDING ON SUSCEPTIBILITY OF SPRUCE TREES TO *Dendroctonus micans*

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Abstract—The effect of lignified stone cell masses (lignin) and mechanical wounding of bark on gallery formation and oviposition by the spruce bark beetle, *Dendroctonus micans*, was determined in plantations of Norway and Sitka spruce. When beetles were implanted onto trees that varied in bark lignin concentration, there was a significant negative relationship between lignin and adult gallery size. Only a few larval galleries were established, all of them on trees with a low lignin concentration. Results confirm the importance of lignin as a preformed defence in living trees. Adults excavated significantly larger galleries in wounded than unwounded bark. Most larval galleries were also established in wounded bark. The concentrations of nitrogen, carbohydrate, and resin and the moisture content of wounded and unwounded bark were measured at the beginning of the experiment. A number of significant changes were induced by wounding, including an increase in the concentration of nitrogen and starch, and decreases in the moisture content and the concentration of free sugars. There was no overall effect of wounding on resin content of bark, although concentrations were significantly lower in new than old wounds. An increase in the nutritional quality of bark following wounding appears to be the main factor influencing attacks on wounded trees by *D. micans*.

Key Words—Spruce, lignin, nutrients, starch, defense, resin.

INTRODUCTION

The spruce bark beetle, *Dendroctonus micans* (Kug.) (Coleoptera: Scolytidae), in contrast to most other economically important bark beetles (Coulson, 1979; Horntvedt et al., 1983; Raffa and Berryman, 1983), is not dependent on adult

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mass attack and subsequent tree death for successful development of its larvae (Grégoire, 1988). This solitary species infests individual trees within a forest stand without necessarily killing them, at least in the short term. The factors that influence the distribution of attack within the stand as well as the fate of attacked trees are of considerable significance in the management of this primary pest.

Host resistance plays a central role in the population dynamics of mass-attack bark beetles and can influence the distribution of attack within forests (Vité and Wood, 1961; Raffa and Berryman, 1987; Reid et al., 1967; Berryman, 1969; Christiansen and Horntvedt, 1983). In conifers, resin is one of the principal defenses against bark beetle attack, especially in pines (Vité and Wood, 1961; Hodges et al., 1979; Hain et al., 1985; Raffa and Berryman, 1987). In spruce, resin flow is generally less copious than in pines (Christiansen and Horntvedt, 1983), and *D. micans* appears to have a relatively high tolerance of the resin and its constituent monoterpenes (Everaerts et al., 1988). Lignified stone cell masses, which are abundant in the bark of some spruce trees but absent in pines, appear, from the results of laboratory experiments, to be an important preformed physical defense against both adults and larvae of *D. micans* (Wainhouse et al., 1990), although their effects have not been directly tested on living trees.

The distribution of attack by *D. micans* within stands may also be affected by the occurrence of mechanical damage to bark caused, for example, by falling trees or timber extraction (Chararas, 1960; Evans et al., 1984). The association between bark wounding and attack by *D. micans* could simply reflect the attraction of beetles to monoterpenes in spruce resin exuding from the wounds (Vasechko, 1978; Wainhouse et al., 1992). On the other hand, wounding may induce changes in bark that favor oviposition and larval survival and development.

In this paper, we report the results of experiments on Norway and Sitka spruce to determine the effect of preformed lignified stone cell masses on adult gallery formation and oviposition. We also report on changes in the nutritive and defensive status of bark induced by mechanical wounding.

METHODS AND MATERIALS

Trees were selected within adjacent compartments of Norway [*Picea abies* (L.) Kar.] and Sitka spruce [*P. sitchensis* (Bong.) Carr.] at the Long Mynd, Shropshire (British National Grid ref. SO 412 902), planted in 1950–1952. The trees were unwounded and free from attack by naturally occurring *D. micans*.

Origin of Adults and Implantation. Beetles were collected either from naturally attacked forest trees or from Norway spruce logs implanted with second

instars. Individual adults, weighing more than 29 mg and therefore assumed to be females (Robinson et al., 1984), were confined under a 15-mm-diameter plastic cap on an area of bark from which a 5-mm-diameter core (to the cambium) had been removed. Following implantation, the trunks were loosely enclosed in Tygan netting to prevent predation by woodpeckers (Picidae).

Lignified Stone Cell Masses. Eight trees, 16–27.5 cm in diameter at 1.3 m [diameter at breast height (dbh)], were selected in each compartment, four with a high and four with a low concentration of lignified stone cell masses (lignin) in bark, based on visual assessment (Wainhouse and Ashburner, 1996). Selection ensured a range of lignin concentrations among experimental trees. Beetles were implanted on trees in October 1989 at 75, 125, and 175 cm above ground. Three beetles were used at each height, spaced equally around the circumference from the north-facing side of the tree.

At assessment the following August, the occurrence of living or dead beetles was recorded and, after removing the outer bark, a tracing was made of the gallery area excavated by adults or larvae. This area did not include the bark affected by the dynamic wound response (Reid et al., 1967; Berryman, 1969; Raffa and Berryman, 1987), which had formed around most galleries by the time of assessment. Gallery area in this and the wounding experiment, was measured by a PC-based image analysis system. Two transverse sections of bark were removed at each height for a visual estimation of percentage lignin (Wainhouse and Ashburner, 1996).

Bark Wounding. For both Norway and Sitka spruce, 18 trees (15–28.3 cm dbh) with a low lignin concentration at bh were wounded on three occasions. Wounds made at different times were randomly assigned to one of three heights (centered at 90, 170, or 250 cm) and four cardinal points of the compass. Wounds were made by removing, to the cambium, 32 cores of bark 1.5 cm diam. and approximately 6 cm apart (Figure 1). This pattern was selected because it circumscribed an area of intact bark through which beetles could tunnel while remaining close to a wound.

Only two of the three wounds made on each tree were used in the experiment because of limited availability of beetles. The wounds used were made either 11 months or 8–10 days before the start of the experiment (September 26–October 10, 1989), at which time an adult was implanted into the center of each wounded area (Figure 1) with a control implantation at a corresponding position on the opposite, unwounded side of the tree. Gallery formation and oviposition were assessed in August 1990. Living or dead beetles were recorded and, after removing the outer bark, a tracing was obtained of the gallery area excavated by each adult or larvae and adult. Larvae were collected for later counting and instar determination.

At the time of beetle implantation, four 1.5-cm-diameter bark cores were taken from the wounded (Figure 1) and unwounded sides of the tree for deter-

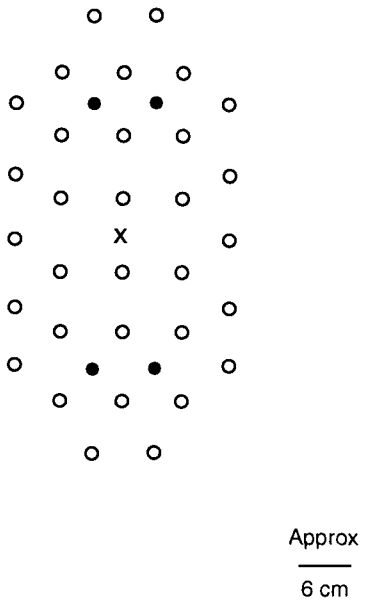

FIG. 1. Pattern of wounding with 1.5-cm-diam. cores (○) and position of samples for chemical analysis (●) and beetle implantation (×).

mination of nitrogen, carbohydrate, and resin concentration. The cores were from fresh bark not affected by the small necrotic area that formed around the circumference of each circular wound. Cores were placed on Dry Ice after sampling before storage at −55°C. Before processing, lichens and loose bark were brushed from the outer surface of cores and in the few samples, where a thick rhytidome was present, this was also removed. Fresh material was used for gravimetric determination of resin content as outlined in Wainhouse et al. (1997). Carbohydrate and nitrogen analysis was on pooled samples dried at 70°C to constant weight. Samples were ground in a rotor-speed mill (0.5-mm sieve perforations) prior to digestion in a sulfuric acid–hydrogen peroxide mixture (Wolf, 1982) to produce a clear colorless solution. Total nitrogen was determined colorimetrically as ammonia by the reaction with salicylate and dichloroisocyanurate using nitroprusside as catalyst. The method employs eight calibration standards and certified reference material was run in every batch of analyses. Total sugar, free sugar, and starch content were determined by the method outlined in Ward and Deans (1993). Moisture content was determined from fresh samples dried to constant weight at 70°C.

Statistical Analysis. Data were analyzed using SAS, Genstat, and CSS

statistical packages. Data on percent lignin and adult gallery size were analyzed by regression. For the wounding experiment, exclusion of some trees that suffered additional natural attack and the failure of some beetles to become established resulted in an unbalanced design and data were analyzed using the SAS "mixed" procedure. For each variate (gallery area and percentage nitrogen, starch, free sugars, total sugars, resin, and moisture content), an initial analysis was done using the "full" model of all factors (species, wound, and age of wound) and interactions and including possible covariates. Transformations were applied as appropriate based on plots of residuals to achieve approximately normal distributions. The final model used included only those factors, interactions, and covariates that were significant ($P < 0.05$) or near significant in the initial analysis. Percent lignin was used as a covariate in all analyses except for gallery area, starch, and resin, with adult weight a covariate only for the analysis of gallery area (Table 1).

RESULTS

Lignified Stone Cell Masses. In this and the bark wounding experiment 8–15% of beetles died without initiating galleries. Approximately 60% of beetles

TABLE 1. MEAN ADULT GALLERY SIZE AND BARK CHARACTERISTICS ESTIMATED BY MULTIVARIATE ANOVAR OF DATA FROM WOUNDING EXPERIMENT[a]

	Wound			Control		
	New	Old	P_1	(mean)	P_2	Covariate
ln gallery area (mm^2)	6.07	5.76	NS	5.62	*	adult weight (+)
Nitrogen (%)[b]	0.58	0.62	**	0.55	***	percent lignin (−)
Starch (%)	0.67	1.29	***	0.29	***	
Free sugars (%)	7.19	8.18	***	8.21	**	percent lignin (−)
Total sugars (%)	7.73	9.36	***	8.25	NS	percent lignin (−)
Resin (%)[c]	1.79	2.25	**	1.99	NS	
Moisture (%)[b]	54.0	52.0	***	54.5	***	percent lignin (−)

[a] Significance of difference between new and old wounds (P_1) and wounded (new + old) and control (P_2). Means are predicted from the model (see text) and adjusted for covariate where appropriate. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

[b] Significant species effects were found, figures presented are overall mean response to wounding and control. Species means are given in Table 3.

[c] Means are back-transformed from % resin$^{-0.85}$ used in the analysis (see text).

that established galleries were dead at assessment, with 40% missing, presumably through reemergence and dispersal. Beetle mortality was not related to lignin concentration, and there were no differences in mortality between the tree species. On Norway spruce, two living beetles were recovered. Mean adult gallery area (log(ln) transformed) was determined for each tree and used in a regression on tree mean percent lignin (Figure 2). Results for both Norway and Sitka spruce were similar with, overall, a significant negative relationship between gallery area and percent lignin ($R^2 = 0.30$, $P < 0.05$).

In a total of five galleries on two Norway spruce trees and a single gallery on Sitka spruce, oviposition resulted in larval establishment. On all three trees, lignin concentration was relatively low (Figure 2). The resulting larval galleries were not included in the regression analysis.

Bark Wounding. Within galleries, approximately 30% of beetles were dead and 5% alive at assessment, with the remaining beetles missing. On some trees, more than one adult gallery was found within a given wounded area or its equivalent on the unwounded side. Some of these galleries appeared to result from second attacks by the original implanted beetle and were therefore included in the total gallery area. On the unwounded side of two trees, beetles reemerged and initiated galleries close to "wounds" made by taking cores for chemical analysis. These galleries were excluded from the data analysis, as were two Sitka spruce trees that were attacked by naturally occurring beetles.

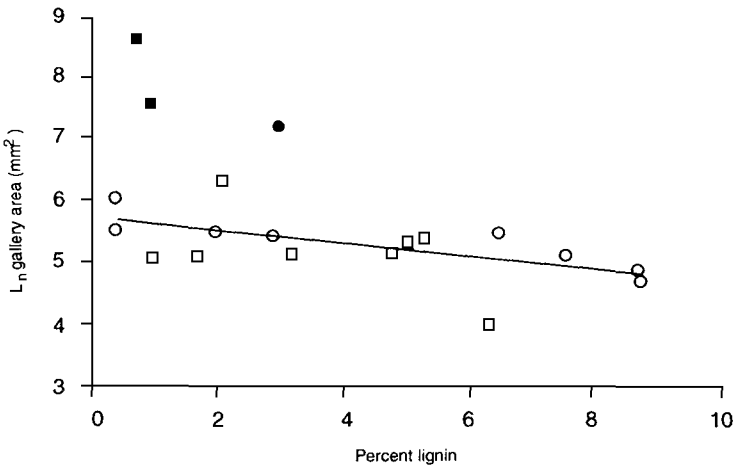

FIG. 2. Relationship between the mean size of galleries formed by adult female *D. micans* on Norway (□) and Sitka spruce (○) and percent lignin in bark, $y = 5.73 - 0.11x$. The mean size of larval galleries on Norway (■) and Sitka spruce (●) is also shown but was not included in the regression.

The main analysis was based on seven variates: log (ln) adult gallery size, percent resin$^{-0.85}$, percent nitrogen, percent moisture content, and percent total sugars, free sugars, and starch. For percent lignin, the power transformation was selected on the basis of a near linear normal probability plot. Mean values, adjusted for covariates, with appropriate significance levels for treatment effects were those predicted from a balanced model (Table 1). Adult galleries were significantly larger within wounded bark (new + old), but there was no significant effect of wound age on gallery size. Larvae became established on four Sitka and nine Norway spruce trees. Three of the larval galleries on Norway spruce contained dead larvae within resin-soaked frass. The remaining galleries contained living larvae whose developmental stage suggested that egg hatch and larval feeding had not commenced until the spring following implantation of the beetles in the previous autumn. There were too few larval galleries for inclusion in the main analysis but summary data, combining species and wound age, are shown in Table 2. These data are consistent with those from adult galleries, indicating a tendency for larvae to form galleries more readily within wounded bark and for more of them to survive than in unwounded bark.

Wounding significantly increased the percentage nitrogen and starch content of bark, with the highest concentrations on the old wounds (Table 1). The concentrations of total sugars and resin were also significantly higher in the old wounds, but there was no overall effect of wounding. The lowest concentration of free sugars was found on new wounds and the lowest moisture content on old wounds. Only two of the variables measured differed significantly between tree species. Norway spruce bark had a higher nitrogen content than that of Sitka spruce, whereas moisture content was higher in Sitka spruce bark (Table 3).

DISCUSSION

In both experiments, 30–40% of beetles died after some initial tunneling within the bark. Few beetles laid eggs and most appeared to redisperse, suggesting that some of the trees were relatively resistant to attack. This conclusion

TABLE 2. LARVAL GALLERIES DEVELOPING ON WOUNDED AND UNWOUNDED BARK[a]

	Wounded	Unwounded
No. of galleries	15	4
Larvae/gallery	123.8 ± 15.2 (12)	84.5 (2)

[a] Data combined for Norway and Sitka spruce and for new and old wounds (see text). Means based on (*N*) observations ± SE.

TABLE 3. SPECIES MEANS FOR PERCENT NITROGEN AND MOISTURE CONTENT

Species	Nitrogen (%)	P^a	Moisture (%)	P^a
Norway	0.60	<0.05	52.1	<0.001
Sitka	0.55		55.4	

[a] Significance of difference between species.

supports earlier observations of unattacked trees within heavily infested stands and the widespread occurrence of abortive attacks (Bevan and King, 1983). Part of that resistance can be attributed to the presence of lignin in bark, which can affect beetle tunneling and oviposition (Wainhouse et al., 1990). Variation in lignin concentration both between and within trees (Wainhouse and Ashburner, 1996; Wainhouse et al., 1997) seems likely to have a significant effect on the distribution and success of attack within spruce forests.

The effects of lignin were also evident in the wounding experiment. Although low lignin trees were selected in order to minimize effects on beetles, there remained sufficient variation in lignin content both within and between trees for it to be a significant covariate for several of the variables analyzed (Table 1). The negative correlation between lignin and both nitrogen and starch content probably reflects the reduced proportion of living tissue in bark with a high lignin content. This could enhance any direct toxic or antifeedant property of lignin, increasing its effectiveness as a defense against *D. micans*.

Wounding itself induced changes in adjacent intact bark that were long lasting and had positive effects on adult gallery size and larval establishment. Probably the most important change was the increase in concentration of both starch and nitrogen, which was higher in the old (11 months) than the new wounds (8–10 days). In previous studies, changes in the starch content of bark following removal of bark strips have been related to the degree to which the wounds prevented downward movement of carbohydrate from sources in the tree crown and upper bole (Miller and Berryman, 1986). The wounding pattern used in our experiments would form a partial barrier to phloem transport but starch content increased rather than decreased compared to control areas. This suggests that the changes were not a passive effect of isolation from the upper crown. The amount of starch in bark has been linked to defense against bark-invading organisms. While starch reserves may (Waring and Pitman, 1980, 1985; Larsson et al., 1983; Lorio and Hodges, 1985; Wright et al., 1979) or may not (Christiansen and Ericsson, 1986) provide a reliable index of resistance, they do at least indicate the potential to respond to infection. This arises because starch is metabolized during a defense reaction (Reid et al., 1967; Christiansen

and Ericsson, 1986), reflecting the often high energy requirements of secondary chemical production (Gershenzon, 1994). The changes caused by wounding in our experiments appear, therefore, to have been an active response that seems likely to have increased the potential for a strong induced defensive reaction. The increase in nitrogen concentration may be related to active repair processes following wounding of bark (Mullick, 1977; Biggs et al., 1984) and is likely to have enhanced the nutritional value of bark for beetles.

Wounding bark reduced the moisture content, as has also been reported for damaged foliage (Hartley and Lawton, 1987). Likely effects of changes in moisture content on *D. micans* are difficult to assess because beneficial effects on larval survival or development have been reported for both low (Berryman, 1972; Wagner et al., 1979; Webb and Franklin, 1978) and high bark moisture content (Storer and Speight, 1996). We would expect nitrogen concentration to be a major determinant of bark nutritional quality and, in our study, it was negatively related to moisture content of wounded bark. In Norway spruce, the preferred host of *D. micans*, nitrogen concentration was higher and moisture content lower than in Sitka spruce.

Changes in the resin content of bark induced by wounding were difficult to interpret in terms of defense. There appears to be an initial decrease in concentration shortly after wounding, but in the 11-month-old wounds concentrations were higher than in unwounded bark. *D. micans* has a high tolerance for resin (Everaerts et al., 1988) and we conclude that the major effect of wounding spruce bark is to increase the nutritive quality of surrounding bark for this bark beetle.

The effect of wounding spruce bark contrasts markedly with that reported for leaves from a number of mainly broad-leaved tree species where damage commonly results in an increase in defensive chemicals and a decrease in nutritive quality (Smith, 1988; Haukioja, 1990). The effects of wounding on bark may be an unavoidable consequence of the need to repair damaged tissue and to maintain an adequate reservoir of carbohydrates to fuel induced defensive responses.

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SEX PHEROMONE OF *Glossina tachinoides*: ISOLATION, IDENTIFICATION, AND SYNTHESIS

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Abstract—Study of lipids from male and female laboratory-reared flies led to the demonstration of a potent contact sex stimulant in extracts and cuticular hydrocarbons of the female tsetse fly *Glossina tachinoides* (Westwood) against conspecific males. Thin-layer and column chromatography indicated that extracts contained hydrocarbons and saponifiable lipids. Biological activity was found in the alkanes from females, including prominent branched-chain alkanes that were detected by gas chromatography (GC). The alkanes were separated and collected by preparative gas chromatography (GC), and only the 37-carbon region showed biological activity. GC-mass spectrometry showed the major peak contained a mixture of isomeric 11,23-, 13,25- plus a minor amount of 11,21-dimethylheptatriacontane. Two racemic isomers were synthesized, and bioassays showed that the greatest activity was possessed by the 11,23- isomer with somewhat less activity in 13,25-dimethyl heptatriacontane. Dose–response data showed ED_{50} at 5 μg per decoy with solvent-washed males, nonspecific females, or corks as decoys. These alkanes released sexual activity in males that comprised most of the behaviors released by a female fly of the same species.

Key Words—Tsetse fly, sex stimulant, pheromone, hydrocarbon, methylalkanes, gas chromatography, Diptera, biting fly.

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INTRODUCTION

Tsetse flies are a hazard to the health of humans and animals because they spread trypanosomiasis, also known as sleeping sickness, and *Glossina tachinoides* is an important vector of this disease in central and west Africa. Knowledge of the structure of the sex pheromone in this species may help in modern biocontrol efforts against this disease vector by ensuring that the correct strain of fly is used in large-scale areawide sterile male releases such as those sponsored by the International Atomic Energy Agency. The synthetic pheromone could also be used to increase the rate of reproduction in colonies and to test sexual behavior in reared males intended for mass release. Female tsetse flies of 26 species and subspecies synthesize long-chain methyl-branched alkanes (Carlson et al., 1993) and alkenes that are often species specific (Carlson and Langley, 1986).

Cuticular hydrocarbons from females may be utilized by conspecific males as sex stimulants and species recognition compounds for the release of sexual behavior. Cuticular hydrocarbon constituents possessed stimulatory activity for conspecific male *Glossina morsitans morsitans* Westwood (Langley et al., 1975). The natural trimethylalkane, 15,19,23-trimethylheptatriacontane, was synthesized and found to be a potent male copulatory stimulant, and less activity was found in 15,19- and 17,21-dimethylheptatriacontane (Carlson et al., 1978). Similarly, a sex stimulant pheromone that activated conspecific males was identified in female *Glossina pallidipes* Austen as a mixture of 11,21- and 13,23-dimethylpentatriacontane, in which both racemic synthetic dimethylpentatriacontanes showed biologically significant activity (Carlson et al., 1984). All three possible diastereoisomers of 13,23-dimethylpentatriacontane were synthesized (Kuwahara and Mori, 1983) and only the 13*R*,23*S* isomer released biological activity with laboratory males at 4.5 μg (ED_{50}), whereas the diastereoisomeric mixture required twice as much (McDowell et al., 1985). Males of *G. austeni* Newstead showed nearly obligatory sexual responses to synthetic 15,19-dimethyltritriacontane (Huyton et al., 1980). Surface extracts of frozen female *G. palpalis palpalis* Robineau-Desvoidy released 60–75% copulatory activity in conspecific males compared to 95% responses to frozen females (Offor et al., 1981). Failure to elicit a greater response at higher doses may have been due to habituation during successive tests on the same males with insufficient recovery periods. Some decoys did not present enough grasping surface for a fly to position itself and copulate since decoys were fastened to the bases of glass vials. Cork decoys provided the best configuration for response (Offor et al., 1981).

Extensive gas chromatography–mass spectrometry (GC-MS) studies of the metylalkanes of several species of tsetse of the Nemorhina (*palpalis*) group showed that the major peak in extracts from *G. tachinoides* females contained

mixtures of the isomers 11,23-, less 13,25-, and much less 11,21-dimethylheptatriacontane (Nelson et al., 1988). Later, numerous samples were analyzed from individual and pooled samples of laboratory and museum collections that gave consistent results in which females had a mixture of large amounts of these three compounds (17–38%, average 29%, ca. 8–15 μg/female), and males of unknown mated status had much less (undetectable to 0.5 μg/male). These quantitative results were described in a systematic comparison of methyl alkanes from 26 species and subspecies of *Glossina* (Carlson et al., 1993). We describe here the isolation by gas–liquid chromatography (GLC) of a fraction obtained from females of *G. tachinoides*, its biological activity, the identification of the major branched-chain alkanes, and dose–response bioassays of several synthetic racemic isomers.

METHODS AND MATERIALS

Biological Material. Female flies were obtained from colonies maintained at CNRS Orsay. Pooled samples of *G. palpalis gambiensis*, *G. morsitans submorsitans*, and *G. tachinoides* were washed with solvent to measure the amount of lipid present and to prepare conspecific extracts for tests conducted in France. The flies were obtained from colonies at Laboratoire de l'Institut d'Elevage et de Médecine Vétérinaire des pays Tropicaux, Maison-Alfort, France, and derived originally from Bobo-Dioulasso. Male *G. tachinoides* were obtained as adults from the colony at CNRS Orsay. After normal feeding procedures, they were used for bioassays at 3–5 days of age. In Austria, male *G. tachinoides* were obtained as adults from the colony at Seibersdorf. They were fed normally for 3–5 days and then held for bioassays without being fed for 1–3 days, since well-fed or starved flies (four or more days after a blood meal) did not respond as well in preliminary tests. Good responses were seen in males that were partially fed and tested on the following two days. Pregnant females from the colonies were thawed after being frozen for two to seven days and then solvent-washed for use as decoys. In Nigeria, wild *G. tachinoides* were collected at the Yankari Game Reserve. Some males were obtained from shipments of pupae from colonies at BICOT (Biological Control of Tsetse Unit, Department of Pest Control Services, Vom, Nigeria). The flies were allowed to emerge and were tested unfed at the laboratory in Kaduna, Nigeria, between 3 and 19 days of age.

Preparation and Analysis of Natural Compounds. Extracts of three species of Orsay colony flies were made with hexane solvent, and samples of hydrocarbons were obtained by column chromatography on silica gel after elution with hexane containing increasing proportions of ether. After removal of solvent, the samples were weighed for comparison of lipid classes. The hydrocarbons

that eluted with hexane were analyzed with a Girdel model 30 gas chromatograph fitted with a nonpolar column (25 m × 0.32 mm ID, 0.15 μm film, CpSil 5 CB, Chrompack, Amsterdam) and a flame ionization detector. Nitrogen was the carrier gas. The hydrocarbons from female *G. tachinoides* were separated by preparative GC on a nonpolar, packed preparative column (2 m, 10% OV-1) installed in the same Girdel instrument equipped with a column effluent splitter. Fifty female equivalents at a time were injected and collected as five fractions during temperature-programmed separations. Fraction I contained condensed hydrocarbons up to Kovats index (KI) (Ettre, 1964) 3000; II contained KI 3065; III contained KI 3235; IV contained KI 3363 to KI 3663; and V contained KI 3763 and 3775 (El Messoussi, 1988).

Electron ionization mass spectra of natural and synthetic compounds were obtained with a Hewlett-Packard 5988 mass spectrometer (MS) interfaced to an HP 5890 GC fitted with an OCI-3 on-column injector and a nonpolar column as above (DB-1, 30 m × 0.25 mm ID, 0.25 μm film, J&W). The carrier gas was helium with a pressure-regulated flow rate of 25 cm/sec. The GC oven was temperature programmed from 60 to 230°C at 20°C/min, and then to 330°C at 5°C/min. The transfer line was maintained at 315°C and the source at 200°C. Manual tuning of the MS to increase high-end ion intensity (m/z 200–500) was necessary in order to obtain satisfactory spectra.

Synthetic Chemicals. The pheromone candidate compounds 11,23-, 13,25-, and 11,21-dimethylheptatriacontane were synthesized (Matsuyama and Mori, 1994) and sent to Gainesville, Florida, where they were made up into small samples of 1.0–3.0 mg for shipment in glass vials without the use of solvent. Weighed quantities of synthetic compounds (liquid at room temperature) were dissolved in appropriate amounts of reagent-grade hexane, usually 1 ml, for treatment of decoys with serial dilutions of five concentrations by treating multiples of 5 μl with a 10-μl glass microsyringe or pipet (Finnpipette, Kemistien OY, Helsinki). Dried extracts were treated the same way by dissolving them in hexane.

Bioassays in France. Males were exposed between 10 AM and 5 PM in tests against frozen, unwashed versus hexane-washed mature females; frozen, unwashed versus washed mature males; aliquots of female and male cuticular extracts; and hydrocarbons from mature females and males that were obtained from open-column chromatography on silica gel. Also tested were preparative GC fractions I–V after collection of the separated hydrocarbons into glass tubes. The tubes were washed with hexane and the contents used for bioassays by treating dead solvent-washed males. Male behavior was described as a progression of stages: response level 1 (RL1) included mounting; RL2 included mounting and orientating to the copulatory position; and RL3 included RL1 and -2 with intensified responses of downward abdomen curving and hypopygium

extension that indicates extreme sexual activation, preparatory to aediagus eversion (Langley et al., 1987).

Bioassays in Austria. Each male was introduced into a 2- × 6-cm plastic tube and forced into three contacts with a pinned decoy over a 3-min test. Decoys were introduced through a hole in the paper bottom of the tube and held close (ca. 1–4 mm) to the bottom. The tube was thumped on the table to bring males into contact with the decoy 3× over a 30-min test. Decoys were hexane-washed corks (6 × 3 × 3 mm) with rounded edges, pregnant *G. tachinoides* females, or frozen hexane-washed *G. austeni*, *G. fuscipes fuscipes*, and *G. palpalis palpalis* pregnant females. The male behaviors were recorded as (−) for no response/escape response, RL1 (mounting and brief contact), RL2 (mounting, grasping, and orientation to the copulatory position), RL3 (RL2 with hypopygium extension), and RL4 (copulatory responses when the hypopygium extension lasted longer than 1 min). The highest score was recorded for each male for each test. Tests were run between 8:30 and 10 AM. In preliminary tests in Austria after 10 AM, nonresponding males were chased around inside the 50-ml plastic bioassay tubes with the test object on a long pin held by the investigator. During this time they would try to avoid contact, although upon contact they would usually respond instantly to treated decoys or to unwashed conspecific females.

Bioassays in Nigeria. Each male was introduced into a tube with a decoy and left for 1 hr exposure to allow comparison of activities of three types of hexane-washed decoys: cork (2 × 2 × 4 mm), pieces of heavy upholsterer's furnishing fabric (2 × 2 × 4 mm), and dead, unfed, solvent-washed males with clipped wings. Males responded poorly to treated fabric and males with clipped wings in preliminary tests. Fresh test males were used whenever possible on each series of treated decoys, but nonresponding males were transferred to another set of decoys and tested again because of the lack of test males.

RESULTS

Separation and Analysis of Hydrocarbons. Surface extracts from several species of flies were separated into fractions, and each fraction was weighed after removal of solvent, with the result that 5–17 μg of hydrocarbons were obtained per female, usually more than in conspecific males (Table 1). The major peak in *G. tachinoides* females comprised 30% of the total alkanes, or about 5 μg/female. Preparative GC collection for bioassays (Table 2) revealed that fraction V contained a major peak that eluted after KI 3750 on a packed nonpolar column, together with a minor peak that eluted slightly later at KI 3775 at the same time as synthetic 15,19,23-trimethylheptatriacontane (El Messoussi et al., 1994).

TABLE 1. WEIGHTS OF HYDROCARBONS AND OTHER LIPIDS OF TSETSE FLIES FROM POOLED SAMPLES

Samples	Files (*N*)	Sex (M/F)	Crude extract (mg)	Hydrocarbons (mg)	Total lipids (mg)	Total alkane (mg)	Alkanes/fly (μg)
G. p. palpalis	990	F	1253	158	1051	14.4	14.5
G. p. palpalis	1178	M	658	45	608	2.4	2.0
G. m. submorsitans	529	F	268	32	508	2.7	5.1
G. m. submorsitans	218	F	757	54	755	3.2	14.6
G. m. submorsitans	1000	M	2928	14	2722	10.9	10.9
G. tachinoides	441	F	138	13	115	7.5	17.0
G. tachinoides	852	M	2117	143	186	9.8	11.5

Analytical GC of hydrocarbons from Orsay *G. tachinoides* (El Messoussi, 1988) were consistent with analytical results from colony material from Bristol, Austria, and from numerous field locations (Nelson et al., 1988, Carlson et al., 1993). The GC-MS of hydrocarbons from males contained major proportions of terminal trimethyl- and 2-methyl alkanes, whereas only females contained internal dimethyl-branched alkanes with 11 methylenes between methyl branches in the majority of compounds. There was a minority of isomers with nine methylenes between branch points. Typical of females was the major peak containing 11,23- and 13,25-dimethylheptatriacontane in a 1.4:1 proportion, which coeluted with a small amount of 11,21-dimethylheptatriacontane (KI 3763, 29%) on a capillary column. These results were consistent with structures proposed by Nelson et al. (1988) for the same species. This peak was followed by a small, incompletely separated double peak. The first half of this peak to elute (KI 3775, total 3%) contained mostly 11,15,23-trimethylheptatriacontane (*m/z* 168/169, 224/225, 239, 351, 365, 421), and 11,15,25-trimethylheptatriacontane (*m/z* 168/169, 196/197, 239, 351, 393, 421) as a minor isomer. The last half (KI 3785) contained mostly 9,13,23-trimethylheptatriacontane (*m/z* 140/141, 211, 224/225, 365, 379, 449) in about half as much as the residual 11,15,23-isomer, and its trailing edge contained a small amount of 7,11,21-trimethylheptatriacontane (*m/z* 112/113, 183, 252/253, 337, 407, 477).

Bioassays in France with Natural Compounds. Mating attempts at RL2 and RL3 were observed consistently in *G. tachinoides* males exposed to live or dead mature *G. tachinoides* females (mean 48% RL2), but not to males or solvent-washed flies of either sex, in a large number of tests. A biologically active material was shown to be present in the epicuticular waxes of females that could be readily removed with hexane solvent, leaving no bioactive residue. Biological

TABLE 2. BIOASSAYS OF *G. tachinoides* MALES AGAINST NATURAL MATERIALS ON SOLVENT-WASHED MALE DECOYS IN FRANCE: PERCENT OF MALES RESPONDING AT RESPONSE LEVEL (RL) 2 OR 3

Materials tested	Tests (*N*)	Dose (FE)	Percent			% of potential score (*X*)[a]
			RL2	RL3	RL2+3	
Frozen, dead female	25	1	40	48	88	75 (2.2)
Frozen, dead male	22	1	0	0	0	0
Washed female	30	1	0	0	0	0
Washed male	30	1	0	0	0	0
Female extract	60	0.5	13[b]	1.3	14.3[b]	16 (0.6)
	30	0.75	17	17	34	28 (0.8)
	31	1	58	29	87	68 (2.0)
	30	2	50	30	80	63 (1.9)
	25	3	53	33	85	67 (2.0)
	30	5	40	47	87	73 (2.2)
	34	8	65	20	85	64 (1.9)
Preparative GC fraction V	23	1	17[c]	0	17[d]	11 (0.3)
	30[e]	5	50	40	90	60 (2.2)
	30[e]	5	50	23	73	57 (1.8)
	25	8	67	17	84	53 (1.7)

[a] Score for RL2 = mounting the decoy, orientating to the copulatory position and gripping the decoy; RL3 = hypopygium extension. *X* = no. of responders at RL2 (×2) plus no. of responders at RL3 (×3)/no. tests.
[b] Slope $P = 0.0001$ over all doses of this isomer in this column.
[c] Slope $P = 0.07$ over all doses of this isomer in this column.
[d] Slope $P = 0.0005$ over all doses of this isomer in this column.
[e] Combined both 5 F-tests: (117/60 = 1.9).

activity was present only in the extracts of females when tested with treated male decoys. Nearly the same level of activity was observed with five female equivalents (47% RL3) as with dead females (Table 2). In tests of the hydrocarbons separated by preparative GC, only fraction V showed activity. Dose responses of fraction V showed the highest activity (mean of 31% RL3) at 5 female equivalents (FE), somewhat less than the whole female extract. However, addition of RL2 + RL3 values showed a mean of 88% male response to unwashed females, 87% to 5 μg of female hydrocarbons, and 91% to fraction V.

Bioassays of Candidate Sex Pheromones in Austria. Mating attempts were released in males when exposed to treated nonspecific pregnant females in several tests (Table 3). To quantify responses for a dose–response curve, bioassays

TABLE 3. BIOASSAYS OF *G. tachinoides* MALES AGAINST 11,23- AND 13,25-DIMETHYLHEPTATRIACONTANE ON WASHED WINGLESS, LEGLESS FEMALE BODY DECOYS IN AUSTRIA: PERCENT OF MALES RESPONDING AT RESPONSE LEVEL 2, 3, OR 4[a]

Dimethylheptatriacontane isomer	Tests (*N*)	Decoy	Amount (μg)	Percent				% of potential score (*X*)
				RL2	RL3	RL2+3	RL4 (*t'* = min)	
11,23-	4	Ga	0.5	50	0	50[b]	0	33 (1.0)
	4	Ga	0.75	50	0	50	0	42 (1.2)
	2	Ga	1.0	0	0	0	0	0 (0)
	2	Ga	4	0	100	100	100 (25′)	100 (4.0)
	5	Gff, Ga	1.5	20	80	100	60 (70′)	93 (3.6)
	4	Gff	5	75	25	100	25 (1′)	75 (2.3)
	5	Gff	10	20	80	100	40 (99′)	93 (2.8)
	4	Gff	20	0	75	75	50 (10′)	83 (2.5)
13,25-	3	Ga	0.5	0[b]	33	33	0	33 (1.0)
	5	Ga	1	60	20	80	20[c]	60 (1.8)
	11	Gff	2	55	36	91	25[c]	73 (2.1)
	2	Ga	4	0	50	50	0	33 (2.0)
	6	Ga	5	17	50	67	50[c]	72 (2.3)
	5	maleGt	10	20	60	80	40 (5.5′)	73 (2.2)
	3	cork	20	67	0	67	0	44 (1.2)
	8	cork	40	75	25	100	25 (4′)	75 (2.3)
Mixture	4	Gff	0.65 ea	25	25	50	0	58 (1.8)
	2	Gff	1 + 0.65[d]	0	100	100	100 (51′)	100 (4.0)
	4	Gff	1 ea	50	25	75	0	67 (2.0)
	3	Ga	2 ea	33	33	66	1 (3′)	67 (2.0)
	3	Ga	4 ea	33	33	66	0	67 (2.0)

[a] Score for RL2 = mounting decoy; orienting to the copulatory position, gripping the decoy and curving the abdomen downward; RL3 = hypopygium extension: RL4 = extended copulatory effort with hypopygium extended for more than 0.5 min. Untreated controls invariably gave no response at any time (N = 25). Decoys are Ga = *G. austeni*, Gff = *G.f. fuscipes*, Gt = *G. tachinoides*, X = no. of RL2 (×2) plus no. of RL3 (×3)/N.

[b] Slope P = 0.10 over all doses of this isomer in this column.

[c] Hypopygium extended for short duration, or not timed.

[d] 1 μg of 11,23 isomer.

of 11,23- and 13,25-dimethylheptatriacontane isomers were scored as the highest of three contacts over a 3-min interval. Dose responses for the 11,23- isomer showed release of copulatory response including hypopygium extension (RL3) in three of four males at 10 μg, while two of two responded at 4 μg, and four of five responded at 1.5 μg. Little response was seen at less than 1 μg (1/5 FE). The scores reflect the trend of males to show less activity with smaller quantities. Dose responses for the 13,25- isomer showed the highest release of full copulatory response (RL3) in three of five males at 10 μg, with fewer responses to declining treatments, and little interest at 1 μg. The scores reflect a downward trend from 2.8 at 10 μg to 1.0 with dead female decoys in which fewer copulatory responses were observed until a threshhold level was seen at about 1 FE. The higher copulatory attempts and scores suggest that the 11,23- isomer is the more active isomer. Limited tests of mixtures suggest some synergism between isomers, with good activity at low doses. Full activity was observed in two tests with a mixture of 1 μg of the 11,23- and 0.65 μg of the 13,25-isomer, with intense interest and hypopygium flexing that extended for 6 min with one male and for 45 min with the other male tested.

Bioassays of Candidate Sex Pheromones in Nigeria. Mating attempts were released in males when they were exposed to treated cork decoys in several tests (Table 4). Mating attempts culminating with hypopygium flexing (RL3) were observed in 27% of tests at 35 μg and 36% at 2.3 μg of the 11,23- isomer. However, in tests with 19-day-old males, some full copulatory responses were observed at 1.2 and 2.3 μg. More than 50% of males exhibited an RL2 response without hypopygium extension with 1.2 μg, and up to a 75% response was observed at 5, 14, and 21 μg. However, with the 13,25- isomer, hypopygium responses were less frequent, although in the last tests with 19-day-old males, instant copulatory responses were observed to decoys treated with 1.4 and 5 μg. The highest RL2+3 score of 1.7 was obtained at 11 μg.

Dose–response data were subjected to probit analysis with PC SAS to determine the existence of a relationship where proportional response is expected. Data for RL2 and RL2+3 were tested. Any significant value for the slope indicates the presence of a dose–response relationship. Cases where the P values are less than 0.15 are indicated on the tables. Both natural female extracts and natural preparative GC fraction V showed significant dose–response relationships for RL2 and for RL2+3, the full copulatory response (Table 2). Bioassays conducted in Austria showed a significant dose–response for RL2+3 (slope $P = 0.10$) for 11,23-dimethylheptatriacontane, and for RL2 for the 13,25- isomer (slope $P = 0.10$), but not for the mixture (Table 3). Bioassays conducted in Nigeria showed significant dose–response relationships for RL2 (slope $P = 0.09$) and RL2+3 (slope $P = 0.15$) for 13,25-dimethylheptatriacontane and in RL2+3 (slope $P = 0.15$) for the 11,23- isomer (Table 4).

TABLE 4. BIOASSAYS OF *G. tachinoides* MALES AGAINST SYNTHETIC 11,23- AND 13,25-DIMETHYLHEPTATRIACONTANE ON CORK DECOYS IN NIGERIA; PERCENT OF MALES RESPONDING AT RL2 OR RL3[a]

Dimethylheptatriacontane isomer	Tests (*N*)	Amount (μg)	Percent			% of potential score (*X*)
			RL2	RL3	RL2+3	
11,23-	1	0.6	100	0	100[b]	67 (2.0)
	11	1.2	54	9	63	49 (1.5)
	11	2.3	54	36	90	72 (2.1)
	7	3.5	43	0	43	38 (1.3)
	4	5	75	0	75	58 (1.8)
	16	7	38	6	44	40 (1.2)
	11	14	73	9	82	61 (2.2)
	11	21	73	9	82	61 (2.2)
	11	28	54	9	63	52 (1.9)
	11	35	64	27	91	70 (2.5)
13,25-	15	0.7	0[c]	0	0[d]	0
	16	1.4	31	6	37	29 (0.9)
	16	2.7	63	0	63	44 (1.3)
	10	5.5	30	10	40	33 (1.0)
	9	11	22	11	33	26 (1.7)
	5	16.5	40	0	4	27 (0.8)
	5	22	60	0	60	40 (1.2)
	5	27.5	60	0	60	40 (1.2)

[a]Score for RL2 = mounting decoy, orientating to the copulatory position, gripping the decoy and curving the abdomen downward; RL3 = hypopygium extension. Untreated controls invariably gave no response at any time ($N = 50$). X = no. of RL2 (×2) plus no. of RL3 (×3)/N.
[b]Dose vs. response: slope $P = 0.15$ over all doses of this isomer in this column.
[c]Dose vs. response: slope $P = 0.09$ over all doses of this isomer in this column.
[d]Dose vs. response: slope $P = 0.11$ over all doses of this isomer in this column.

DISCUSSION

Male copulatory behavior was investigated in France in *G. tachinoides* by comparing Orsay colony males with those of three other tsetse species, *G. m. submorsitans*, *G. palpalis gambiensis*, and *G. f. fuscipes* in bioassays against frozen conspecific and nonspecific females. The results were consistent with those of Huyton et al. (1980), in which bioassays showed that the effect was independent of age and physiological status of females, and thus, was based largely on pheromonal stimulation rather than movement or sound. These preliminary assays showed that contact sex pheromones released conspecific behavior somewhat similarly in all five species. Presentation to males of natural sex pheromone in the form of an epicuticular lipid extract in the absence of a three-

dimensional object produced inconsistent results in previous studies (El Messoussi, 1988). The present bioassays of treated decoys with natural *G. tachinoides* extracts and fractions were consistent with previous findings in *G. morsitans*. Chemoreceptors responsive to female sex pheromone are situated on the tarsi of sexually responding males and are the first structures to touch a female fly (Langley et al., 1987).

Our recent GC-MS results were consistent with mass spectra of individuals, with pooled samples of this species that showed consistent presence of these isomers, regardless of origin of the flies. (Nelson and Carlson, 1986, Carlson et al., 1993), and with all three synthesized isomers. The later-eluting, previously unidentified, trimethylheptatriacontanes contained one dominant isomer with an interruption of three and seven methylenes, although smaller amounts of three isomers with interruptions of three and nine were found. Preparative GC collections of the natural major dimethyl alkanes would inevitably contain small amounts of these minor trimethyl isomers. Although we have no way of assessing their contribution to biological activity, we assume that their effect was minor. The major trimethyl isomer could be constructed by adding one methyl group to 11,23-dimethylheptatriacontane, which is apparently the main contributor to sex stimulant activity.

Shortly after the synthetic sex pheromone candidates became available, a limited opportunity to obtain bioassay information became possible in Nigeria and subsequently in Austria. To systematize the bioassays, a scoring system, originally developed in Nigeria, was used in which the highest-scoring of three contacts per 3-min test were recorded when each behavior above RL1 was observed (Offor et al., 1981). We feel that this scoring is consistent with the original tests of this species conducted previously in France.

Tests of males in Austria showed that cork decoys were not stimulatory at high doses, nor were dead starved males with shrunken abdomens. Therefore, pregnant *G. fuscipes fuscipes*, *G. austeni*, or *G. p. palpalis* females were hexane-washed for use as decoys. Males often would continue copulatory attempts with large bioactive decoys for 3–10 min and sometimes 60 min, even after the cover tube was removed. Sibling males 5 days old were fed and held for two days before use or partially fed under normal laboratory blood feeding procedures for good responses the next day. In Nigeria, males were used repeatedly for several days, and about the same order of response was seen for at least a week, despite the lack of feeding after capture. It was surprising that some sexual responses were released in weakened, unfed males that had been starved for 19 days and were near death.

Bioassays of synthetic compounds in Nigeria and Austria with laboratory-reared males gave similar results, i.e., more responses to the 11,23- than to the 13,25-isomer. No highly significant dose–response relationships were found, but in the region of 0.5–2 FE (2.5 and 10 μg, respectively) of synthetic com-

pounds or mixture of compounds, the results were nearly as positive as for killed females. For example, in Austria, full responses were obtained from each of two males tested at 4 μg, which included lengthy copulatory attempts that could last for an hour. These responses were indistinguishable from activity with a conspecific dead female. We believe that such responses indicate that the activity of these compounds duplicates the responses observed with dead natural *G. tachinoides* females. The observation of declining activity at high treatments was not unusual with males of other species, suggesting habituation or that the decoys become "too greasy" at more than 5 FE from addition of liquid hydrocarbon (Langley, unpublished observations). The effect of testing these synthetic compounds as racemic mixtures, rather than as single enantiomers is also unknown. Synergism between compounds is possible, as shown when good activity was obtained in limited tests of mixtures of both compounds at low treatments. We do not expect, however, that other substances could be found that would dramatically increase the activity of these sex stimulants.

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INFLUENCE OF SOME FATTY ACIDS ON OVIPOSITION BY THE BRUCHID BEETLE, *Callosobruchus maculatus*

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Abstract—The cowpea seed beetle, *Callosobruchus maculatus*, will lay its eggs on many potential hosts and inert surfaces. Oviposition on glass beads is stimulated by coating them with individual fatty acids. Nevertheless, female beetles reject mung seeds less frequently than beads treated with either an extract of mung seeds or, especially, an extract of mung seeds plus oleic acid. The addition of oleic acid to the extract resulted in a change in the sequence of oviposition behavior, notably an increase in a raised body position indicative of hosts of low acceptability. Fatty acids are present in the epicuticular waxes of legume seeds; wax extract of mung bean contains 32.4% fatty acid and 14 alkanes, whereas a wax extract of chickpea contains 5% fatty acid and 18 alkanes. Thus, chickpea may be a less acceptable host for oviposition than mung bean because of physical differences and/or because of chemical differences, including a reduced total level of fatty acid or the high proportion of oleic acid it is reported to contain. It is concluded that an appropriate mixture of fatty acids in the epicuticular waxes stimulates oviposition but that an elevated level of oleic acid in conjunction with others is deterrent.

Key Words—*Callosobruchus maculatus*, oviposition behavior, host acceptance, behavior sequences, epicuticular lipids, waxes, fatty acids, oleic acid, Coleoptera, Bruchidae.

INTRODUCTION

Callosobruchus maculatus (F.) (Coleoptera: Bruchidae), the cowpea seed beetle is a pest of stored pulses (Dobie, 1981). Under storage conditions, ovipositing

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beetles select seeds from the same or different species (Birch et al., 1989). The role of seed size, shape, and chemistry in this selection behavior is not fully understood (Thanthianga and Mitchell, 1990; Parr et al., 1996, 1998).

Fatty acids are a common component of pulse cotyledons, although the relative mix of fatty acids in seed oils varies (Kay, 1979). They are also thought to occur in the epicuticular waxes of legume seeds (Gunn, 1981) and could be important mediators of host acceptance by bruchid beetles (Birch et al., 1989). Acetone extracts of seed waxes have been shown to stimulate oviposition in *C. maculatus* (Credland and Wright, 1988). However, some seed oils and solutions of fatty acids have also been reported to deter oviposition in bruchids (Don-Pedro, 1990; Hill and Schoonhoven, 1981).

The presence of a bruchid egg on a seed increases the probability that a female bruchid will reject that seed (Parr et al., 1996). This rejection may be partially explained by the perception of the chemical marker, known as the oviposition deterrent pheromone (ODP), that *C. maculatus* deposits when ovipositing (Credland and Wright, 1990). The chemical composition of the ODP of *C. maculatus* has been investigated by Sakai et al. (1986); they showed it contains about 30% v/v oleic acid, the fatty acid thought to be partly responsible for the ODP's deterrent effects. Oleic acid has also been found in the ODPs of other insects, for example, it is being considered as a protectant to stop *Lobesia botrana* ovipositing on grape vines (Gabel and Thiéry, 1996).

In this paper we investigate whether the proportions of fatty acids in acetone extracts from the epicuticular wax of two species of legume (mung bean *Vigna radiata* L and chickpea *Cicer arietinum* L.) are different, whether individual fatty acids have differential effects on oviposition and, in particular, whether oleic acid has a deterrent effect on oviposition by *C. maculatus*. With this information, it may be possible to associate differences in the previously established acceptability of chickpea and mung bean with differences in the fatty acid composition of their epicuticular waxes.

METHODS AND MATERIALS

Insects. The *C. maculatus* biotype Campinas was collected in Brazil in 1975. Since that time it has been cultured on cowpeas (Credland, 1987). Experimental insects were reared at a density of one larva per seed, isolated, and mated using the method of Parr et al. (1996). This provided a ready supply of newly emerged and standardized females.

Plant Material. Material was obtained from local suppliers of mung beans (a single but unknown commercial cultivar) and chickpeas (a single unknown kabuli type cultivar). Seeds were inspected and those with damaged testae were excluded.

Extraction and Analysis of Seed Surface Compounds. Volumes of seeds

providing a comparable surface area, based upon the approximation that both legume seeds are spherical, were used to produce extracts of seed surface waxes. First 1600 ml of mung beans or 4750 ml of chickpeas (approximately 24,000 and 7500 seeds, respectively) were washed with 200 ml of acetone for 60 sec, and subsequently rinsed with a further 50 ml. The extracts were each reduced by rotary evaporation at 55°C under reduced pressure to 1 ml, split into six equal aliquots and further reduced to dryness by passing nitrogen gas over the sample. Samples were weighed using a Sartorius 4501 micro balance to calculate the dry weight of material extracted from each seed. The samples were stored under nitrogen at −80°C until use. Prior to analysis, samples of the seed extracts were treated in a number of ways. Aliquots of crude extracts were spotted and dried on TLC plates (Merck Art. 5724) and developed in acetone–hexane (27 : 73 v/v). Fatty acid standards were run in parallel and the plates were stained with concentrated sulfuric acid. This determined that the position of fatty acids on the plates lay between 6 and 9 cm from the origin, approximately halfway from the origin to the solvent front. All the fatty acids moved as a single spot and so they could not be differentiated by TLC. The subfraction containing fatty acids was eluted from the absorbent with acetone and dried with nitrogen gas. Extract and subfractionated samples were derivatized with 10 μl Methyl-8 reagent (Pierce Chemical Co.) at 70°C for 30 min, which converted fatty acids to their more volatile methyl esters. Underivatized samples of extracts were dissolved in an equivalent volume of ethyl acetate.

In addition to characterizing the fatty acid composition of extracts, a number of seed wax hydrocarbons were also identified. Subsamples that appeared to be rich in hydrocarbons were insoluble in ethyl acetate and were dissolved in hexane.

The extracts from the seed surface were analyzed by gas chromatography–mass spectrometry (GC-MS): GC, Perkin-Elmer 8500; MS, Finnigan-MAT ion trap detector. Chromatography was achieved on a 25-m × 0.22-mm-ID capillary column with 0.25-μm film thickness BPX5 (SGE Ltd.) using an oven temperature program of 200°C (1 min), 200–340°C (4°C/min), 340°C (10 min), and a helium carrier gas head pressure of 20 psi. The injection volume was 1 μl into a split/splitless injector at 340°C. The effluent from the column was split between an FID, to record quantitative data (Misra and Ghosh, 1991), and the ion trap detector, to record electron impact mass spectra (scan rate, 1/sec; m/z range 50–650 with automatic gain control on). Fatty acids were characterized from their molecular ions, comparison of the mass spectra with published data (Aulsoos et al., 1992) and, when possible, comparison of the mass spectrum and retention time with authentic standards. Carbon chain lengths of alkanes were approximated by calibrating retention times with an even-chain *n*-alkane series (Supelchem).

Bioassays. Two bioassays were undertaken to elucidate the bioactivity of

fatty acids. The first investigated what effect fatty acids had on stimulating oviposition, while the second investigated whether manipulating the fatty acid composition of seed waxes affected the acceptability of an artificial seed.

Oviposition on Beads Coated with Different Fatty Acids. The concentration of fatty acids applied to artificial seeds was based on the amounts extracted from the surface of the seeds. GC-MS analysis of the mung bean surface waxes showed that they contained 32.34% by volume of fatty acid, which is approximately 1.49×10^{-4} mg fatty acids per seed. Preliminary experiments had shown the minimum amount of extract that, when coated onto glass beads, would elicit behavior similar to that obtained on mung beans was 20 seed equivalents per bead. Beads coated with such an extract were termed artificial seeds. Therefore, to ensure that the total amount of fatty acids coated onto each glass bead (5 mm diam.) would be approximately the same as that found on a seed, fatty acids were applied to beads at 2.97×10^{-3} mg/bead, which is equivalent to 20 times the amount extracted from a single seed.

Nine even-carbon-numbered chain-length fatty acids, myristic (C_{14}), palmitic (C_{16}), stearic (C_{18}), arachidic (C_{20}), behenic (C_{22}), lignoceric (C_{24}), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic acid ($C_{18:3}$) (Sigma 99% Analar, purity verified by GC-MS) as well as a mixture of these compounds were tested for their ability to stimulate egg laying in *C. maculatus*. The mixture contained all the even carbon-numbered fatty acids in the proportions in which they were found to be present in the TLC subfractionated mung bean extract. Three beads (5 mm diameter) each coated with only one of the fatty acids, three coated with the mixture, and three uncoated control beads (total of 33 beads) were placed individually into separate wells of a 60-well plate (Nunc HLA plate) in a randomized pattern. Two female beetles were released into each plate and could move freely among the wells and beads. The number of eggs laid on each of the 33 beads was recorded after 6, 24, 36, 48, and 72 h and at the time of death. Twenty replicate assays were performed. The total numbers of eggs laid by the females on each treatment (three beads coated with the same material) were compared using a Kruskal-Wallis one-way analysis of variance test, and an *a posteriori* Student-Newman-Keuls (SNK) multiple comparison of mean ranks.

Oviposition Behavior on Artificial Seeds. The effect of oleic acid on oviposition behavior was tested by adding the acid to an extract of mung. A mung extract was made from 470 ml of seeds (approximately 7000 seeds). This extract was split into two equal aliquots, one of which was coated onto 175 glass beads. Oleic acid (0.26 mg) was dissolved into the second aliquot, which was applied onto another 175 glass beads. This second treatment contained 50.3% of its total fatty acid composition as oleic acid, the same proportion of oleic acid that is found in the oil of kabuli chickpeas (Kay, 1979), a poorly accepted host of *C. maculatus*.

Bruchid behavior was observed through a binocular microscope and recorded manually onto paper (Parr et al., 1996, 1998). The experimental arena was an enclosed glass Petri dish (25 mm diameter, 15 mm high), warmed to 27°C, containing sand and a single centrally placed glass bead that was partially submerged in the sand. The recording of behavior started when a female was released into the arena and continued until an egg was laid. The female was removed from the bead immediately after oviposition. The bead was removed and replaced by another, similarly coated, and recording recommenced 30 sec later. Behavior was recorded for a minimum of 30 min. If a female was in contact with the seed at the end of 30 min, recording was continued until a female had either oviposited or left the seed.

The behavior of 20 females was recorded on each of the glass bead treatments and compared to the behavior previously recorded on mung beans using defined categories of behavior pattern (Table 1) (Parr et al., 1996). The number of eggs laid by females and rejections (leaving a host without ovipositing) were compared among treatments by Kruskal-Wallis tests and multiple comparisons of mean ranks by SNK. Behavioral data were pooled for all females, and transitional frequencies between pairs of behavior patterns were calculated. These were used to construct a 22 × 22 × 3 contingency table, in which the first variable is the 22 possible "preceding" behavior patterns, the second variable is the 22 possible "following" behaviors, and the third variable is the host [mung data from Parr et al. (1996), artificial mung, artificial mung plus oleic acid]. Log-linear analysis was used to determine which interactions among the three variables could best account for the observed transitional frequencies. This method of analysis has been used to construct models that describe oviposition behavior (Parr et al., 1996, 1998). An index of dissimilarity quantifies the differences between expected and observed tables (Messina and Dickinson, 1993). Standardized residuals quantifying the dissimilarity between each expected cell and its corresponding observed cell were calculated. Transitions that occurred more often than predicted by the model, indicated by high positive residuals, were used to construct a table of significant transitions. The frequencies of transitions were calculated as conditional (transitional) probabilities and the transitional frequencies as percentages of the total number of transitions. The frequencies of behavior patterns were calculated as a percentage of the total number of behavioral events observed.

RESULTS

Seed Surface Waxes. Fatty acids were identified from GC-MS analysis of TLC fractionated samples. A reduction in compound diversity after fractionation reduced the "noise" on the baseline of both GC and MS traces, which expedited

TABLE 1. BEHAVIOR PATTERNS OF FEMALE *C. maculatus*

Number	Behavior pattern
1	Contact with seed made by the antennae and/or palpi
2	Contact with seed made by the tarsi
3	Walking
4	Walking with the thorax, head, and antennae raised (approximately 45 degrees above the horizontal)
5	Walking with the antennae and palpi lowered and antennating/palpating the substrate
6	Walking/standing with the antennae and palpi lowered and antennating/palpating the substrate and the ovipositor extended
7	Walking/standing with the antennae and palpi lowered and antennating/palpating the substrate and the ovipositor lowered to the substrate
8	Standing (not walking, neither antennae nor palpi lowered, ovipositor not extended)[a]
9	Standing with the thorax, head, and antennae raised (approximately 45 degrees above the horizontal)
10	Standing with the palpi lowered and palpating the substrate
11	Standing with the antennae and palpi lowered and antennating/palpating the substrate (ovipositor not lowered to the substrate)[a]
12	Standing with the palpi lowered and palpating the substrate, and the ovipositor lowered to the substrate
13	Grooming antennae
14	Grooming mouthparts
15	Grooming tarsi (and rest of legs)
16	Grooming of body parts other than antennae, mouthparts, tarsi, and rest of legs
17	Grooming of the antennae, with the ovipositor lowered to the substrate
18	Grooming of the tarsi and rest of legs, with the ovipositor lowered to the substrate
19	Grooming of the mouthparts, with the ovipositor lowered to the substrate
20	Ovipositor lowered to the substrate
21	Ovipositing
22	Off-seed behavior

[a] Exclusions are cited in these cases to avoid possible confusion with other descriptors in the table.

the analysis and quantification of those compounds present in low concentrations. The subfractionated mung bean sample was found to contain 17 fatty acids (Table 2). Six were simple saturated fatty acids with even chain lengths in a series from C_{14} to C_{24}, three were unsaturated fatty acids ($C_{18:1}$ = oleic acid, $C_{18:2}$, $C_{18:3}$), which came off close to each other in a single peak, and the remainder had odd-numbered chain lengths between C_{17} and C_{25}. The fatty acids with odd-numbered chain lengths were thought to be products of the partial decomposition of other fatty acids and fatty acid derivatives. The extract from mung beans contained at least 32.3% of its volume as fatty acid (estimated from

TABLE 2. IDENTITIES AND QUANTITIES OF FATTY ACIDS EXTRACTED FROM MUNG BEAN SEEDS

Compound name	Retention time (sec)	Quantity in extract (mg/g)
Myristic acid (C_{14})	204	0.323
Palmitic acid (C_{16})	322	68.884
Unknown (C_{17})	370	13.259
Unknown (C_{17})	399	1.617
Mixture of oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$)	473	168.168
Stearic acid (C_{18})	491	27.230
Unknown (C_{19})	555	5.498
Unknown (C_{19})	591	0.647
Arachidic acid (C_{20})	698	5.498
Unknown (C_{21})	811	0.323
Behenic acid (C_{22})	926	13.32
Unknown (C_{23})	1031	0.647
Lignoceric acid (C_{24})	1152	6.468
Unknown (C_{25})	1412	3.234
Unknown (C_{25})	1514	0.970
Unknown (C_{25})	1552	3.881
Unknown (C_{25})	1820	2.264

the derivatized crude extract). It was difficult to quantify exactly the fatty acids in the chickpea extract because peaks were frequently small compared to background peaks in the baseline. However, we did have qualitative evidence of the occurrence of odd- and even-number chain-length fatty acids (C_{14}, C_{16}, $C_{18:1,2,3}$, C_{20}, C_{23}, C_{24}), which in total amounted to only about 5% of extract volume, as estimated by comparisons of MS fragmentation traces.

Analysis of underivatized samples showed that hydrocarbons were common components of seed waxes. Both mung bean and chickpea extracts were found to contain series of both odd- and even-numbered carbon chain length *n*-alkanes and isoalkanes in the range of C_{23} to C_{33}. The waxes of chickpea and mung bean contained 18 and 14 hydrocarbon compounds, respectively, corresponding to 66.1% and 19.2% v/v of the extracts. Two *n*-alkanes, C_{27} (R_t = 18.79) and C_{29} (R_t = 22.56) were particularly abundant in the chickpea extract.

Role of Fatty Acids as Stimulants. The fatty acids significantly influenced the total number of eggs laid ($\chi^2_{(df=10)} = 123.40$, $P < 0.001$). The SNK tests showed that four of the nine fatty acids stimulated oviposition to a greater level than occurred on the untreated control beads (Table 3). Behenic acid appeared to stimulate the fastest rate of oviposition (0.145 eggs/hr) and the highest number of eggs laid (Table 3, Figure 1). Arachidic acid, lignoceric acid, and oleic acid

TABLE 3. MULTIPLE COMPARISON (STUDENT-NEWMAN-KEULS OF MEAN RANK) OF TOTAL NUMBERS OF EGGS LAID ON FATTY ACID-COATED BEADS

Compound name	Homogeneous subgroup[a]	Eggs laid in lifetime of two females (mean ± SE)
Behenic acid (C_{22})	a	35.25 ± 3.40
Arachidic acid (C_{20})	b	10.75 ± 1.22
Lignoceric acid (C_{24})	b	9.25 ± 1.44
Fatty acid mixture	bc	7.90 ± 1.41
Oleic acid ($C_{18:1}$)	bc	7.40 ± 0.96
Linolenic acid ($C_{18:3}$)	cd	3.20 ± 0.69
Stearic acid (C_{18})	cd	3.00 ± 0.59
Myristic acid (C_{14})	d	2.85 ± 0.71
Linoleic acid ($C_{18:2}$)	d	2.50 ± 0.59
Palmitic acid (C_{16})	d	1.95 ± 0.43
Control	d	0.40 ± 0.13

[a] Values in the same column that are significantly different at the 5% level ($P < 0.05$) (SNK) are indicated by different letters.

stimulated production of the same number of eggs as did the fatty acid mixture. The numbers of eggs laid on beads treated with the other fatty acids (linolenic acid, stearic acid, myristic acid, linoleic acid, and palmitic acid) were similar to those laid on the untreated control beads (Table 3).

Role of Fatty Acids as Deterrents. Females laid significantly more eggs on mung beans (7.2 ± 0.75; mean ± SEM) and extract-coated beads (8.5 ± 0.45) than on beads coated with extract + oleic acid (3.9 ± 0.68) in the 30-min observation period ($\chi^2_{(df=2)} = 17.64$, $P < 0.001$). Rejections occurred significantly more frequently on beads treated with mung extract + oleic acid (9.45 ± 1.72) than on beads treated with mung extract (2.9 ± 0.63) or mung seeds (0.7 ± 0.17) ($\chi^2_{(df=2)} = 26.47$, $P < 0.001$).

The results of the log-linear contingency table analysis are summarized in Table 4. The analysis indicates that three-way interactions significantly structure the transitional frequencies, i.e., the degree of association between preceding and following behaviors was different on each class of host. However, the index of dissimilarity of model 8, which assumes all pairwise interactions but no three-way interaction, is 6.7. In other words, only 6.7% of the expected values would need to be reallocated to obtain the observed data. The importance of the three-way interaction is probably fairly small, as is indicated by the low value of the index of dissimilarity.

Model 7, assuming no interaction between preceding and following behaviors (i.e., no sequence) can be used to indicate the transitions that have an

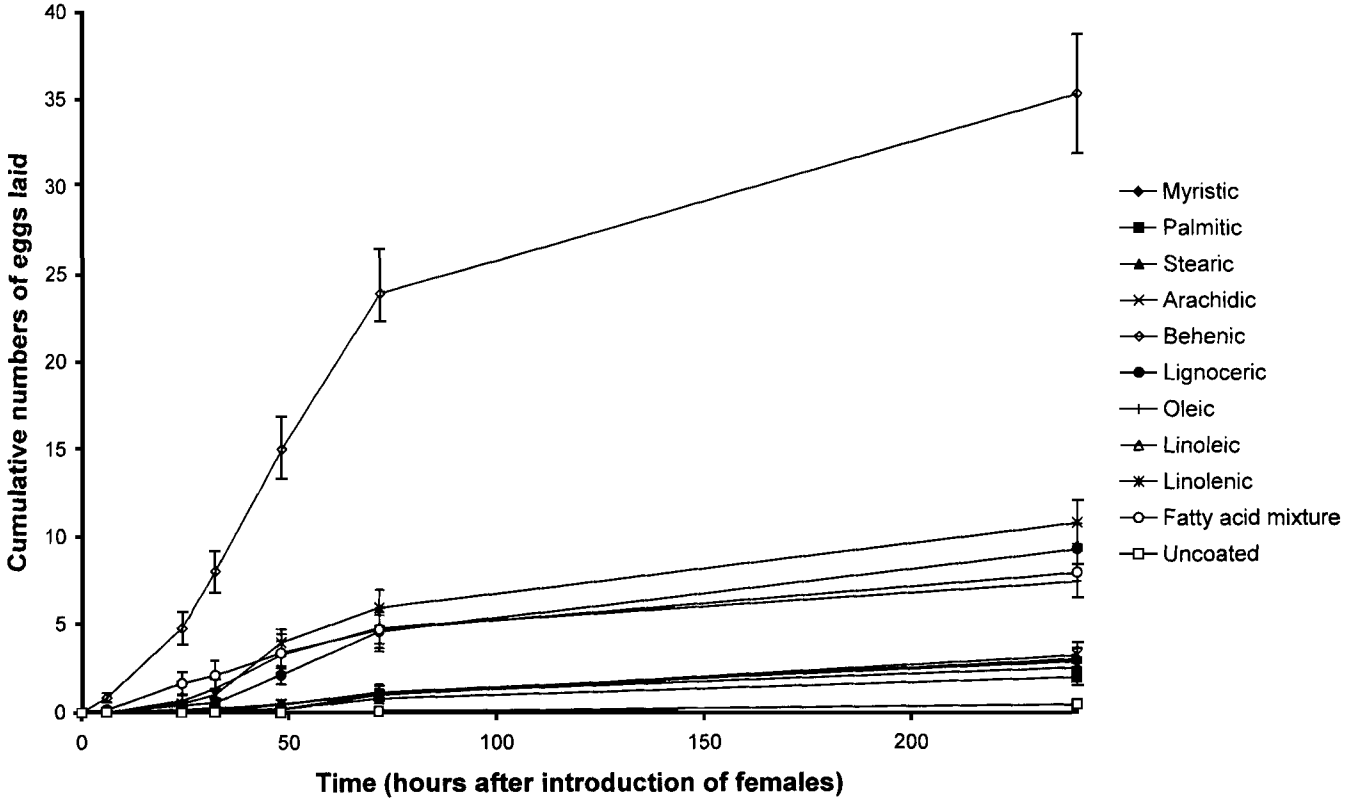

FIG. 1. The course of oviposition on fatty acid-coated beads. Plotted values are mean cumulative numbers of eggs laid by two females. Bars represent standard errors.

TABLE 4. FIT OF DIFFERENT MODELS TO TRANSITIONAL DATA OBTAINED BY OBSERVING OVIPOSITIONAL BEHAVIOR OF *C. maculatus*

Model	Model log (Exp_{PFH}) =, and (model no)[a]	*df*	$\chi^2_{df,0.05}$	*G* test statistic	Significance (*P*)	Index of dissimilarity
Complete independence	$u + u_P + u_F + u_H$ (1)	1040	1116	20,078	<0.001	64.84
	$u + u_P + u_F + u_H + u_{PF}$ (2)	841	910	1,509	<0.001	16.84
Partial	$u + u_P + u_F + u_H + u_{PF}$ (3)	998	1073	19,478	<0.001	64.60
	$u + u_P + u_F + u_H + u_{FH}$ (4)	998	1073	19,483	<0.001	64.29
	$u + u_P + u_F + u_H + u_{PF} + u_{PH}$ (5)	678	740	914	<0.001	11.55
Conditional	$u + u_P + u_F + u_H + u_{PF} + u_{PH}$ (6)	678	740	909	<0.001	11.33
	$u + u_P + u_F + u_H + u_{PH} + u_{FH}$ (7)	956	1029	18,883	<0.001	63.44
No second order	$u + u_P + u_F + u_H + u_{PF} + u_{PH} + u_{FH}$ (8)	515	570	471	NS	6.66

[a] The parameter u represents the overall mean effect, u_P, u_F, and u_H the main effects of variables *P* (= preceding behavior), *F* (= following behavior), and *H* (= host) (see text for details). Parameters u_{PF}, u_{PH}, and u_{FH} represent the interactions between the pairs of variables *PF*, *PH*, and *FH*. Parameter u_{PFH} represents the three-way interaction (i.e., second-order effects).

expected value noticeably lower than those of the observed. The standardized residuals for this model singled out the transitions that made the greatest contribution to the sequence (Table 5). Nineteen significantly positive transitions were exhibited on all hosts, 26 were demonstrated on mung beans, 40 on the extract, and 49 on the extract- + oleic acid-coated beads. Sixteen transitions were significantly associated with extract- + oleic acid-coated beads alone. These included transitions to and from the exhibition of a raised body posture, transitions between grooming behaviors, and transitions representing loops back to states from which oviposition was less likely to occur. Percentage frequency, percentage transitional frequency, and conditional probability data confirmed the increased preponderance of the raised body posture in the behavior of beetles on extract- + oleic acid-coated beads. This body posture is known to be exhibited on low acceptability hosts (Parr et al., 1996). For example, the behavior "walking with the thorax, head, and antennae raised" comprised 0.21% of all behaviors on mung, 0.72% on extract-coated beads, and 3.34% on extract- + oleic acid-coated beads. Looking more closely at particular transitions, the transition between "walking with the thorax, head, and antennae raised" and "standing with the thorax, head, and antennae raised," had a percentage transitional frequency of 0.35% on extract- + oleic acid-coated beads (conditional probability: 0.1), but did not occur on the other hosts. The transition "walking with the antennae and palpi lowered and antennating/palpating the substrate" to "walking with the thorax, head, and antennae raised" was exhibited most often on extract- + oleic acid-coated beads (1.43%, 0.09; percentage transitional frequency and conditional probability), less often on extract-coated beads (0.17%, 0.01), and least often on mung (0.07%, 0.004).

A general increase in the combined percentage frequency of nonkey behaviors, a feature associated with diminishing host acceptability (Parr et al., 1998) was found from mung (6.19%), to extract-coated beads (8.26%), to extract- + oleic acid-coated beads (19.53%).

DISCUSSION

The epicuticular waxes of mung bean seeds were found to contain large quantities of fatty acids and alkanes, whereas chickpea waxes contained a relatively small amount of fatty acid but were rich in alkanes. The fatty acids present in the waxes were the same as those normally found in the cotyledons of both species and those in the mung bean wax were also found in the same proportions as those that might have been expected to occur in seed oil extracted from the cotyledons (Kay, 1979). This concurrence is unlikely to have been caused by leaching of the seed because the extraction period was short (Deriddj et al., 1996) and seeds with damaged testae were excluded. Furthermore, there

TABLE 5. SIGNIFICANTLY POSITIVE (FREQUENT) TRANSITIONS BETWEEN BEHAVIOR PATTERNS IN OVIPOSITIONAL BEHAVIOR OF *C. maculatus*[a]

	1	2	3	4	5	6	7	8	9	10
1										
2										
3		abc		b	bc			a		
4			bc		c				c	
5	abc		abc					abc		abc
6					abc		abc			
7						abc				
8					c	b				b
9				c						
10						bc		bc		
11										b
12						abc	a			
13										
14								bc		
15			a	c				c		
16										
17										
18										
19						a				
20						abc				
21										
22			abc	bc						

[a] A matrix of significantly positive transitions (higher than three times the calculated threshold for positive residuals) in behavior, exhibited by *C. maculatus* females on mung (a), extract-coated beads (b), and extract- + oleic acid-coated beads, determined from the model $u + u_P + u_F + u_H + u_{PH} + u_{FH}$ (model 7). The 22 preceding behaviors are shown on the horizontal axis and 22 following behaviors are shown on the vertical axis (see Table 1).

is good evidence that free fatty acids are common components of epicuticular waxes (Eigenbrode and Espelie, 1995). It seems reasonable to deduce then that the fatty acid composition of the chickpea wax is the same as that recorded for its seed oil.

Although long chain ($>C_{16}$) free fatty acids comprise a large proportion of the surface lipids of some plants, these compounds have infrequently been

TABLE 5. CONTINUED

11	12	13	14	15	16	17	18	19	20	21	22	
											abc	1
											abc	2
												3
												4
			b	b								5
	c								c			6
												7
			bc									8
												9
			a									10
												11
								c	bc			12
			c	bc		b						13
		bc		abc								14
		bc	c		c		c					15
		a	bc	c								16
							a					17
						c		c				18
	abc						bc		bc			19
	abc							abc				20
									abc			21
										abc		22

shown to affect insect behavior. In one example, long-chain fatty acids have been shown to be feeding stimulants to *Locusta migratoria* (Chapman, 1977). Later studies by Woodhead and Chapman (1986) indicated a structure–activity relationship in which a greater length of the carbon chain of waxes was correlated with increased phagostimulatory activity. If ovipositing female bruchids can similarly perceive differences between fatty acids, it would allow them to make choices among potential hosts for their progeny.

The pattern of odd- and even-carbon-numbered hydrocarbons found in the surface waxes of mung bean and chickpea is similar to those found in the waxes of *Phaseolus aureus* (Wolfee and Kwolek, 1971) and two *Acacia* sp. (Horn et al., 1964), that were reported to contain odd- and even-carbon-numbered normal paraffins in the ranges of C_{26} to C_{33} and C_{21} to C_{29}, respectively. In *Phaseolus aureus*, C_{29} and C_{31} predominated, while in *Acacia* the most common *n*-alkanes were C_{27} and C_{29}, as in chickpea. *n*-Alkanes have been shown to affect insect behavior (Städler, 1986; Eigenbrode and Espelie, 1996).

The present study shows that a number of fatty acids presented to beetles in isolation at ecologically meaningful concentrations stimulated oviposition. Behenic acid (C_{22}) was the most stimulatory, followed by arachidic acid (C_{20}), and lignoceric acid (C_{24}). There is some evidence of a structure–activity relationship in that the fatty acids with the longer chain length stimulated the bruchids to lay more eggs than those acids with shorter chain lengths, comparable with the work of Woodhead and Chapman (1986).

Oleic acid ($C_{18:1}$) stimulated oviposition when applied alone to beads. However, when added to an "acceptable" extract, small amounts of oleic acid reduced the acceptability of an artificial seed, a glass bead coated with the extract, to ovipositing females.

The log-likelihood analysis showed that the host type influenced the frequency with which insects switched between behaviors. Although similar numbers of eggs were laid on mung bean and mung bean extract-coated beads, a number of other variables (occurrence of significant transitions, frequency and transitional frequencies of behaviors) suggested that the extract-coated beads were not as acceptable as real seeds. Nonetheless, the addition of oleic acid reduced an extract's acceptability significantly. For example, the increased frequency of rejection and the action of raising the body may reflect the removal of host-related receptors from the proximity of the current oleic acid-enriched host towards more relevant stimuli including other potential hosts (Bernays, 1996). Similar behaviors were found in an earlier study to be caused by the presence of an egg on a seed (Parr et al., 1996).

Oleic acid appears to have different effects on behavior depending on the concentration and mix in which it occurs. It has been shown to act as a deterrent when applied at a concentration $\frac{1}{33}$ of the minimum concentration of fatty acid (in oils) that had been reported to cause deterrent effects when applied to seeds (Pereira, 1983). Yet it can stimulate oviposition in isolation and may stimulate oviposition as a component of a natural mix of fatty acids. This implies that it may act as a "dual-function" contact stimulant to oviposition (Birch et al., 1996). Current evidence for the prevalence of dual-function chemicals in insect–plant interactions is sparse (Finch, 1977; Kostal, 1992). It has been shown that a number of compounds produced by *Brassica* crops may act as dual-function

attractants–repellents to *Delia floralis* and that insects may use quantitative chemical information when effecting a host choise (Birch et al., 1996).

The adjustment of the fatty acid composition of the extract to elevate its oleic acid content and to make the manipulated extract more like that from a chickpea was imperfect, and therefore our results must be treated with caution. The reduced acceptability of an extract containing an elevated level of oleic acid when compared to the unaltered extract may be caused by a number of factors in addition to the increasing proportion of oleic acid. The manipulated extract contained more fatty acid on a volume basis and more chemical material. In addition, chickpea waxes contain less fatty acid and more alkanes, both of which may be important factors in reducing its susceptibility (Eigenbrode and Espelie, 1995). Nonetheless, there is some evidence that seeds that contain a greater relative proportion of oleic acid in their seed oils, and therefore perhaps in their epicuticular waxes, are less acceptable hosts to *C. maculatus*. *Cicer arietinum*, *Lens culinaris*, *Vicia faba*, and *Glycine max*, which contain 30% and more oleic acid, are less acceptable oviposition sites than *Vigna unguiculata*, *V. angularis*, *V. radiata*, *Phaseolus vulgaris*, and *Cajanus cajan*, which contain less than 15% (Kay, 1979; Wasserman, 1986; Giga and Smith, 1987; Messina and Mitchell, 1989; Huignard, personal communication). This may be of considerable importance as "the door is now open for the genetic engineering of crops with epicuticular lipid phenotypes designed to limit pest damage" (Eigenbrode and Espelie, 1995).

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EFFECT OF FEMALE AGE ON DAILY CYCLE OF SEXUAL PHEROMONE EMISSION IN GREGARIOUS EGG PARASITOID *Anaphes listronoti*

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Abstract—The temporal pattern of pheromone emission by *Anaphes listronoti* females was established in a four-arm olfactometer by observing, at 2-hr intervals, the response of <2-hr-old males to odor produced by individual virgin females of <1, 1, and 2 days of age. This is the first evidence of a sexual pheromone in a Mymaridae. Under a 16L:8D photoperiod and 24°C constant temperature, the responsiveness of males to females of different ages varied significantly during the photophase. When <1-day-old females were used, males made significantly more final choices in the pheromone field than odorless fields at 4 and 6 hr after the onset of the photophase, and their walking speed was significantly higher from 4 to 12 hr, suggesting that females began to release a long-range pheromone during this period. Final choices and high walking speed were observed earlier with 1- and 2-day-old females than with <1-day-old females, and there was a significant decrease in male responses at 6 hr after the onset of the photophase, suggesting a bimodal temporal pattern of sexual pheromone emission.

Key Words—Sexual pheromone, daily cycle, male attraction, female age, olfactometer, egg parasitoid, Hymenoptera, Mymaridae, *Anaphes listronoti*.

INTRODUCTION

Upon emergence, adult parasitoids must find the opposite sex to copulate. Both sexes have developed mechanisms to increase the likelihood of encountering the

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other sex. Males may search for emergence, feeding, or oviposition sites and use stimuli emitted by the female (Godfray, 1994). Mating at emergence sites is likely to occur in gregarious parasitoids because the sexes emerge in proximity to each other. In such circumstances, mate finding is done through tactile and visual stimuli (Assem and Jachmann, 1982; Tripathi and Singh, 1990; Yoshida and Hidaka, 1979), sound (Assem and Putters, 1980), or short-range pheromone (Swedenborg et al., 1993).

Anaphes listronoti Huber (Hymenoptera: Mymaridae) is a short-lived egg parasitoid of the carrot weevil *Listronotus oregonensis* (LeConte) (Coleoptera: Curculionidae). The parasitoid has a life expectancy of 4 days at 23°C (Collins and Grafius, 1986a). The distribution of newly emerged adult parasitoids depends on the spatial distribution of carrot weevil eggs. Host eggs are aggregated in carrot fields in clutches of one to seven eggs with a mean of 2.0 eggs per oviposition site. More than one laying site may occur on a single plant (Collins and Grafius, 1986b; Whitcomb, 1965). In a parasitized host egg site, the probability of having both sexes of *A. listronoti* is high because on average more than two adults emerge per parasitized egg (Collins and Grafius, 1986b; Cormier et al., 1996). Most of the parasitoid emergence occurs during the first 2 hr of light with males emerging first (Collins and Grafius, 1986a). *Anaphes listronoti*, like most hymenopterous parasitoids, has an arrhenotokous type of reproduction where unfertilized eggs produce males and fertilized eggs give rise to females (Doutt, 1959). When mated, the female thus has the ability to determine the sex of her progeny, while unmated females also produce progeny but are restricted to producing males. Mating begins soon after emergence, and 92% of *A. listronoti* females mate in the first 2 hr (Collins and Grafius, 1986c). Because mating at the emergence site is widespread among gregarious species, such as *A. listronoti*, the emission of a long-range sexual pheromone is expected to be uncommon (Godfray, 1994).

Collins and Grafius (1986c) described the courtship and mating behavior of newly emerged *A. listronoti* in Petri dishes. They found that males were attracted to females; however, the stimuli involved are unknown. Because males and females were close to one another, tactile, visual, auditory, and chemical stimuli may have been used by both sexes. However, a long-range sexual pheromone may be involved because, in the field, 91% of *A. listronoti* females are considered mated (Collins and Grafius, 1986b) and in 19% of the emergence sites no males are present (D.C., unpublished data).

We examined male response to odor produced by a virgin female to determine the temporal pattern of pheromone emission under light and dark conditions and the periodicity of pheromone emissions of 0-, 1-, and 2-day-old females during the photophase. Because *A. listronoti* mating occurs soon after emergence, we hypothesized that a large proportion of females would release sexual pheromone to attract neighboring males and that, as time elapsed, an increasing

number of females would temporarily cease to emit pheromone since oviposition becomes the dominant activity 24 hr after emergence, even for unmated females (Collins and Grafius, 1986a).

METHODS AND MATERIALS

Insects. A laboratory colony of *A. listronoti* was established from field-collected parasitized *L. oregonensis* eggs in 1990 in the Holland Marsh, Ontario. The colony was replenished annually and was maintained at 25 ± 1°C under a 16L:8D photoperiod. *Anaphes listronoti* and *L. oregonensis* were reared according to the methods described by Boivin (1988) and Martel et al. (1975), respectively. Female parasitoids were obtained by exposing adults of both sexes in the ratio of one male to two females to 10 carrot weevil eggs per female. As unmated females produce only males (Clausen, 1940), we exposed, in separate Petri dishes, only virgin females in order to obtain males. After three days, eggs were individually transferred in 300-μl polyethylene capsules (Beem) and incubated at 24 ± 1°C under a 16L:8D photoperiod. To have newly emerged males every 2 hr during each experimental period, cohorts of males were incubated under the same photoperiodic regime but with photophase delayed in a series of 2-hr periods. In order to standardize individuals, only solitary developing individuals were kept at emergence and used in the experiments. Females were individually kept in a 3-cm × 5-mm-ID glass tube enclosed by a piece of fine mesh PeCap polyester (Tetko Inc. No. 7-105/52) at each extremity. Each tube was deposited on moistened filter paper in separate 9-cm Petri dishes and incubated at 24 ± 1°C under a 16L:8D photoperiod. Males were kept in their emerging capsules until use. Males and females were never in contact with conspecifics or host eggs before and during experiments.

Olfactometer. The response of <2-hr-old males to female pheromone was measured using a four-arm olfactometer (Pettersson, 1970; Vet et al., 1983) with modifications described by Vigneault et al. (1997). The sharpest boundaries and an absence of turbulence were obtained at an airflow of 150 ml/min. A video camera fixed 45 cm above the olfactometer provided video signals to a computer. Males in the exposure chamber were illuminated by a circular fluorescent light (40 W) located below the chamber. To eliminate the directional effect of light from above, the exposure chamber was surrounded by a covered cylinder, 50 cm high, which allowed light only from below. To restrict light in the exposure chamber and eliminate any visual contact between males and females, a 25-cm-long Tygon tube, covered with black tape, was looped and connected the exposure chamber to the tube containing the female. During the photophase, illumination to females was provided by three parallel fluorescent tubes (30 W each) hung 1 m above the female. During the scotophase, females

were manipulated under an infrared light (250 W), and only females were kept in the dark by wrapping their chamber with aluminum foil.

General Experimental Procedure. Five minutes before each test, one virgin unfed female was transferred with a fine moistened paint brush in a clean glass tube enclosed with a piece of polyester at each extremity and connected to one arm of the olfactometer. This period enabled the female to acclimate to new environmental conditions and to fill the corresponding field in the exposure chamber with female odor. The remaining arms of the olfactometer contained only humidified air (odorless fields) and served as controls. Males were individually transferred in a 1-cm-long polyethylene tube enclosed with pieces of small mesh polyester and were accliminated to the exposure chamber for a minimum of 5 min before a test. The tube was opened and immediately placed in the introduction hole at the center of the exposure chamber. Males were exposed to the female odor while exiting the tube and entering the exposure chamber. The test started as soon as the male entered the olfactometer, and male response was observed for 5 min. Males that did not enter the exposure chamber within 5 min were gently placed with a fine brush in the center of the olfactometer, and the experiment started 60 sec later to minimize the effect of disturbance. After a female was tested, she was returned to the first glass tube and placed in the incubator until the next test was performed 2 hr later. After three or four replications, the whole system was dismantled, washed with hot soapy water, rinsed with ethanol, and air dried for 30 min. Before the next series of tests, the olfactometer was rotated 90° clockwise. Males were tested only once. Experiments were conducted in a climate-controlled room maintained at 24 $\pm$ 1°C.

System Bias. To determine if parasitoid behavior was similar to each field, we tested male response to humidified air in all four fields. Sixteen replicates were carried out.

Diel Periodicity. Male response to newly emerged females was determined during the first day of the female's life (day 0) at 2-hr intervals. Individual females were used as soon as their wings were fully expanded and cleaned, about 15 min after emergence. For each female, the first observation was carried out about 1 hr after the beginning of the photophase, because in this study females emerged 56.7 $\pm$ 3.4 min (N = 32) after the onset of the photophase. The pheromone emission of 16 females was observed throughout the photophase. To limit potential cumulative damage to females due to frequent manipulation, we used a new cohort of 15 females to evaluate pheromone emissions during the first scotophase. Observations started 2 hr after the onset of the scotophase and were made at 2-hr intevals for 8 hr.

Effect of Female Age. The effect of female age on pheromone emission was determined by observing 16 newly emerged females (day 0) during the first three days. Each day, pheromone emission of individual females was determined

at 2-hr intervals during the first 10 hr of the photophase. To ensure female survival during the experiment, they were fed from the end of the first day to the end of the experiment with a honey–beer diet that has been shown to increase their longevity (Collins, 1982).

Data Recording and Statistical Analysis. Male response in the olfactometer was recorded using a real-time videotape tracking system (Vigneault et al., 1997). The arena was divided into six zones: one zone for each field (zone 1–4), a 6-mm-radius zone centered at the introduction hole (zone 5), and a 2-mm zone just inside the inner wall of the arena (zone 6). Data from zones 5 and 6 were excluded from statistical analysis as the borders of the exposure chamber may influence male behavior (Berry and Holtzer, 1990; Bigler et al., 1988). To detect pheromone emission, three parameters were measured during a 5-min period: the number of males making a final choice, the time spent per field, and the walking speed of males. A male was considered to have made a final choice if he exited one of the arms for more than 60 sec. If this occurred, the experiment was stopped and the remaining time was added to the time spent in the chosen field. The chi-square test was used to compare final choices made by males (Ho: 25% of final choice in each field). The time spent in the odorless fields was pooled and compared with three times the time spent in the odor field. The Wilcoxon matched-pairs signed-rank test based on relative time (Ho: 50% of time spent in pheromone = 50% of time spent in odorless fields) was used to test between pheromone and odorless fields (Daniel, 1990). The time spent per field was reported only for the diel periodicity experiment to support our discussion. In all other experiments, this parameter did not provide useful information and was not included in the results section. In each field, walking speed was calculated as the total distance traveled divided by the total time, including time spent motionless. When necessary, data on walking speed were transformed by means of square root to normalize distribution of means (Steel and Torrie, 1980). A two-way analysis of variance (GLM procedure) for repeated measurements with treatments and time of the photophase as main factors was performed on walking speed (SAS Institute, 1988). Differences in the mean walking speed in pheromone fields were compared between hours and ages by the Student's t test performed under the least-squares means (adjusted means) statement of proc GLM (SAS Institute, 1988).

RESULTS

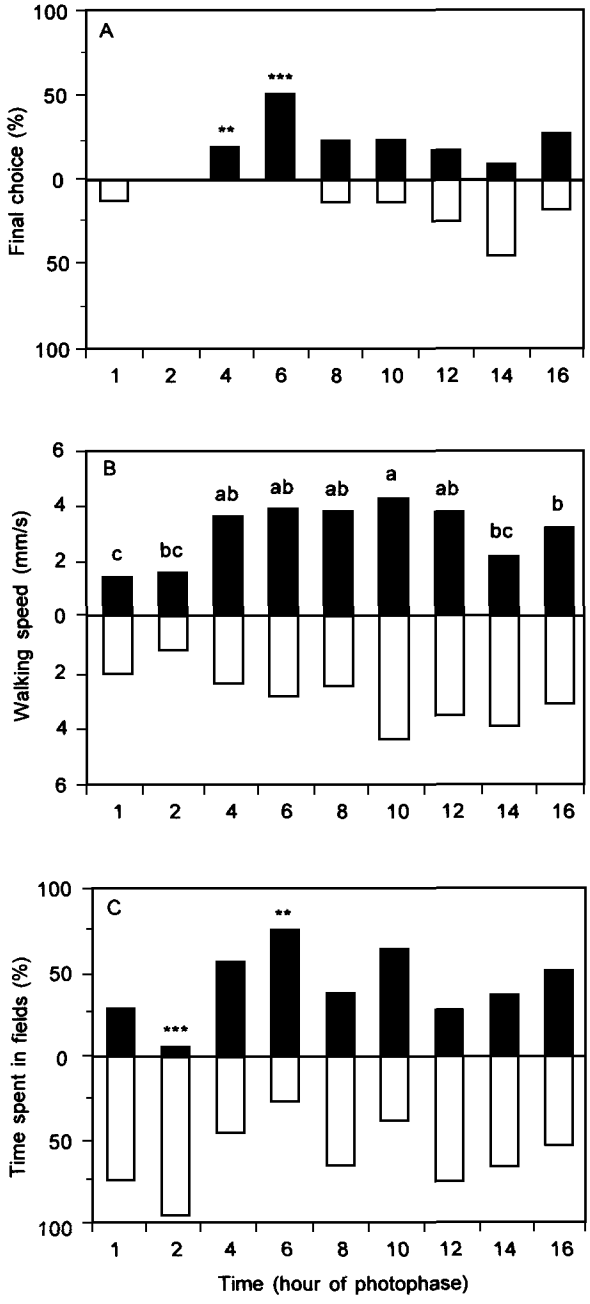
System Bias. In the absence of pheromone odor, the number of males making a final choice ($\chi^2_3 = 3.00$; $P = 0.3916$) and the walking speed ($F_{3,22} = 0.73$; $P = 0.5436$) were not significantly different among fields, indicating that each field of the olfactometer had similar physical properties.

Diel Periodicity. Few final choices were made by males during the first 2-hr, but they were more frequent in pheromone and odorless fields from the 4th to 16th hr of the photophase (Figure 1A). The percentage of males making a final choice was significantly higher in pheromone than in odorless fields at 4 hr ($\chi^2_1 = 9.00$; $P = 0.0027$) and 6 hr ($\chi^2_1 = 21.00$; $P < 0.001$) after the onset of the photophase. The walking speed in pheromone odor and odorless fields did not differ at each time interval ($F_{1,158} = 0.84$; $P = 0.3599$) but did differ with the time of the photophase ($F_{8,158} = 4.63$; $P < 0.0001$), reaching its highest value by 10 hr (Figure 1B). The time spent per field was higher in the odorless and pheromone fields, respectively, at 2 hr ($P = 0.0005$) and 6 hr ($P = 0.0087$) after the beginning of the photophase (Figure 1C).

During the first scotophase of females, few males made a final choice (Figure 2A). Six hours after the onset of scotophase, the percentage of males making a final choice was significantly higher in pheromone than odorless fields ($\chi^2_1 = 6.00$; $P = 0.0143$), whereas no significant difference was observed at 8 hr ($\chi^2_1 = 0.33$; $P = 0.5657$). The walking speed was similar in pheromone and odorless fields at each time interval ($F_{1,72} = 0.48$; $P = 0.4915$). However, the walking speed was affected by the time of the scotophase ($F_{3,72} = 10.81$; $P < 0.0001$), males moving faster during the second half of the scotophase (Figure 2B).

Effect of Female Age. Male behavior was affected by female age, indicating that pattern of pheromone emission changed with age. On day 0, significant differences in the percentage of final choice were observed between 4 and 8 hr after the onset of the photophase (Figure 3). On days 1 and 2, final choices were made earlier than on day 0 and were observed until 10 hr after the onset of the photophase with no significant difference observed on day 1 at 6 hr ($\chi^2_1 = 0.00$; $P = 1.0000$) and on day 2 at 6 hr ($\chi^2_1 = 0.67$; $P = 0.4131$) and 8 hr ($\chi^2_1 = 0.11$; $P = 0.7401$), suggesting a bimodal temporal pattern of pheromone emission (Figure 3). Both female age ($F_{2,333} = 3.86$; $P = 0.0221$) and time of photophase ($F_{5,333} = 6.33$; $P < 0.0001$) affected the walking speed with interaction between the two factors ($F_{10,333} = 2.4$; $P = 0.009$) (Figure 4). Walking speed was not significantly different between pheromone and odorless fields

FIG. 1. Response of <2-hr-old *Anaphes listronoti* males to odor emitted by one virgin female during the first photophase of life. The black and white bars show the means in pheromone and odorless fields, respectively. (A) Percentage of final choice (** and ***, significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively, chi-square test); (B) walking speed (means followed by the same letter did not differ significantly at $P > 0.05$ between pheromone fields, ANOVA followed by comparison of means); (C) time spent per field (** and ***, significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively, Wilcoxon matched-pairs signed-ranks test).


A
Final choice (%)
100
50
0
50
100
**
***
1 2 4 6 8 10 12 14 16
B
Walking speed (mm/s)
6
4
2
0
2
4
6
c
bc
ab
ab
ab
a
ab
bc
b
1 2 4 6 8 10 12 14 16
C
Time spent in fields (%)
100
50
0
50
100
***
**
1 2 4 6 8 10 12 14 16
Time (hour of photophase)

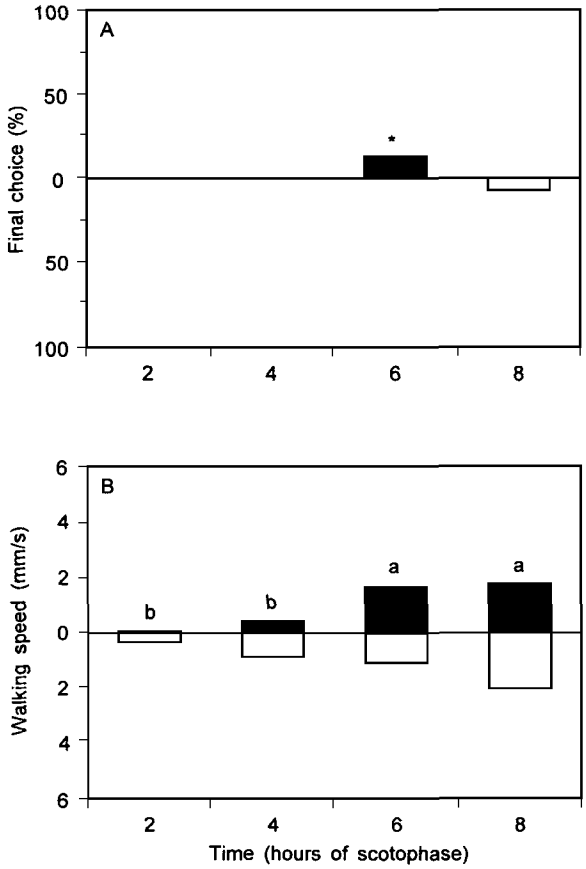
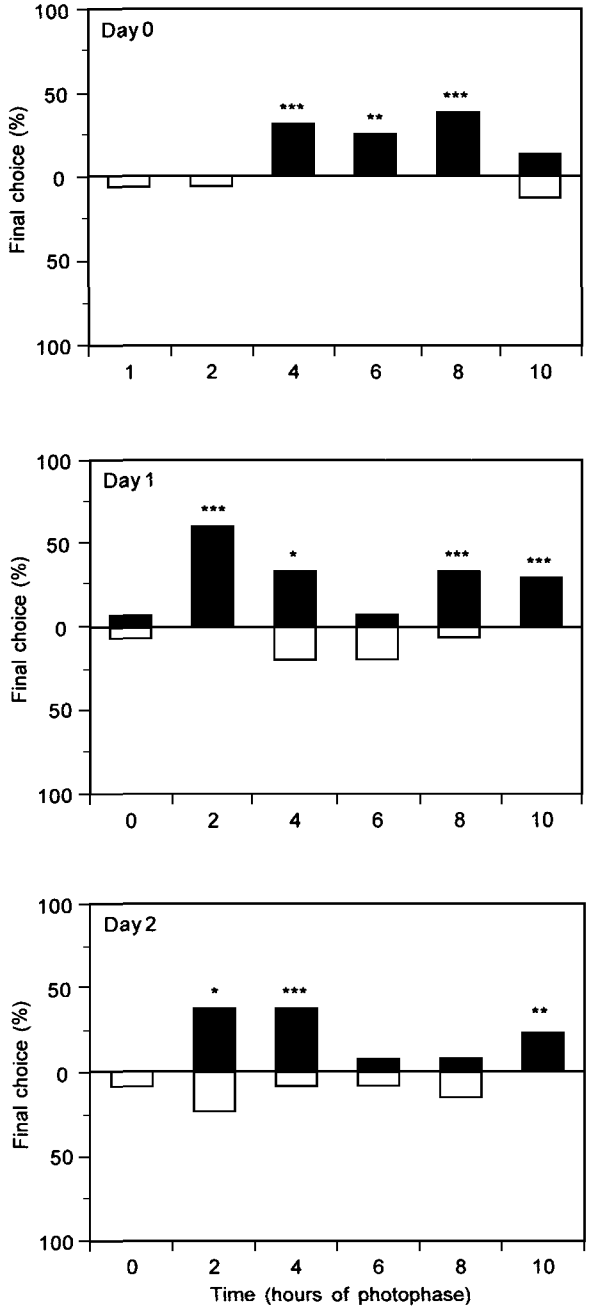


FIG. 2. Response of <2-hr-old *Anaphes listronoti* males to odor emitted by one virgin female during the first scotophase of life. The black and white bars show the means in pheromone and odorless fields, respectively. (A) Percentage of final choice (* significantly different at $P \leq 0.05$, chi-square test); (B) walking speed (means followed by the same letter did not differ significantly at $P > 0.05$ between pheromone fields, ANOVA followed by comparison of means).

FIG. 3. Percentage of <2-hr-old *Anaphes listronoti* males making a final choice to odor emitted by one virgin female during the first three days of life (*, **, and ***, significantly different at $P \leq 0.05$, $P < 0.01$, and $P \leq 0.001$, respectively, chi-square test). The black and white bars show the means in pheromone and odorless fields, respectively. On day 0, the first tests were performed 15 min after emergence (1 hr after the onset of the photophase).


Day 0
Day 1
Day 2
Final choice (%)
100
50
0
50
100
***
**
*
1
2
4
6
8
10
0
Time (hours of photophase)

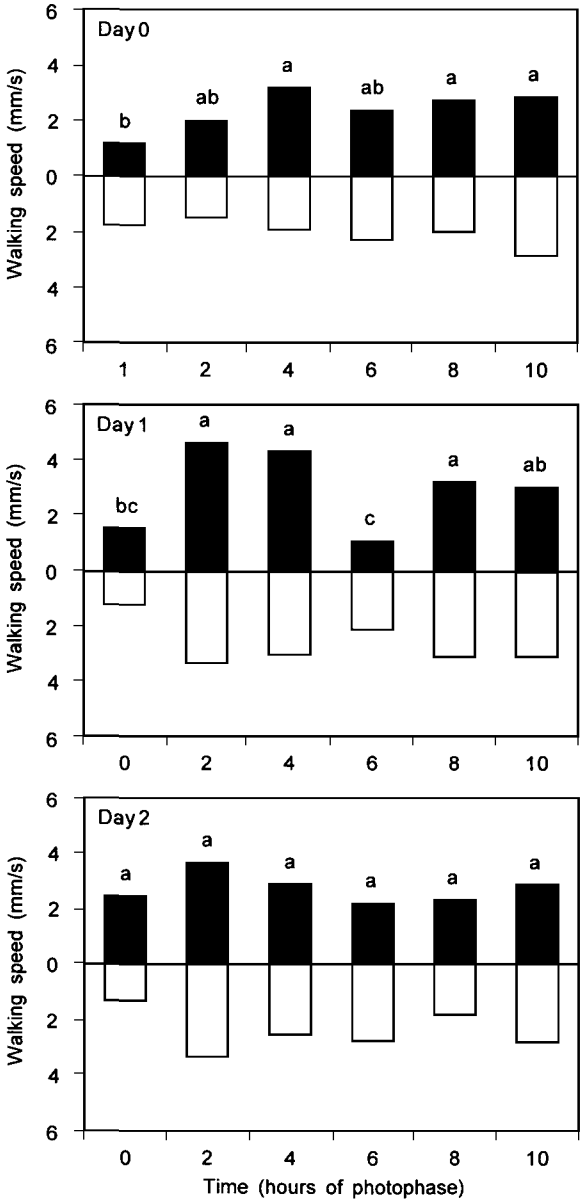


FIG. 4. The walking speed of <2-hr-old *Anaphes listronoti* males to odor emitted by one virgin female during the first three days of life (means followed by the same letter did not differ significantly at $P > 0.05$ between pheromone fields, ANOVA followed by comparison of means). The black and white bars show the means in pheromone and odorless fields, respectively. On day 0, the first tests were performed 15 min after emergence (1 hr after the onset of the photophase).

($F_{1,333} = 3.13$; $P = 0.08$). Walking speed was significantly higher during day 1 compared with day 0 ($P = 0.0065$), and values for day 2 were similar to values for day 0 ($P = 0.1012$) and day 1 ($P = 0.3946$). On day 1, the highest walking speed was observed earlier than on day 0, and the lowest values were observed at 0 and 6 hr after the onset of the photophase, suggesting a bimodal temporal pattern in pheromone emission at day 1. On day 2, the walking speed was not statistically different between hours of the photophase.

Generally, male behavior changed considerably in the presence of a virgin female releasing sexual pheromone. Males showed typical wing-fanning and increased their activity.

DISCUSSION

Sexual pheromones in hymenopterous parasitoids have been observed in Aphelinidae (Rao and DeBach, 1969), Aphidiidae (Gordh and DeBach, 1978), Braconidae (Vinson, 1972), Chalcididae (Leonard and Ringo, 1978), Cynipidae (Read et al., 1970), Eucharitidae (Gordh and DeBach, 1978), Eulophidae (Takahashi and Sugai, 1982), Eurytomidae (Pittara and Katsoyannos, 1985), Ichneumonidae (Robacker et al., 1976), Pteromalidae (Yoshida, 1978), Scelionidae (Schwartz and Gerling, 1974), and Trichogrammatidae (Pintureau and Toonders, 1983). This is the first report, however, of the occurrence of a sexual pheromone for a species belonging to the family Mymaridae, although Bakkendorf (1934) mentioned the possible existence of odor involved in mate finding in the mymarid *Cleruchus pluteus*.

Sexual pheromone alone was an adequate stimulus to elicit male sexual behavior, as our set-up eliminated any visual or tactile stimuli that could be used by parasitoids during courtship (Assem, 1986). Therefore, we suppose that the wing-fanning behavior of *A. listronoti* males during courtship observed by Collins and Grafius (1986c) was triggered by the sexual pheromone emitted by the female.

No difference was observed in the walking speed of males in the pheromone and odorless fields at any time interval. Upon perception of the sexual pheromone, they continued to walk quickly for a certain time after they left the pheromone odor field. The locomotion of *A. listronoti* males after leaving a pheromone plume may be an adaptive behavior that increases the probabilities of their reentering readily the pheromone plume in order to locate a mate. It is also possible that the odorless fields may have been contaminated by the volatile female sexual pheromone (Giles et al., 1996).

The response of males suggests a diel periodicity in the emission of a long-range sexual pheromone with a peak early in the photophase. The absence of male response at the beginning of the photophase suggests that for newly emerged females it may take a few hours to biosynthesize the long-range pheromonal

components. During this time, the pheromone could be released in a quantity not concentrated enough to attract males over a long distance. If *A. listronoti* females emitted a high concentration of pheromone shortly after emergence, we would have observed a reduction in the percentage of final choice and in the walking speed but an increase of the time spent by males in the odor plume (Baker et al., 1981). Because the time spent by males in the pheromone field was not higher than in odorless fields, the reduction of male response was probably not due to a high concentration.

In the gregarious *A. listronoti*, both sexes are sexually mature at emergence (Collins and Grafius, 1986c), and most mating could be expected to occur at the emergence site, as is observed for other gregarious parasitoids (Godfray, 1994). Because the likelihood of finding a mate at the emergence site is high, we suppose that, upon emergence, *A. listronoti* females release a short-range pheromone to arrest neighboring males in the mating site and, within 4 hr of emergence, start releasing pheromone eliciting long-range attraction of more distant males. Short- and long-range attraction to females have been suggested for the braconids *Macrocentrus grandii* (Swedenborg and Jones, 1992a; Swedenborg et al., 1993) and *Cotesia flavipes* (Kimani and Overholt, 1995) and the Aphidiidae *Aphidius nigripes* (McNeil and Brodeur, 1995).

Male behavior during the first half of the scotophase suggests that no volatile pheromone was released but, in the second half of the scotophase, the number of final choices and the walking speed indicate some emission of pheromone. The emission during the scotophase for a species that is sexually diurnal is not unusual. In some Lepidoptera species where mating occurs during the night, the emission of sexual pheromone during the day has been observed (Haynes et al., 1983; Raina et al., 1986). In *A. listronoti*, mating probably occurs principally during the day because the emission during the photophase was clearly demonstrated.

The response of males on days 1 and 2 indicates that females were still releasing long-distance sexual pheromone. We anticipated a smaller number of females emitting pheromone because *A. listronoti* is arrhenotokous and oviposition of unmated females occurs mostly during the two days following emergence (Collins and Grafius, 1986a). Release of pheromone and oviposition are probably not mutually exclusive. In some Hymenoptera, cuticular hydrocarbons have been reported as sexual pheromone components (Bartelt et al., 1982; Swedenborg and Jones, 1992b). It is possible that *A. listronoti* females synthesize cuticular hydrocarbons that are spread over the cuticle and volatilize from the body surface while females search for host eggs.

The bimodal temporal pattern of pheromone emission observed on days 1 and 2 suggests that, in nature, pheromone emission would occur in the morning and then later in the afternoon. In field situations, peaks in the number of males caught with female-baited traps have been observed for other parasitoid species. The majority of males in the aphid parasitoids *A. nigripes* and *A. colemani* were

caught, respectively, between 06:00 and 10:00 hr and before noon (McNeil and Brodeur, 1995; Reed et al., 1994). However, additional observations at time intervals shorter than 2 hr and field studies are required to verify a bimodal temporal pattern of *A. listronoti* pheromone emission during the photophase.

Swier et al. (1977) observed relationships between age and onset of pheromone emission in lepidopterous females and suggested that older females increase their probability of attracting males by advancing the time of pheromone emission. In *A. listronoti*, unmated older females emitted pheromone earlier, increasing their probability of attracting more distant males. However, the decreased number of final choices and the reduced walking speed observed on day 2, compared with day 1, suggest that some females either stopped emitting sexual pheromone, released it during a shorter time period, or decreased the concentration of pheromone emission.

Considerable attention has been directed toward monitoring parasitoid species in the field (Brodeur and McNeil, 1994; Decker et al., 1993; Swedenborg et al., 1993). *Anaphes listronoti* is presently monitored by exposing host eggs in the field or by sampling carrot plants for the presence of parasitized eggs. These methods establish the presence of the parasitoid in a field; however, they only estimate its abundance because several eggs may be parasitized by the same female. Direct counting of adult parasitoids which makes use of a trapping method with live females could provide a means of monitoring the presence and the abundance of *A. listronoti* in a field situation.

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NEONATE *Plutella xylostella* RESPONSES TO SURFACE WAX COMPONENTS OF A RESISTANT CABBAGE (*Brassica oleracea*)

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Abstract—Behavior of neonate *Plutella xylostella* was observed and quantified during the first 5 min of contact with cabbage surface waxes and surface wax components deposited as a film (60 $\mu g/cm^2$) on glass. The time larvae spent biting was greater and the time walking was less on waxes extracted from the susceptible cabbage variety, Round-Up, than on an insect-resistant glossy-wax breeding line, NY 9472. The waxes of both cabbage types were characterized and some of the compounds present at higher concentrations in the glossy waxes were tested for their deterrent effects on larvae by adding them to the susceptible waxes. Adding a mixture of four *n*-alkane-1-ols or a mixture of α- and β-amyrins to wax from susceptible cabbage reduced the number of insects biting and, among those biting, reduced the time biting and increased the time walking in a dose-dependent manner. Among individual *n*-alkane-1-ols, adding C_{24} or C_{25} alcohols reduced the number of insects biting but only adding C_{25} alcohol reduced the time spent biting among those insects that initiated biting. Adding a mixture of five *n*-alkanoic acids did not affect biting, but increased the time spent palpating and decreased walking time. Among individual *n*-alkanoic acids, only adding C_{14} significantly increased the time palpating. If the observed responses were gustatory, the results indicate that some primary wax components, including specific long-chain alkyl components, have allelochemical activity influencing host acceptance behavior by a lepidopteran larva.

Key Words—Surface waxes, triterpenoids, amyrins, alcohols, fatty acids, diamondback moth, glossy wax, deterrent, host selection, insect-plant interactions, host plant resistance.

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INTRODUCTION

From an ecological and evolutionary perspective, plant surface attributes are expected to be important factors influencing host selection by phytophagous insects (Chapman, 1977). Plant surface waxes may play a role in this process. Several studies show that they influence herbivore behavior (reviewed in Woodhead and Chapman, 1986; Juniper, 1994; Eigenbrode and Espelie, 1995), but it is not always clear whether the primary wax components, or plant secondary compounds associated with the waxes, are the active factors. Some reports show that wax components themselves have allelochemical activity (Klingauf et al., 1971; Bernays et al., 1976; Greenway et al., 1978; Sherwood et al., 1981; Mori, 1982; Phelan and Miller, 1982; Woodhead, 1983; Herbach, 1987; Varanda, 1992; Udayagiri and Mason, 1997), but rarely have these studies included wax components known to vary among host plants that elicit different behavior by an insect herbivore (Bernays et al., 1976; Woodhead, 1983). More studies are needed to examine the responses of insects to typical surface wax components with potential relevance in host selection.

Brassica oleracea with glossy surface waxes are resistant to *Plutella xylostella* L. (Lepidoptera: Plutellidae), and this resistance is associated with different neonate larval behavior on the plant surfaces (Eigenbrode et al., 1991a). *P. xylostella* larvae spent more time walking and searching and less time biting and palpating on surface waxes extracted from an insect-resistant cabbage that has a glossy wax caused by the mutation g_a (Dickson and Eckenrode, 1980; Dickson et al., 1984) than they do on waxes extracted from a susceptible cabbage with a typical waxy bloom (Eigenbrode et al., 1991b). This effect appears to be allelochemical because the waxes as bioassayed did not differ in surface wax morphology. Primary wax components appear to be the active compounds, rather than volatiles or polar compounds that might be on the plant surface, because the extracts were obtained with hexane and subjected to evaporation under reduced pressure at 40°C. The glossy waxes had smaller proportions of *n*-alkanes, secondary alcohols, and ketones and greater proportions of *n*-alkanoic acids (fatty acids), *n*-alkane-1-ols (primary alcohols), and triterpenols as compared with typical *B. oleracea* waxes (Eigenbrode et al., 1991b). The components comprising a greater proportion in waxes from the susceptible cabbage may include stimulants that increase biting, palpating, and spinning and decrease walking, while those comprising a greater proportion in waxes from resistant glossy plants may include deterrents that reduce acceptance behavior by the larvae. The present study was designed to test the deterrent effects of some of the surface wax components elevated in a glossy wax cabbage with the gl_a gene.

METHODS AND MATERIALS

Plants and Insects. Surface waxes were obtained from two cabbage genotypes grown in the field with irrigation in March and April 1994 in Tucson, Arizona. The susceptible typical-wax cabbage was the commercial hybrid Round-Up (Fery Morris), and the resistant glossy-wax cabbage was breeding line NY 9472 expressing gl_a, obtained from Dr. M. H. Dickson, Cornell University.

P. xylostella neonates were obtained from a colony maintained on artificial diet at the University of Arizona and derived from the Geneva 88 colony (A. M. Shelton, Cornell University). Eggs were collected on aluminum foil and allowed to hatch at room temperature. Within approximately 1 hr of hatch, insects descending from the foil sheet on silk strands were used in behavioral bioassay.

Bioassay Procedure. The bioassay procedure was similar to the one used by Eigenbrode et al. (1991a). Wax mixtures were deposited as an amorphous film (60 $\mu g/cm^2$ unless otherwise noted) on glass slides (4-cm × 4-cm) by rapid evaporation of a hexane solution. Neonate *P. xylostella* were confined on this film within a 4-cm-diam. circular arena bounded by a bead of silicone grease (Dow-Corning). Individual neonates were observed with a dissecting microscope during their first 5 min of contact with each test mixture, and the total times in behavioral categories (Table 1) were recorded on a computer (Arena program, Eigenbrode and McInnis, unpublished; Eigenbrode et al., 1989). Observations

TABLE 1. CATEGORIES USED TO QUANTIFY NEONATE *P. xylostella* BEHAVIOR IN BIOASSAYS

Behavioral category	Defnition
Biting	Contracting mandibular muscles, visible through cuticle, while mouthparts are in contact with substrate
Spinning	Deliberate side-to-side movement of head while spinning a strand and anchoring the strand of silk at the extremes of this movement
Palpating	Touching mouthparts repeatedly to the substrate but not biting
Searching	Raising the front half of the body from the substrate and moving the body from side to side
Walking	Forward movement
Other	Unidentifiable; stationary; or in contact with barrier on observational arena; together accounting for less than 5% of total time

were conducted in a controlled temperature cabinet (28°C). Illumination was omnidirectional from a fiberoptic ring light attached to the microscope objective.

Response of neonate larvae to wax extracts of NY 9472 and Round-Up was quantified to confirm previously reported relatively reduced biting and increased walking on waxes from the resistant plant (Eigenbrode et al., 1991a). The wax extracts were analyzed (methods below), and representatives of primary alcohols, fatty acids, and triterpenoids (three classes of components found in higher concentrations in the NY 9472 waxes as compared with Round-Up waxes) were bioassayed by adding them as purified compounds to Round-Up waxes. The procedure was designed to detect any deterrent effects of these components in the context of a surface wax mixture as they would be encountered on the plant. The waxes from susceptible cabbage stimulate biting, palpating, and spinning silk, providing a baseline response against which deterrence of tested components can be detected.

Two types of tests were conducted. In the first, susceptible waxes were augmented with mixtures of the components within each of the three classes. The mixtures of primary alcohols and fatty acids were in equal proportions by weight, and the mixture of amyrins was the 1:8 α:β ratio obtained from a natural source (see below). Each mixture was added to susceptible wax extracts in a range of amounts to approximate the concentration of the respective class in NY 9472 waxes. In the second type of test, individual primary alcohols and fatty acids were used to augment Round-Up waxes by 10%. None of the treatments mimics the exact quantitative composition of the resistant NY 9472 waxes, nor were the amounts of wax adjusted to reflect the lower amounts of waxes found on NY 9472 versus Round-Up (Eigenbrode et al., 1991b). The primary objective was to identify components that act as deterrents in a wax mixture.

The control in all bioassays was the susceptible wax extract alone (control I). In some experiments, a second control (control II) was used to measure the effect of dilution of susceptible waxes by the test mixtures. Control II consisted of the susceptible waxes applied to the glass plate in an amount reduced proportional to the maximum dilution by the test mixture.

Test Compounds and Mixtures. Individual (>98% purity) primary alcohols and fatty acids were obtained commercially (Sigma, St. Louis). Amyrins were extracted and purified from germinating *Pisum sativum*, following the methods of Takayuki and Tsuyoshi (1975). Peas (450 g, three days after imbibition) were ground in a mortar and pestle and soaked in 2 liters of methanol for 24 hr. The methanolic extract was filtered and partitioned two times against equal volumes of hexane. The hexane fraction was concentrated and separated on silica gel G columns (2 cm × 10 cm) with a 9:1 hexane–ether mobile phase. Fractions were monitored with GC-MS to isolate amyrins to 98% purity. The α- and

β-amyrin ratio was 1 : 8 in the purified mixture, and the contaminants appeared to be structurally related based on their mass spectra.

Wax Extracts and Analysis. Plant waxes were extracted from NY 9472 and Round-Up cabbage at the preheading stage with 20-sec hexane washes of mature leaves at room temperature. To avoid extracting internal plant constituents, cut petioles and damaged leaves were not immersed in the solvent. Waxes of all leaves of 10 plants of each type were pooled. For analysis, each wax extract was derivatized with *N,O*-bis(trimethylsilyl)acetamide to generate trimethylsilyl ethers and esters of alcohols and acids. The derivatized mixture was dissolved in hexane and analyzed by gas chromatography (GC). For quantification, the derivatized lipids were injected along with an *n*-hexadecane internal standard onto a fused silica capillary column (100% methylpolysiloxane, HP Ultra-1, 15 m, 0.2 mm ID, 0.250 μm film) on a Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionization detector. The oven temperature program was 80°C for 1 min, 15°/min to 260°C, hold 10 min, then 5°C/min to 320°C and hold 15 min. Response factors were determined from known standards (*n*-pentacosane, *n*-heptacosane, *n*-nonacosane, *n*-triacontane, and trimethyl-silyl esters and ethers of 1-tetradecanoic acid, 1-hexadecanoic acid, 1-octadecanoic acid, 1-hexacosanoic acid, 1-docosanol, 1-octacosanol, and 1-triacontanol), or approximated for components for which standards were not obtained. Identification of the components was on the basis of mass spectra of representative samples run on a Hewlett Packard 5890 gas chromatograph equipped with a HP 5973 quadrapole mass selective detector and on the retention times of standards. Relative composition of the wax components was calculated on a percent basis.

Experimental Design and Statistical Analysis. Thirty insects were observed on the wax extracts from glossy and normal-wax cabbage. Forty insects were observed on treatments involving Round-Up waxes augmented with pure compounds. Three to five insects were observed on each of several individual preparations of the treatments within an experiment. Because all wax treatments appeared similar, it was possible to conduct the experiments using a single-blind design in which the observer was not aware of the treatment. Biting was the most variable behavioral category, and some insects did not bite at all during a 5-min observation. Therefore, a two-step analysis was used. First, the proportion of insects biting during the 5-min observation was compared among treatments using χ^2. Second, only the subset of insects biting in 5 min was included in a multivariate analysis of variance (MANOVA) (Harris, 1985) with times (standardized to seconds per minute) in each behavior as dependent variables and wax treatment as the independent variable. Wilk's lambda MANOVA statistic assessed the significance of treatment effect on all behavioral categories simultaneously and individual ANOVA *F* statistics were used to assess the influence

of each behavior. Times in each behavior were transformed to $\log(x)$ to normalize distributions and stabilize variances before analysis.

RESULTS

A smaller proportion of insects initiated biting during 5 min on waxes from glossy cabbage NY 9472 than on waxes from susceptible Round-Up cabbage (Table 2). Among those insects biting within 5 min, time allocated to behavioral categories differed on the two waxes, largely because of reduced biting and increased walking on glossy waxes (Figure 1).

The waxes on the two types of cabbage differed in composition. The concentrations of primary alcohols, fatty acids, and amyrins were elevated in the glossy cabbage waxes (Table 3) as reported for another NY line expressing gl_a (Eigenbrode et al., 1991a). In addition, this analysis showed esters, alkenes, and some unidentified components also are elevated in NY 9472 glossy waxes. Individual wax components selected for subsequent bioassay are indicated in Table 3.

The proportion of insects biting on waxes from susceptible Round-Up was reduced when these waxes were augmented with a mixture of primary alcohols (Table 2). Among those insects biting within 5 min, time allocated to the behavioral categories differed significantly; specifically, biting was reduced and walking was increased on waxes augmented with primary alcohols (Figure 2). There was an approximate dose-related response to primary alcohol concentration in total time spent biting and walking.

The number of insects biting differed significantly among Round-Up waxes augmented with the individual primary alcohols and the control (Table 2). The number of insects biting on waxes augmented with C_{24} and C_{25} alcohols was lower compared to the control and waxes augmented with C_{26} and C_{27} alcohols. Among those insects biting during 5 min, time allocated to behavioral categories differed significantly in response to adding different alcohols (Figure 3); specifically, biting was greatly reduced on waxes with added C_{25} alcohol, and searching was reduced on waxes with added C_{24} alcohol. There was a tendency towards reduced biting, spinning, and palpating in response to adding alcohols with odd-numbered chain lengths (C_{25} and C_{27}) as compared with the even-numbered chain-length alcohols (Figure 3).

The proportion of insects biting on susceptible waxes was not significantly reduced by augmenting with a mixture of fatty acids (Table 2). Among insects biting during 5 min, time allocated to behavioral categories differed significantly; specifically, spinning and palpating were increased and walking was decreased in a dose-dependent manner in response to augmenting with the mixture of fatty acids (Figure 4).

TABLE 2. PROPORTION OF *P. xylotella* NEONATES BITING DURING 5 MINUTES ON EXTRACTED SURFACE WAXES OF RESISTANT AND SUSCEPTIBLE CABBAGE AND ON WAXES OF SUSCEPTIBLE CABBAGE AUGMENTED WITH PURIFIED WAX COMPONENTS

Experiment and treatments	Test mixture ($\mu g/cm^2$)[a]	% of test compounds in mixture[b]	N	Prop. biting	P[c]
Cabbage wax extracts					
Waxes of susceptible Round-Up	60		30	0.97	
Waxes of resistant glossy NY 9472	60		30	0.83	0.047
Susceptible wax with added mixture of primary alcohols					
Control I	60	0	40	0.93	
Control II	36	0	40	0.76	
Primary alcohols C_{24}, C_{25}, C_{26}, C_{27}	60	5	40	0.83	
Primary alcohols C_{24}, C_{25}, C_{26}, C_{27}	60	10	40	0.50	
Primary alcohols C_{24}, C_{25}, C_{26}, C_{27}	60	20	40	0.43	
Primary alcohols C_{24}, C_{25}, C_{26}, C_{27}	60	40	40	0.53	<0.001
Susceptible wax with added individual primary alcohols					
Control I	60	0	40	0.83	
C_{24}	60	10	40	0.70	
C_{25}	60	10	40	0.57	
C_{26}	60	10	40	0.83	
C_{27}	60	10	40	0.93	0.008
Susceptible wax with added mixture of fatty acids					
Control I	60	0	40	0.76	
Control II	48	0	40	0.76	
Fatty acids C_{14}, C_{16}, C_{18}, $C_{18:1}$, C_{26}	60	1	40	0.73	
Fatty acids C_{14}, C_{16}, C_{18}, $C_{18:1}$, C_{26}	60	5	40	0.57	
Fatty acids C_{14}, C_{16}, C_{18}, $C_{18:1}$, C_{26}	60	10	40	0.50	
Fatty acids C_{14}, C_{16}, C_{18}, $C_{18:1}$, C_{26}	60	20	40	0.60	0.119
Susceptible wax with added individual fatty acids					
Control I	60	0	40	0.73	
Control II	54	0	40	0.85	
C_{14}	60	10	40	0.58	
C_{16}	60	10	40	0.64	
C_{18}	60	10	40	0.75	
$C_{18:1}$	60	10	40	0.44	
C_{26}	60	10	40	0.75	0.034
Susceptible wax with added amyrins					
Control I	60	0	40	0.90	
α- and β-amyrin (8:1)	60	1	40	0.51	
α- and β-amyrin (8:1)	60	3	40	0.56	
α- and β- amyrin (8:1)	60	5	40	0.37	<0.001

[a] Amount of test mixture applied to glass for bioassay.
[b] % by weight by which susceptible waxes were augmented with the test compound or test mixture.
[c] P value for χ^2 based on expected equal proportion biting on all treatments within an experiment.

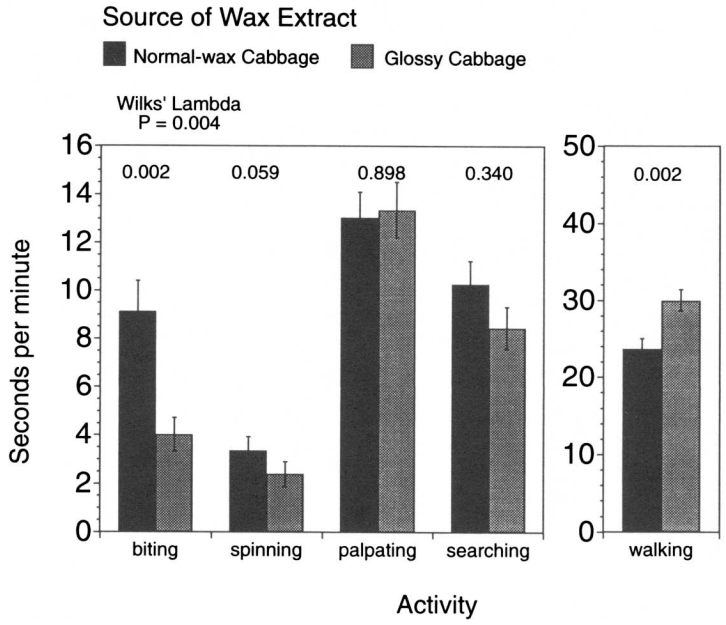

FIG. 1. Proportion of time (seconds per min averaged over 5-min observation) spent by neonate *P. xylostella* in different activities on waxes extracted from resistant, glossy NY 9427 and susceptible, normal-wax Round-Up cabbage. The Wilk's lambda *P* value was used to test for significance of all behavioral categories as response variables to the treatments. Individual *P* values are for ANOVAs for each behavioral category. Of the 30 insects, those biting during the assay were included in the analysis: normal-wax, 29; glossy, 25.

TABLE 3. RELATIVE COMPOSITION (% BY WEIGHT) OF NY 9472 AND ROUND-UP CABBAGE SURFACE WAXES AND COMPOUNDS TESTED FOR DETERRENT EFFECTS ON *P. xylostella* LARVAE

Component class and individual component (order of elution within component class)	Carbon chain length	Round-Up normal-wax (%)	NY 9472 glossy wax (%)	Tested for deterrent effect
Aldehydes				
Tetracosanal	24		0.49	
Hexacosanal	26	0.33		
Octacosanal	28	0.23		

TABLE 3. CONTINUED

Component class and individual component (order of elution within component class)	Carbon chain length	Round-Up normal-wax (%)	NY 9472 glossy wax (%)	Tested for deterrent effect
Alkanes				
Heneicosane	21		0.15	
Tetracosane	24		0.38	
Pentacosane	25	0.17	0.98	
Heptacosane	27	0.65	1.94	
Nonacosane	29	45.18	1.61	
Triacontane	30	0.10	0.29	
Hentriacontane	31	4.26		
Alkenes				
Nonacosene	29	0.17	1.74	
Amyrins				
α-amyrin		0.00	0.59	*
β-amyrin		0.20	0.98	*
Fatty acids				
Tetradecanoic acid	14	0.08	0.19	*
Pentadecanoic	15	0.09	0.11	
Hexadecanoic acid	16	0.07	0.65	*
Octadecenoic acid	18:1	0.03	0.24	*
Octadecanoic acid	18	0.09	0.49	*
Eicosanoic acid	20		0.13	
Docosanoic acid	22	0.07	0.38	
Tetracosanoic acid	24	0.08	1.35	
Hexacosanoic acid	26	0.75	6.23	*
Ketones				
Nonacosanone	29	25.05	2.55	
Primary alcohols				
Docosanol	22	0.04	0.97	
Tricosanol	23	0.12	0.24	
Tetracosanol	24	0.16	2.97	*
Pentacosanol	25	0.18	0.79	*
Hexacosanol	26	0.76	20.66	*
Heptacosanol	27	0.97	1.98	*
Octacosanol	28	0.31	0.57	
Secondary alcohol				
13- and 14-Heptacosanol	27	0.10	0.74	
14- and 15-Nonacosanol	29	13.41		
Secondary diol				
14,15-Nonacosandiol	29	0.63		
Wax esters		1.91	38.02	
Unidentified		3.70	11.58	
Total		100.00	100.00	

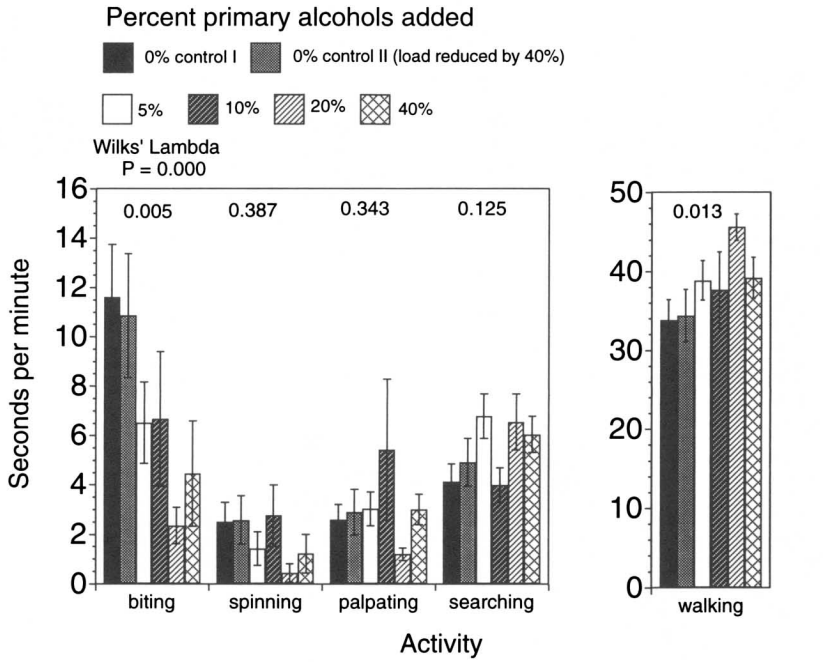

FIG. 2. Proportion of time (seconds per min averaged over 5-min observation) spent by neonate *P. xylostella* in different activities on waxes of susceptible cabbage augmented with a mixture of primary alcohols (C_{24}, C_{25}, C_{26}, C_{27}) at a range of concentrations. The Wilk's lambda *P* value was used to test for significance of all behavioral categories as response variables to the treatments. Individual *P* values are for ANOVAs for each behavioral category. Of a sample of 40 insects, those biting during the assay were included in the analysis: control I, 37; control II, 23; 5%, 33; 10%, 20; 20%, 17; 40%, 21.

The proportion of insects biting on waxes augmented with individual fatty acids differed significantly, largely due to a reduced proportion biting on waxes with added $C_{18:1}$ fatty acid (oleic) (Table 2). Among those insects biting during 5 min, time allocated to behavioral categories differed. Only palpation differed among the treatments, and the greatest effect was increased palpating in response to added C_{14} fatty acid (Figure 5). There were no other significant effects of individual fatty acids on larval behavior.

The proportion of insects biting was significantly reduced on waxes from susceptible Round-Up augmented with the mixture of α- and β-amyrins (Table 2). Among those insects biting during 5 min, time allocated to behavioral categories differed significantly; biting and spinning were reduced, and walking

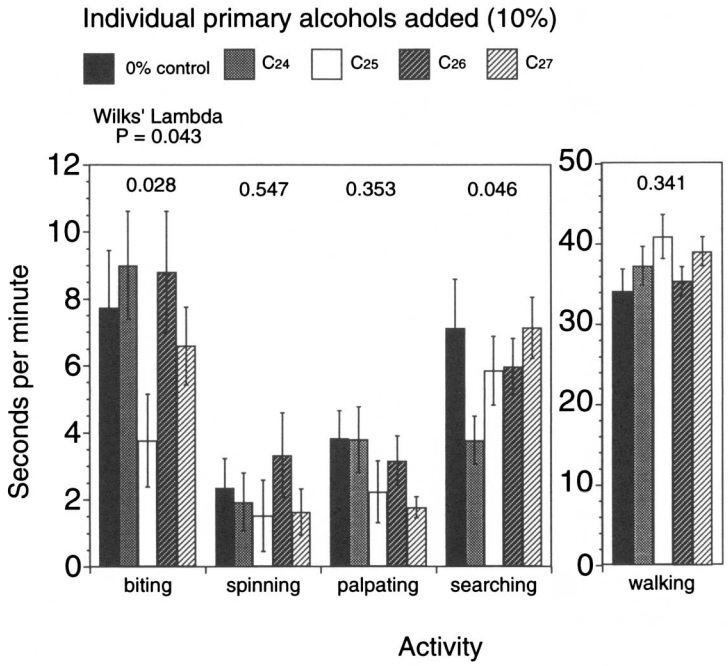

FIG. 3. Proportion of time (seconds per min averaged over 5-min observation) spent by neonate *P. xylostella* in different activities on waxes of susceptible cabbage augmented by 10% with individual primary alcohols. The Wilk's lambda *P* value was used to test for significance of all behavioral categories as response variables to the treatments. Individual *P* values are for ANOVAs for each behavioral category. Of a sample of 40 insects, those biting during the assay were included in the analysis: control, 33; C_{24}, 28; C_{25}, 33; C_{26}, 33; C_{27}, 37.

was increased on waxes with added amyrins (Figure 6). A dose–response effect is apparent for all three behavioral categories.

Control II, which measured the effect of dilution of the susceptible waxes with tested components was not substantially different from control I, except in the alcohol mixture experiment. In that experiment, control II consisted of susceptible waxes reduced by 40%, resulting in a film of only 36 $\mu g/cm^2$, which reduced the number of insects biting (Table 2). Therefore, with the exception of the waxes augmented by 40% with primary alcohols, the effects detected in these bioassays can be attributed to allelochemical activity rather than to dilution of compounds in the susceptible waxes.

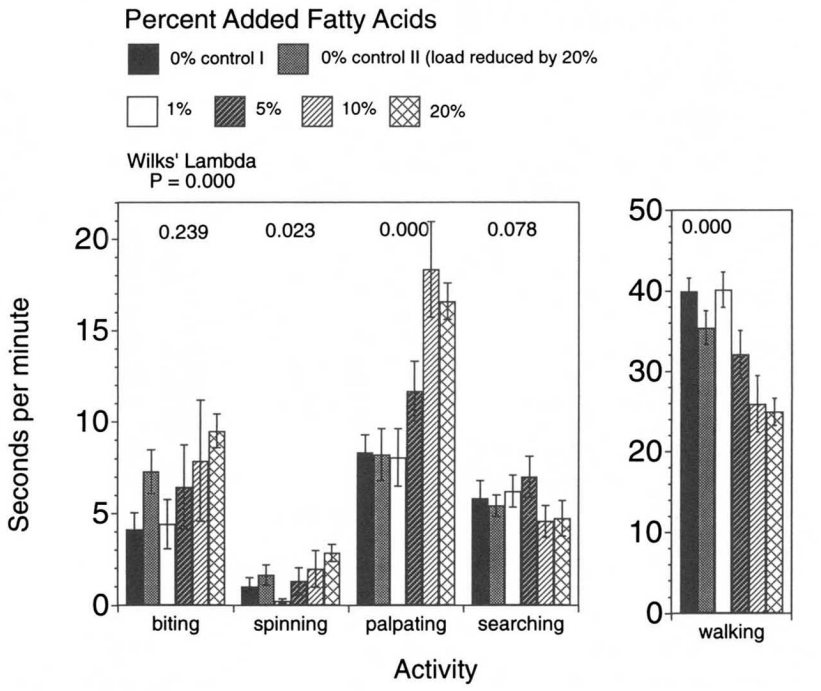

FIG. 4. Proportion of time (seconds per min averaged over 5-min observation) spent by neonate *P. xylostella* in different activities on waxes of susceptible cabbage augmented with a mixture of fatty acids (C_{14}, C_{16}, C_{18}, $C_{18:1}$, C_{26}) at a range of concentrations. The Wilk's lambda *P* value was used to test for significance of all behavioral categories as response variables to the treatments. Individual *P* values are for ANOVAs for each behavioral category. Of a sample of 40 insects, those biting during the assay were included in the analysis: control I, 30; control II, 30; 1%, 29; 5%, 20; 10%, 20; 20%, 24.

DISCUSSION

Waxes of resistant glossy cabbages and susceptible cabbages differ allelochemically, as indicated by reducing biting and increased walking by *P. xylostella* neonates on glossy waxes (Eigenbrode et al., 1991a). The higher concentrations of amyrins and primary alcohols in glossy waxes must contribute to this larval response. Augmenting waxes from susceptible cabbage with mixtures of amyrins or primary alcohols reduced larval biting and increased walking on these waxes in an approximately dose-dependent manner. On waxes from susceptible cabbage augmented with primary alcohols or amyrins to concentrations

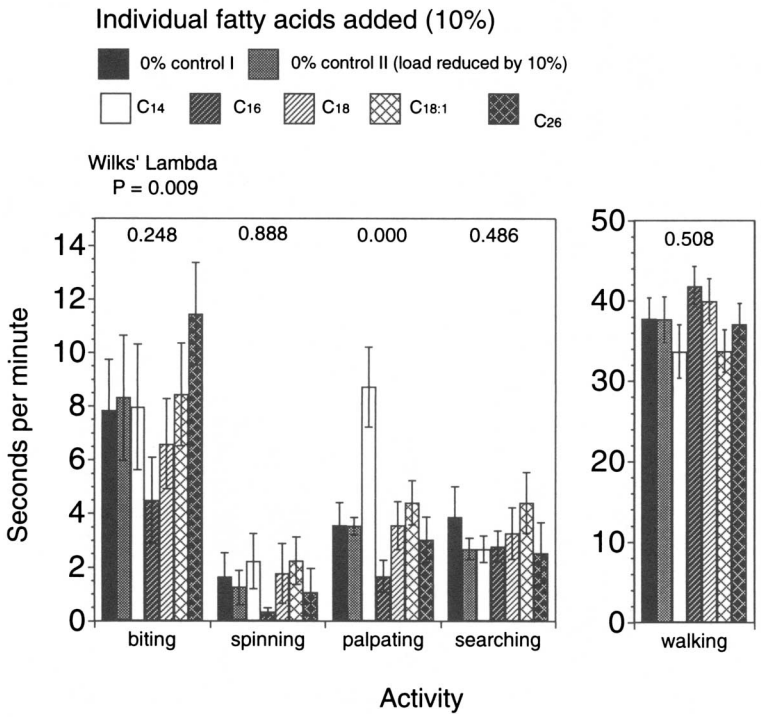

FIG. 5. Proportion of time (seconds per min averaged over 5-min observation) spent by neonate *P. xylostella* in different activities on waxes of susceptible cabbage augmented by 10% with individual fatty acids. The Wilk's lambda *P* value was used to test for significance of all behavioral categories as response variables to the treatments. Individual *P* values are for ANOVAs for each behavioral category. Of a sample of 40 insects, those biting during the assay were included in the analysis: control I, 29; control II, 34; C_{14}, 23; C_{16}, 26; C_{18}, 30; $C_{18:1}$, 18; C_{26}, 30.

near those occurring in glossy waxes, larval biting and walking rates were similar to those on waxes from glossy plants.

In contrast to the primary alcohols and amyrins, the greater concentration of fatty acids in glossy waxes may not contribute to reduced biting and increased walking on these waxes. Adding a test mixture of five of these fatty acids to waxes from susceptible cabbage increased palpation and spinning and decreased walking by *P. xylostella*, indicating these compounds could be stimulants or arrestants.

Among individual primary alcohols, only C_{25} alcohol substantially deterred

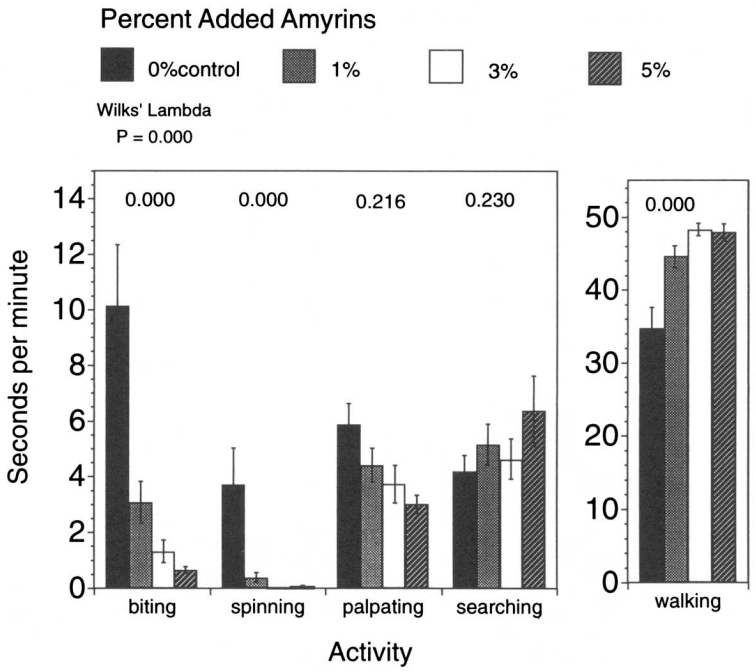

FIG. 6. Proportion of time (seconds per min averaged over 5-min observation) spent by neonate *P. xylostella* in different activities on waxes of susceptible cabbage augmented with a mixture of α- and β-amyrin (8 : 1) at a range of concentrations. The Wilk's lambda *P* value was used to test for significance of all behavioral categories as response variables to the treatments. Individual *P* values are for ANOVAs for each behavioral category. Of a sample of 40 insects, those biting during the assay were included in the analysis: control I, 36, 1%, 20; 3%, 22; 5%, 15.

biting when added to wax from susceptible cabbage and may have contributed most to the effect of the mixture. Individual amyrins were not tested, and it remains to be determined whether their activities differ. Among individual fatty acids tested, only C_{14} fatty acid individually increased palpation, so this component likely accounts for increased palpation in response to the mixture. None of the individual fatty acids affected walking, so the effect of their mixture on walking remains unexplained. Finally, $C_{18:1}$ fatty acid reduced initiation of biting but had no effect on time allocation among those insects biting. Thus, the effects of fatty acids on *P. xylostella* larvae are complex and not simply related to responses of the insects to intact glossy waxes.

The data do not fully explain the larval response to waxes from glossy cabbages because the treatments employed do not mimic glossy waxes. First,

not all potential deterrents (those in higher concentrations in NY 9472 waxes), including some fatty acids and primary alcohols, were tested. Second, the concentrations in augmented mixtures differed from those in glossy waxes. C_{24}, C_{25}, and C_{27} alcohols occur in lower proportions than the C_{26} alcohol in glossy waxes, and the ratio of amyrins is 3:1 α:β, not the 8:1 ratio used in these experiments. Last, potential deterrents were tested by augmentation, but potential stimulants were not tested by removal (e.g., ketones that are in lower concentrations in glossy waxes). Nonetheless, the data indicate that cabbage surface waxes with elevated amyrins and primary alcohols, especially C_{25}, should elicit reduced acceptance by neonate *P. xylostella* and contribute to resistance to this insect.

Responses of *P. xylostella* to individual primary alcohols are consistent with a general hypothesis that atypical or rare wax components should deter herbivores, while more common ones should stimulate them (Woodhead and Chapman, 1986). Primary alcohols occur widely in plant waxes, but those with odd-numbered chain lengths always occur in lower concentrations than those with even-numbered chain lengths (Walton, 1990). In the present study, the odd-numbered chain-length alcohols, C_{25} and C_{27}, generally reduced biting, spinning, and palpating more than the even-numbered chain-length alcohols.

Responses of *P. xylostella* to individual fatty acids are not consistent with the hypothesis that rare, shorter-chain, fatty acids should be more deterrent, as has been found for other insects (e.g., Sherwood et al., 1981). Rather, effects of fatty acids tested were not clearly related to chain length.

The deterrent effects of amyrins towards *P. xylostella* are consistent with evidence that these and other saponins are deterrent or toxic to insects (Gershenzon and Croteau, 1991). Amyrins inhibit feeding by *Locusta migratoria* (Bernays and Chapman, 1976), and the structurally related ursolic acid is deterrent to *Schizaphis graminum* (Varanda, 1992). Concentrations of amyrins in surface waxes are correlated with resistance to *Stephanitis pyrioides* in *Rhododendron* (*Azalea*) (Balsdon et al., 1995) and with aphid resistance in *Sorghum* and *Rubus occidentalis* (Heupel, 1985; Robertson et al., 1991). Amyrins are major wax components in waxes of some plant species (e.g., Baas and Figdor, 1978; Smith and Severson, 1992), but are absent or minor components of waxes of the crucifer species examined so far (Eigenbrode et al., 1991a; Jenks et al., 1995). A deterrent effect of amyrins above certain concentrations in surface waxes might help *P. xylostella* larvae avoid nonhost plants in a mixed canopy.

P. xylostella responses in these bioassays were not necessarily mediated by gustation and could have been affected by physical properties of the mixtures tested. For example, shorter chain-length or unsaturated alkyl compounds have lower melting points than longer-chain or saturated homologs, and this could influence the physical properties of the mixtures containing them at the bioassay temperature (28°C). Physical characteristics of the mixtures were not measured,

however, nor is it obvious how to measure subtle differences in physical properties that might be detected by neonate insects.

Whether discrimination is by gustation or some other means, the data demonstrate behavioral activity of plant surface wax components and show that some of these components can deter insect feeding behavior. This adds to the growing evidence that plant surface wax components can mediate insect-plant interactions.

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PYGIDIAL GLAND OF *Azteca* NR. *bicolor* AND *Azteca chartifex*: MORPHOLOGY AND CHEMICAL IDENTIFICATION OF VOLATILE COMPONENTS

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Abstract—The large paired reservoirs of the pygidial gland of *Azteca* nr. *bicolor* and *A. chartifex* represents the largest exocrine structure in the abdomen. The glands produce a secretion with a strong smell, which the ants release when they are disturbed. Analyses of the secretions by gas chromatography–mass spectrometry (GC-MS) revealed a mixture of iridoids and ketones. *A.* nr. *bicolor* contains 2-heptanone (8%) and a mixture of three iridodial isomers, with *trans-trans*-iridodial as the major component (32%). *A. chartifex* contains 6-methyl-5-hepten-2-one (13%) and the three isomeric iridodials with *cis-trans*-iridodial as the principal component (32%).

Key Words—*Azteca* spp., morphology, iridoids, pygidial glands, gas chromatography–mass spectrometry.

INTRODUCTION

Azteca ants (Dolichoderinae) occur exclusively in the neotropics, being distributed from Mexico to the north of Argentina (Harada and Benson, 1988). All species of *Azteca* are arboreal. Some species have a symbiotic relationship with

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certain trees of *Cecropia* sp. while others construct carton nests in trees and shrubs.

The species of *Azteca* that possess a symbiotic relationship with *Cecropia* trees have their nests located in cavities inside the branches and trunk of the tree and feed mainly on special structures called Müllerian bodies, produced by the *Cecropia*. The ants in return keep the tree clear of any invading invertebrates. They swarm out to attack any small herbivores. The Kayapó Indians of Brazil use these ants to protect their cultivated crops from pests, such as leaf-cutting ants (Overall and Posey, 1990).

Ants use volatiles from exocrine glands for communication (Attygalle and Morgan, 1984). In dolichoderine ants, two glands are responsible for the production of volatiles involved in their communication system: Pavan's gland, situated between the 6th and 7th abdominal sternites, is the source of the trail pheromone (Wilson and Pavan, 1959). The pygidial gland occurs between the 6th and 7th abdominal tergites. In dolichoderine ants it is very large, and has sometimes been confusingly called the "anal gland" (Pavan and Ronchetti, 1955).

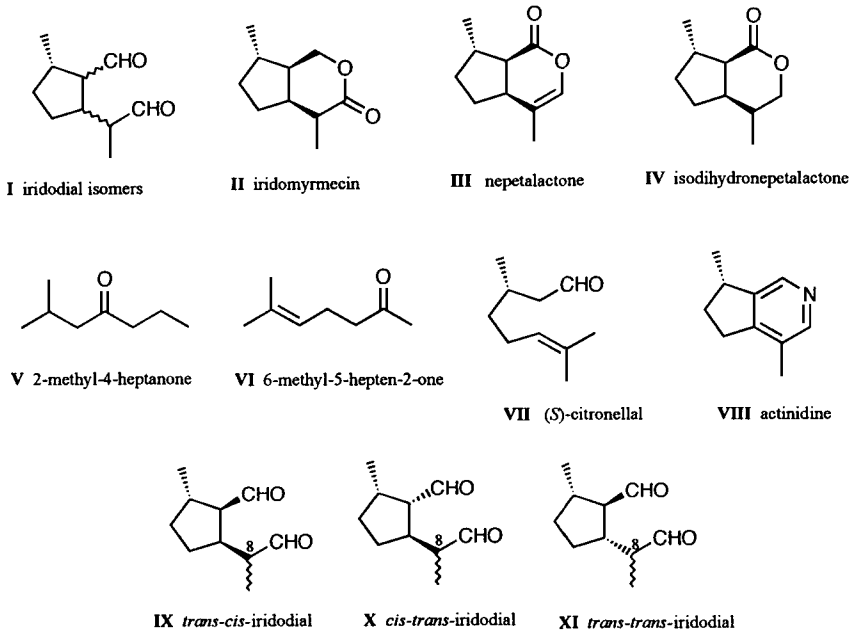
The secretion from the pygidial glands of dolichoderine ants is generally characterized by a mixture of iridoids (I–IV) and ketones (Figure 1). The iridoids seem to be used for defense, being repulsive to a number of insects whereas the ketones; for example, 2-methyl-4-heptanone (V) and 6-methyl-5-hepten-2-one (VI), elicit alarm behavior in conspecific individuals (Hölldobler and Wilson, 1990).

Knowledge of the chemical nature of volatile components present in the secretion from the pygidial glands of *Azteca* ants has until now been restricted to three species, *A.* nr. *nigriventris*, *A.* nr. *instabilis*, and *A.* nr. *velox* (Wheeler et al., 1975). Iridodial and cyclopentyl ketones (2-acetyl-3-methylcyclopentene, *cis*-1-acetyl-2-methylcyclopentane, and 2-methylcyclopentanone) were identified in the pygidial gland of these species (Wheeler et al., 1975).

In this paper, we report on the chemical characterization of the volatile compounds from the pygidial gland secretions of two *Azteca* species: *Azteca* nr. *bicolor* and *A. chartifex*. With the identification of the compounds present in the exocrine secretions of these species, we hope to increase knowledge of the chemistry of volatiles present in the secretion from these glands. Their secretions may help in chemical taxonomy and diagnosis of the species, since there are no well-defined morphological characters that differentiate the species (Harada, 1982).

METHODS AND MATERIALS

Live ant colonies of *A.* nr. *bicolor* (Formicidae: Dolichoderinae) were collected from a *Cecropia peltata* tree near the Federal University of Alagoas



I iridodial isomers II iridomyrmecin III nepetalactone IV isodihydronepetalactone

V 2-methyl-4-heptanone VI 6-methyl-5-hepten-2-one VII (*S*)-citronellal VIII actinidine

IX *trans-cis*-iridodial X *cis-trans*-iridodial XI *trans-trans*-iridodial

FIG. 1. Structures of compounds commonly encountered in the pygidial gland secretion of dolichoderine ants.

(Maceió-Alagoas, Brazil) and colonies of *A. chartifex* were collected in the Amazon region and sent to Maceió where the colonies of the two species were kept in the laboratory. Whole abdomens of *A. chartifex* were removed and sealed in soft glass capillary tubes (15 mm × 1.8 mm ID × 0.2-mm wall thickness) (Morgan, 1990), and pygidial glands of *A.* nr. *bicolor* were dissected under a binocular microscope with the aid of a pair of fine forceps and also sealed in soft glass capillary tubes.

For morphological studies, abdominal tips of *A.* nr. *bicolor* were fixed in 2% glutaraldehyde, buffered at pH 7.3 with 50 mM sodium cacodylate and 150 mM saccharose. After postfixation in 2% osmium tetroxide, they were dehydrated in acetone and embedded in Araldite, Semi-thin sections of 0.5 μm were stained with methylene blue and thionine.

Gas chromatography–mass spectrometry (GC-MS) was conducted with a Hewlett Packard 5890 gas chromatograph directly coupled to a 5970 B Mass Selective Detector (quadrupole mass spectrometer with 70 eV electron impact ionization). The system was controlled by, and data accumulated on, a Hewlett Packard Series 300 computer with HP 59970 C ChemStation. Mass spectra were scanned from *m/z* 35 to *m/z* 550. Scan rate was about 2.4 sec/scan.

Samples of *A.* nr. *bicolor* were chromatographed on an immobilized polydimethylsiloxane phase coated in a fused silica column (12 m × 0.32 mm ID × 0.32-μm film thickness). Samples of *A. chartifex* were chromatographed on a different capillary column coated with the same phase, but of a different length (14 m × 0.32 mm ID × 0.32-μm film thickness). Helium was used as carrier gas at 1 ml/min. The sample was heated in the injector to 150°C for 2 min before crushing the capillary as described by Morgan and Wadhams (1972). The oven was programmed from 30°C at 8°C/min to 200°C. The split vent was closed before crushing the sample and reopened 30 sec later.

RESULTS

The paired pygidial gland of *A.* nr. *bicolor* consists of two large reservoirs that open between the 6th and 7th abdominal tergites. On each side there is a cluster of a few tens of secretory cells (Figure 2). These rounded secretory cells with a diameter of approximately 10 μm open into the lateral wall of the reservoir through an accompanying narrow duct, according to the type-3 secretory cells in the classification of Noirot and Quennedey (1974). The reservoirs have a diameter of approximately 100 μm and represent the major exocrine structure in the abdomen. They are characterized by a wrinkled lining of flattened epithelial cells. The general organization of the pygidial gland is similar to that of other dolichoderine ants (Dazzini Valcurone and Fanfani, 1982; Billen, 1986). Because of their large size, much secretion can be stored in the gland's reservoir ready for release in an encounter.

Chemical analysis of the pygidial gland contents of *A.* nr. *bicolor* and *A. chartifex* revealed a mixture of iridoids and oxygenated compounds (Figure 3A and B). The number above each peak corresponds to the compounds listed in Table 1, identified by interpretation of their mass spectra. Identification of 2-heptanone, 6-methyl-5-hepten-2-one, isopulegol, *cis-trans*-iridodial (X), *trans-trans*-iridodial (XI), and *trans-cis*-iridodial (IX) were all confirmed by injection of synthetic standards into the GC-MS. Iridodial was synthesized by Dr. Neil J. Oldham according to the method of Clark et al. (1959). This method gives mainly the *trans-trans*-iridodial (Oldham, 1994). Based on Oldham (1994), who showed that the elution order of iridodial isomers from a nonpolar phase capillary column gas chromatography is *cis-trans-*, then *trans-trans-*, and *trans-cis-*, it was possible to identify the relative stereochemistry of the natural iridodials. Each of these compounds can exist as two epimers at C-8; only one epimer of each pair was present in both species, but the order of elution of these epimers is not known, so the stereochemistry of the C-8 methyl group is not known. 2-Acetyl-3-methylcyclopentene, 2-formyl-3-methylcyclopentaneacetaldehyde, 2-formyl-3-methylcyclopenteneacetaldehyde, 2-(3-methylcyclopentyl)propio-

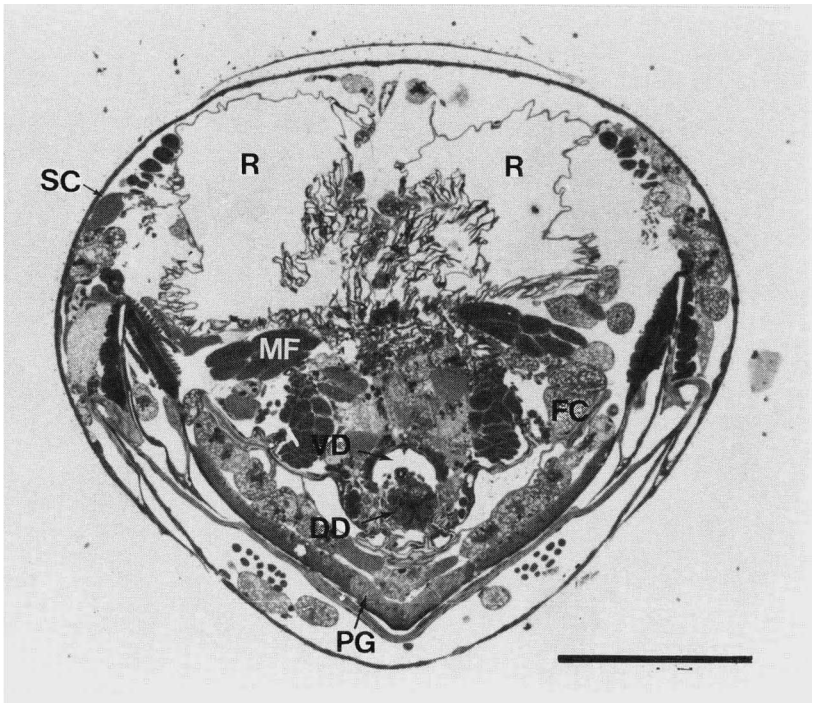


FIG. 2. Semi-thin cross section through the abdominal tip of an *Azteca* nr. *bicolor* worker, showing the large paired reservoirs (R) of the pygidial gland. DD: Dufour gland duct, FC: fat cells, MF: muscle fibers, SC: secretory cells pygidial gland, PG: Pavan's gland, VD: venom gland duct (scale bar 100 μm).

naldehyde, and an isomer of nepetalactol were identified by comparison of their mass spectra with those published in the literature. Actinidine (VIII) was also found to be present in the gland contents from three abdomens of *A. chartifex*, in which the amounts of iridodials were considerably reduced. As we have found that actinidine can be formed by heating iridodials with amino acids and we were in this case using whole abdomens for chromatography, these three specimens were omitted from consideration.

Although the iridodial isomers are common to both *A.* nr. *bicolor* and *A. chartifex*, the composition of the gland contents are species-specific. *Trans-trans*-iridodial (32%), and *cis-trans*-iridodial (21%) are the principal components in the gland of *A.* nr. *bicolor*. *Cis-trans*-iridodial (32%), and *trans-cis*-iridodial (27%) dominate the contents of the pygidial gland of *A. chartifex*. 6-Methyl-5-hepten-2-one and a small amount of isopulegol are also present in

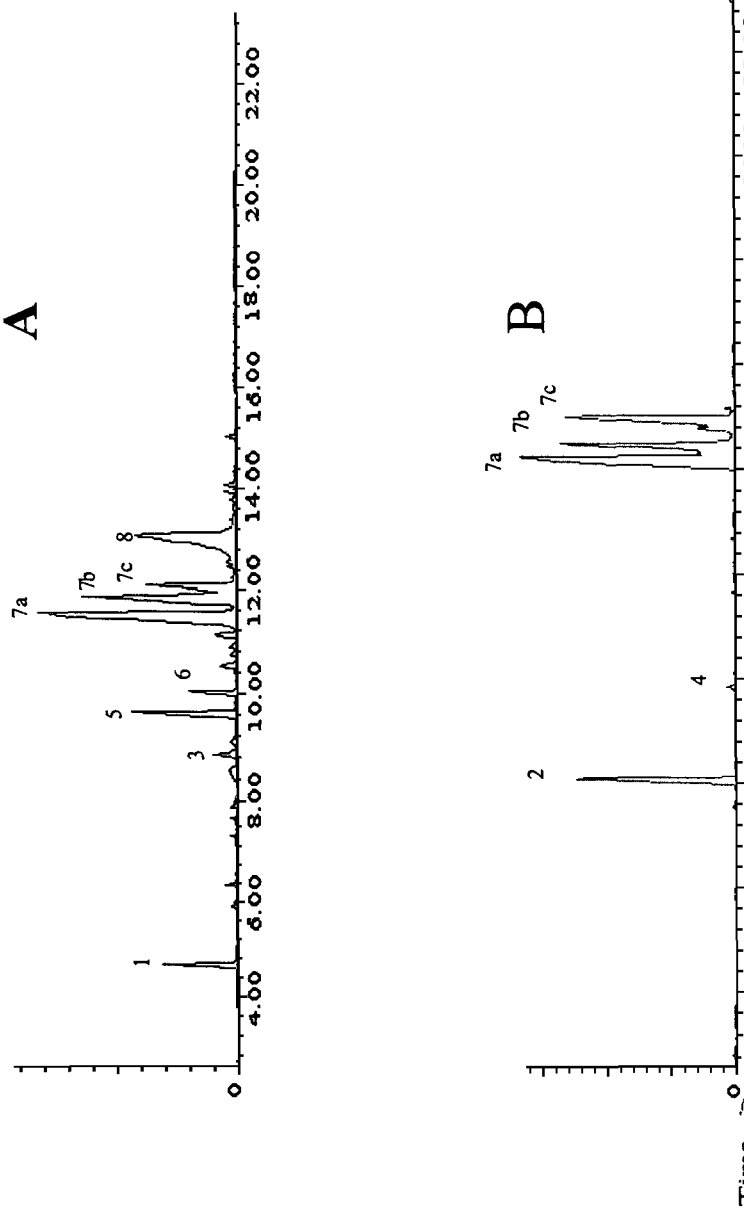


FIG. 3. Total ion chromatograms showing the volatiles from the pygidial gland of (A) *Azteca* nr. *bicolor* worker and (B) *A. chartifex* worker. The number above each peak corresponds to those compounds named in Table 1. Retention times were altered by use of two capillary columns with a similar stationary phase but different lengths between two sets of data.

TABLE 1. MEAN PERCENTAGE OF VOLATILES PRESENT IN PYGIDIAL GLANDS OF *Azteca* nr. *bicolor* ($N = 10$) AND *A. chartifex* ($N = 8$)

Peak number in Figure 3A	Peak number in Figure 3B	Compound	*A.* nr. *bicolor* (% ± SD)	*A. chartifex* (% ± SD)
		2-Acetyl-3-methylcyclopentene	0.2 ± 0.6	
1		2-Heptanone	7.5 ± 4.9	
	2	6-Methyl-5-hepten-2-one		13.1 ± 4.4
		2-Formyl-3-methylcyclopentane-acetaldehyde	trace[a]	
3		2-Formyl-3-methylcyclopentene-acetaldehyde	1.7 ± 2.9	0.2 ± 0.4
		2-Methyl-1-cyclopentene-carboxaldehyde		0.2 ± 0.7
		2-(3-methylcyclopentyl)propion-aldehyde	0.9 ± 1.2	
	4	Isopulegol		0.3 ± 0.4
5		Unknown 1	0.3 ± 0.8	
		Unknown 2	0.3 ± 0.6	
6		Unknown 3	8.4 ± 4.3	
		Unknown 4	1.5 ± 2.0	
7a	7a	*cis-trans*-Iridodial	21.4 ± 11.8	31.8 ± 13.0
7b	7b	*trans-trans*-Iridodial	31.7 ± 10.0	27.1 ± 10.6
7c	7c	*trans-cis*-Iridodial	15.0 ± 9.2	26.6 ± 10.4
		Isomer of nepetalactol	4.2 ± 3.5	trace
8		Unknown 5	6.8 ± 6.7	

[a]Less than 0.1%.

A. chartifex. The glands from *A.* nr. *bicolor* do not contain isopulegol; however, 2-heptanone and four unidentified compounds were present as minor components. The mass spectra of these unidentified compounds show a *m/z* 43 ion as a base peak, which suggests that they are methyl ketones; however, the mass spectra were otherwise too featureless to be able to identify these compounds.

Volatile ketones from the pygidial gland of *A.* nr. *velox* and *A.* nr. *nigriventris* species (Wheeler et al., 1975) produced typical alarm behavior in both species. Nonetheless, in contrast with Wheeler et al. (1975), we found with live colonies of *A.* nr. *bicolor* that few workers were attracted to the filter paper containing diluted or concentrated solutions of pygidial gland contents and synthetic 2-heptanone. Moreover, the attracted ants did not show any sign of alarm behavior.

DISCUSSION

Iridoids and ketones in the pygidial glands of dolichoderine ants are characteristic of this subfamily. The most common iridoids encountered are iridodial and isodihydronepetalactone (IV) (Attygalle and Morgan, 1984). These compounds are thought to be derived from (*S*)-citronellal (VII). 2-Heptanone, 2-methyl-4-heptanone, and 6-methyl-5-hepten-2-one are also common pygidial gland components.

Besides their occurrence in the pygidial glands of dolichoderine ants, iridoids are also present in plants and some other insects. In plants, they function either as feeding or olfactory attractants and in insects they serve as defensive compounds (Harborne, 1993). On the other hand, the ketones, especially 2-heptanone and 6-methyl-5-hepten-2-one, were found to function as very effective allomones for cockroaches in the genera *Palyzosteria* and *Neostylopiga* (Wallbank Waterhouse, 1970; Trave and Pavan, 1956), as well as for beetles in the genus *Dyschirius* (Moore and Brown, 1979).

Although the functions of the compounds from the pygidial gland of *A.* nr. *bicolor* and *A. chartifex* remain unknown, we suggest that, like cockroaches and beetles, the ants may use some or all of them as allomones against herbivores.

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CHEMICAL DEFENSES OF FRESHWATER MACROPHYTES AGAINST CRAYFISH HERBIVORY

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Abstract—We measured feeding preferences of the crayfish *Procambarus clarkii* for fresh tissue from four species of freshwater macrophytes (*Habenaria repens*, *Saururus cernuus*, *Ceratophyllum demersum* and *Typha angustifolia*). We then determined the role of plant chemical defenses in generating these preferences by incorporating crude aqueous and organic extracts from each species into palatable foods and comparing feeding on these foods to feeding on control foods lacking these extracts. Tissue toughness, dry mass and ash-free mass per volume, and percentages of carbon, nitrogen, and phenolics were also measured for each of the four macrophytes. Although it had a low nutritional value, *Ceratophyllum* was the preferred food when it was offered as fresh tissue; it did not produce a chemically deterrent extract. The lipophilic crude extract from *Typha* significantly deterred crayfish feeding, but this highly nutritious plant was preferred when offered in an agar-based diet lacking structural defenses. *Habenaria* and *Saururus* were low preference foods that did not appear to be structurally defended; each species contained both lipophilic and water-soluble extracts that significantly deterred feeding. Fractionation of the lipophilic crude extract from *Saururus* indicated the presence of at least three deterrent compounds. From the orchid *Habenaria*, we isolated and identified a novel bis-*p*-hydroxybenzyl-2-alkyl-2-hydroxysuccinoate metabolite, habenariol, that appeared to explain most of the feeding deterrent activity present in the lipophilic extract of this species. The concentration of the metabolite in frozen collections of this plant doubled if we allowed the material to thaw before placing it in extraction solvents.

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Key Words—Chemical activation, chemical defense, freshwater macrophytes, plant-herbivore interaction, *Saururus*, *Habenaria*, orchid, *Procambarus clarkii*, crayfish.

INTRODUCTION

Despite a history of debate concerning both the role of herbivores in freshwater systems (Shelford, 1918; Frohne, 1956; Lodge et al., 1998) and the occurrence of chemical defenses in freshwater plants (Lamberti and Moore, 1984; Ostrofsky and Zettler, 1986), very few studies have directly tested the effects of macrophyte secondary chemistry on herbivore activity (Buchsbaum et al., 1984; Cronin, 1998; Bolser and Hay, 1998). Even fewer studies have identified specific antifeedant compounds produced by freshwater macrophytes (Newman et al., 1992). Freshwater vascular plants do contain a diverse array of unusual secondary metabolites that could function in defense (McClure, 1970; Ostrofsky and Zettler, 1986), and several authors have suggested that chemical defenses against herbivory may be important in structuring freshwater plant communities (Smock and Stoneburner, 1980; Otto and Svensson, 1981; Ostrofsky and Zettler, 1986; Suren, 1989; Newman et al., 1990). Despite this broad discussion in the literature, we know of only one rigorous demonstration of a freshwater macrophyte being chemically defended against herbivory; the glucosinolate–myrosinase system in watercress, well known as an herbivore deterrent in terrestrial crucifers, acts as a potent chemical defense against herbivory by aquatic invertebrates (Newman et al., 1992, 1996).

Recent reviews have concluded that there is not enough information to assess the importance of antiherbivore chemical defenses in freshwater plants (Lamberti and Moore, 1984; Lodge, 1991; Newman, 1991). Given the role of plant chemical defenses in affecting the ecology and evolution of terrestrial (Rosenthal and Berenbaum, 1992; Fritz and Simms, 1992) and marine (Hay and Steinberg, 1992; Paul, 1992; Hay, 1997) communities, there is a clear need for studies of freshwater macrophytes that determine the frequency, magnitude, and mechanisms of plant chemical defenses against herbivory.

In this paper, we report findings from an investigation of chemical defenses and other plant traits affecting crayfish feeding on four species of freshwater macrophytes. Because previous studies show that both prey nutritional quality and prey defensive traits influence consumer feeding preferences (Mattson, 1980; Duffy and Paul, 1992; Hay et al., 1994), we examined the relationship between plant defensive traits and nutritional value, as well as how these traits affected crayfish feeding preferences. We quantified macrophyte protein, nitrogen, carbon, and phenolic content, as well as ash-free dry mass, toughness, and the effects of lipophilic and water-soluble chemical extracts on crayfish feeding. Finally, for species that appeared to contain potent chemical defenses, we bioassayed chemical fractions to learn more about the chemistry responsible for the

deterrent activity of the crude extracts. For the aquatic orchid *Habenaria repens*, this led to the isolation and identification of habenariol, a previously unknown secondary metabolite that significantly suppressed crayfish feeding.

METHODS AND MATERIALS

Study Organisms. We used the Louisiana red crayfish *Procambarus clarkii* (Decapoda: Cambaridae) as a bioassay organism for testing chemical defenses of freshwater plants because: (1) crayfishes are generalist feeders that include a wide variety of freshwater plants in their diets (Gaeveskaya, 1969; Hobbs, 1993), (2) they can have dramatic effects on the distribution and abundance of freshwater macrophytes (Lodge and Lorman, 1987; Creed, 1994; Lodge et al., 1994), and (3) they feed well in a variety of laboratory assays (Chambers et al., 1991; Lodge, 1991). The four macrophytes selected for this study included the submerged macrophyte *Ceratophyllum demersum* (Ceratophyllaceae) and the three emergent species, *Habenaria repens* (Orchidaceae), *Saururus cernuus* (Saururaceae), and *Typha angustifolia* (Typhaceae).

Feeding Assays. More than 100 *Procambarus clarkii* were obtained from a local aquaculture facility and housed individually in 34 × 28 × 13-cm plastic tubs with 2 liters of dechlorinated tap water. The crayfish were fed commercial trout food daily, and the water was changed every three to five days. The same 100+ crayfish were used for all of the assays outlined below (actual numbers in use at any given time varied slightly because mothers with young were not used in assays and there were a few deaths). This necessitated using the same individuals for multiple assays, but never twice for the same treatment or same assay. The assays below involved about 850 replicates, resulting in each crayfish being involved in about 8.5 assays. Their assignment to different assays was haphazard.

Plants were collected on October 16, 1996, from three locations in North Carolina, USA: Lake Waccamaw State Park and two small ponds in Morehead City. Twenty-five individuals of each species were collected from a broad area at each site, placed in coolers, and brought to the University of North Carolina's Institute of Marine Sciences. The feeding assay was set up as soon as plants arrived at the institute. Similar amounts (200 ± 10 mg blotted wet mass) of the four species were weighed to the nearest milligram and secured with rubber suction cups to the bottom of a tub. Equivalent pieces were taken from the same plants and placed in a separate, adjacent tub; these pieces served as controls for changes in plant mass due to factors other than crayfish grazing (Peterson and Renaud, 1989; Renaud et al., 1990). Tissue from each individual plant was offered to only one crayfish, thus attaining independence among replicates. Leaves were used from *Habenaria*, *Saururus*, and *Typha*, whereas whole shoots (including meristems) of *Ceratophyllum* were used. Once all species were hap-

hazardly placed in each pair of containers, a crayfish was added to one container in each pair and allowed to feed for 20–25 hr. All plant fragments greater than 10 mm in length were then recovered, identified, blotted on paper towels, and weighed. Replicates in which no feeding had occurred (i.e., when no fragments were present) were excluded from the analyses, resulting in a sample size of 17. Herbivore damage to each species was calculated as the initial mass (multiplied by the ratio of the final to initial mass of the control piece, thus correcting for changes occurring in the absence of grazing) minus the final mass of each piece.

To assess how plant morphology and structure influenced crayfish feeding in the assay described above, we destroyed plant structure by freeze-drying the plants, grinding them to a fine powder, and using these powders to make agar-based diets where the different species would be equivalent in structure and morphology. *P. clarkii* was then offered a choice of these foods. These agar-based foods were made using the following recipe: 1.5 g plant powder was mixed with 7 ml distilled water and added to a boiled solution of 0.37 g agar in 11.75 ml distilled water. This mixture was quickly poured into a mold beneath which lay fiberglass window screen. When the mixture solidified, it adhered to the window screen and was sliced into individual squares that we offered to crayfish simultaneously with equal amounts of agar-based diets made from the other macrophyte species (see Hay et al., 1994, for a more detailed description of the methods). Consumption was quantified as the number of holes in the screen mesh completely cleared of food. The assay ran for 6.5 hr. After excluding replicates in which none or all of the available food was eaten, or in which uneaten chunks of food were dislodged from the screens, we obtained a final sample size of 22.

Results of the multiple-choice feeding assays were analyzed with a nonparametric Friedman's test, which does not require independence among the multiple choices within a replicate (Roa, 1992).

Analysis of Plant Traits. To assess the qualitative role of plant nutritional value in determining crayfish preferences, we measured selected traits of the four species and compared these data to preferences of *P. clarkii* for fresh and reconstituted tissues of the four species. Toughness was measured by determining the mass required to penetrate fresh leaves completely ($N = 5$) with a fine-pointed needle (see Duffy and Hay, 1991, for methods). Dry mass per volume of plant was determined by drying 7–10 replicate samples of known volumes for three days at 60°C, and dividing milligrams of dry mass by milliliters of fresh plant. The same samples were then burned in a muffle furnace for at least 7 hr at 500°C. We then divided the dry mass lost during burning by the volume of the sample to obtain milligrams of ash-free dry mass per milliliter of fresh plant (a crude measure of food mass per bite).

Carbon and nitrogen content of leaves or shoots (five samples of each species) used in the original fresh plant choice assay were analyzed with a

Perkin-Elmer 2400 Series II CHN analyzer using an acetanilide standard (C: 71.09%; H: 6.71%, N: 10.36%). Phenolic content of leaves or shoots (four samples of each species) used in the original fresh plant choice assay were determined using the Folin-Ciocalteau method (Folin and Ciocalteau, 1927). Leaves or shoots were individually freeze-dried and ground. A subsample (5 mg) was taken from each piece. Each subsample was extracted in 1 ml 50% MeOH for 24 hr at 1°C, spun in a centrifuge, and 100 μl of the supernatant was added to 7.9 ml distilled water. After agitating this mixture, 0.5 ml Folin-Ciocalteau reagent was added. After 2 min, 1.5 ml sodium carbonate solution was added to the sample, and the time was recorded as zero. Two hours were allowed for color development, and absorbance at 760 nm was recorded using a Spectronic 21D spectrophotometer. A standard curve was generated using tannic acid.

We measured soluble protein content of the four macrophytes using the method of Bradford (1976). As in the above analyses, pieces of leaves or shoots used in the original fresh plant choice assay were freeze-dried and ground, and 5 mg samples were taken. Samples were digested in 1 ml NaOH (1 mol) for 24 hr at 1°C, centrifuged, and 100-μl aliquots of the supernatant were added to 5 ml samples of Bradford reagent. After 10–15 min, absorbances of the samples at 595 nm were measured in a Spectronic 21D spectrophotometer against bovine serum albumin (BSA) standards.

Feeding Assays Testing Crude Extracts. To determine whether chemical defenses affected the feeding choices of *P. clarkii*, we incorporated crude chemical extracts from frozen collections of the plants into palatable foods made of freeze-dried, finely ground broccoli and lettuce (referred to as "brocclett") in an agar matrix. We then measured crayfish feeding on the extract-treated foods relative to control foods in paired choice assays (see similar methods in Hay et al., 1994). We determined the volume of frozen plant to extract by calculating a conversion factor from volume (milliters) of frozen plant to dry mass (grams) for each species, and then calculating the volume equivalent to 1.5 g (the amount of brocclett to which the extract was to be added). This procedure ensured that the ratio of extract to dry mass of the test food was equivalent between the actual plant tissue and the experimental diet used to test the plant extract. The conversion factor (grams of dry plant to milliliters of frozen plant) was determined by measuring volumes of nine samples of frozen plant, drying the samples for three days at 60°C, weighing them to the nearest milligram, and dividing the dry mass by the volume of each sample.

To extract each species, we homogenized frozen materials in 100% methanol (MeOH) and added dichloromethane (DCM) until a mixture of 2:1 DCM–MeOH was reached. After 15–30 min, the mixture was gravity-filtered and extracted two additional times with fresh 2:1 DCM–MeOH. The remaining tissue was extracted three times with 7:3 MeOH–distilled water, using a vacuum-filtration each time. Organic solvents were removed from the crude lipo-

philic and water-soluble extracts by rotary evaporation until only water remained. Both extracts were combined and partitioned between DCM and distilled water.

Lipophilic (DCM-soluble) and water-soluble extracts were tested separately for feeding deterrence. Lipophilic extracts were dried via rotary evaporation, redissolved in a small amount of anhydrous ethyl ether, and added to 1.5 g of brocclett. Ether was removed from the powder via rotary evaporation. The resulting powder, coated evenly with plant extract, was used to make an agar-based food as described previously, with the exception that treatment and control foods (control = brocclett coated with ether alone) were poured into separate, but parallel, openings in a mold. After removal of the mold, screens could be cut into strips containing equal amounts of extract-treated and control foods (see Figure 1 in Hay et al., 1994, for a schematic of these foods). The test strips were offered to 25–30 individual crayfish. In similar tests of water-soluble plant chemistry, water-soluble extracts were reduced to less than 7 ml using a Savant Speed-Vac. This extract was then added to 1.5 brocclett, and this mixture was used in the test food recipe as described above. A control recipe was prepared with only distilled water and offered simultaneously to crayfish. We monitored animals periodically and attempted to remove the test food when roughly half of either choice was consumed. Mean durations (± 1 SE) of tests were 17.6 $\pm$ 1.3 hr (lipophilic) and 17.1 $\pm$ 1.4 hr (water-soluble). Differences in consumption of treated vs control foods were analyzed using paired t tests.

Bioassay-Guided Fractionation. We chose *Saururus* and *Habenaria* for further chemical investigation based on results from crayfish assays testing fresh plants, agar-based diets, and crude chemical extracts from our four macrophytes. The lipophilic (DCM-soluble) extract from freeze-dried, powdered *Saururus* was fractionated over size and polarity gradients using 60–200 mesh Sephadex LH-20 beads eluting with 1 : 1 DCM–MeOH. A total of 32 fractions were collected, and those containing similar compounds (by thin layer chromatographic analysis) were recombined to produce five separate fractions. The five recombined fractions were tested for activity against crayfish feeding using the "brocclett" method, which was described previously for tests of crude extracts. Test concentrations of the fractions were calculated to be equivalent to natural concentrations (on a dry mass basis). Fractions exhibiting statistically significant deterrence of feeding were further fractionated using preparative silica thin-layer chromatography (1000-μm plates), and the resulting preparative plate fractions (acquired by removing the silica bands from the plate and extracting the compounds) were tested individually for activity against crayfish feeding.

Because a previous study suggested that chemical defense in another aquatic macrophyte (watercress) was activated by enzymes released during cell damage (Newman et al., 1990), and because we wanted to avoid possible chemical degradation associated with freeze-drying (Cronin et al., 1995), we chose to ex-

tract frozen *Habenaria* tissue (freezing breaks cells) to obtain crude lipophilic extract for our chemical studies. The DCM-soluble extract was initially fractionated by flash chromatography on silica gel by gradient elution with solvent mixtures from 100% hexane to 100% ether. Fractions containing similar compounds, as indicated by thin-layer chromatography, were recombined to yield a total of seven fractions. These fractions were tested for deterrent activity against crayfish feeding at natural concentrations (dry mass basis). Fractions that were significantly deterrent (or showed a strong, nonsignificant trend) were further purified by silica gel high-performance liquid chromatography (HPLC). Because of the appearance of an overall reduction in deterrent activity of the fractionated extracts compared to the original test of the crude lipophilic extract, compounds purified via HPLC were subsequently tested at concentrations calculated to be twice the natural level in order to counter possible degradation or loss of compounds due to handling.

Through the above process, we isolated a novel bis-*p*-hydroxybenzyl-2-alkyl-2-hydroxysuccinoate, habenariol (Wilson et al., 1998), that strongly depressed crayfish feeding. Highly purified habenariol was further tested in crayfish feeding assays at concentrations of 10, 15, and 20 mg/g (0.8, 1.2, and 1.6 mg/ml, respectively). The highest concentration tested was later determined to occur in *Habenaria* tissues that had been frozen and thawed before being placed in solvents, allowing cell rupture and mixing of cell contents (as should occur when herbivores feed).

Thin-layer chromatography performed on extracts of fresh, whole *Habenaria* tissue did not indicate the presence of appreciable concentrations of habenariol; however, extracts of frozen tissue did contain habenariol, and extracts from frozen tissue allowed to thaw for 20 min before extraction (thus allowing plant enzymes to warm to temperatures where they could be active) appeared to contain even high concentrations. Therefore, we assessed the possibility that this metabolite was activated via cell damage. A similar process is known to occur in both a seaweed (Paul and Van Alstyne, 1992) and a freshwater macrophyte (Newman et al., 1990). To evaluate the possibility of an activated chemical defense, we split a frozen collection of *Habenaria* in half, extracted one portion immediately (the solvents and low temperature should have retarded or prevented enzymatic activity) and extracted the other portion after allowing it to thaw for 20 min (we assumed this would allow enzymes in ruptured cells to warm to temperatures where they would function). We then tested natural concentrations of the two lipophilic crude extracts (frozen and thawed) in separate assays with appropriate controls. The deterrent effects of the two extracts were compared with an unpaired t test on the differences in consumption of control versus extract-treated foods in each replicate. Habenariol concentrations in frozen vs. thawed leaves ($N = 4$ each) also were quantified using silica HPLC (eluting solvents: 6:4 ethyl acetate–2,4,4-trimethyl pentane).

RESULTS

Plant Feeding Assays. Crayfish consumed much more fresh tissue from *Ceratophyllum* than from the other three macrophytes (Figure 1A, $P < 0.001$). However, when plants were freeze-dried, finely ground, and embedded in an agar matrix (thus removing structural or morphological differences between spe-

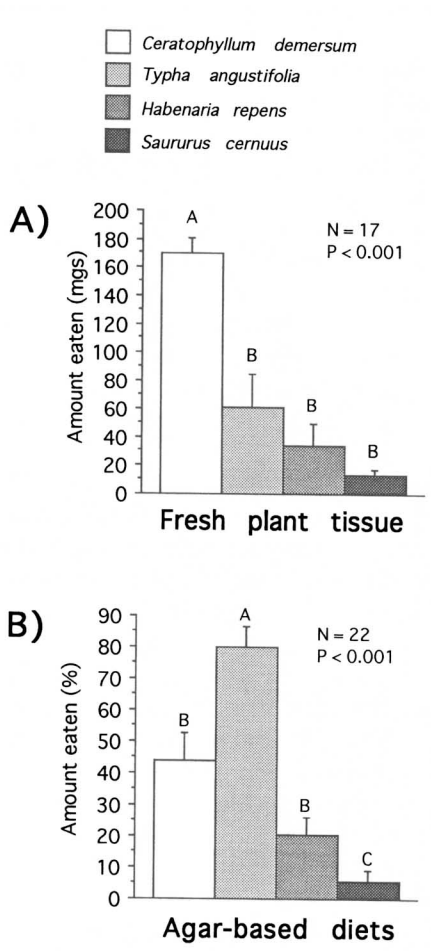

FIG. 1. Results of feeding assays offering crayfish a choice of four freshwater macrophytes: (A) as fresh plant tissue (200 mg of each species offered), and (B) as finely powdered tissue incorporated into agar-based diets. *P* values were generated by Freidman's tests based on rank preferences, and letters above bars indicate statistically different ranks between species.

cies), crayfish preferred *Typha* and, to a lesser degree, *Ceratophyllum* (Figure 1B, $P < 0.001$). Consumption of *Habenaria* and *Saururus* was low in both fresh tissue and agar-based assays, although in the agar-based assay the difference in amount eaten between *Habenaria* and *Ceratophyllum* was not statistically significant.

Macrophyte Traits. Table 1 shows dry mass, ash-free dry mass, carbon, nitrogen, protein, and phenolic concentrations as well as toughness of the macrophytes examined in this study. *Typha* ranked highest in several measures of nutritional value (ash-free dry mass/volume and percentage of nitrogen) and lowest in total phenolics. *Typha* was also extremely tough relative to the other plants. Although *Ceratophyllum* was the preferred food in assays using fresh tissues, it had the lowest carbon, nitrogen, and protein content and thus appeared low in nutritional value. Despite being avoided by crayfish, *Habenaria* and *Saururus* were not physically tough relative to the other species, and *Habenaria* had a high protein content. *Habenaria* and *Saururus* both contained relatively high concentrations of phenolics.

Neither lipophilic nor water-soluble extracts from *Ceratophyllum* significantly affected crayfish feeding (Figure 2). The lipophilic extract from *Typha* reduced feeding by 48% relative to a control diet ($P_{2\text{-tail}} = 0.006$, Figure 2). *Habenaria* and *Saururus* contained both lipophilic and water-soluble extracts that significantly deterred feeding relative to control diets (Figure 2). *Habenaria* lipophilic extract decreased feeding by 68%, and the water-soluble extract decreased feeding by 64%. *Saururus* lipophilic and water-soluble extracts each decreased feeding by 70%.

Bioassay-Guided Fractionation. The initial LH20 fractionation of lipophilic *Saururus* extract yielded only one significantly deterrent fraction (fraction 3, Figure 3, top), although strong deterrent trends were exhibited by fractions eluted immediately before and after fraction 3, suggesting that some overlap of compounds may have occurred. Further separation of the deterrent fraction via preparative thin-layer chromatography yielded at least three different lipophilic metabolites or groups of metabolites (as indicated by distinctive UV absorbance and staining characteristics) that were active against crayfish feeding (Figure 3, bottom).

Three of the seven silica flash column fractions from the lipid extract of *Habenaria* showed deterrent trends (Figure 4, top) and were further separated via silica HPLC. Two of the three fractions yielded no deterrent compounds when tested at twice natural concentration (by dry mass); the third fraction consisted of one major component, subsequently called habenariol (Wilson et al., 1998), that reduced crayfish feeding by a significant 66% (peak 2, Figure 4, bottom) and a minor component that reduced feeding by a significant 42% (peak 3, Figure 4, bottom) relative to control diets when tested at twice natural concentration. The structure of habenariol was determined by combined

TABLE 1. SELECTED MACROPHYTE TRAITS[a]

Plant	Tissue toughness (g)	Dry mass/vol. (mg/ml)	Ash-free dry mass/vol. (mg/ml)	Carbon (% dry mass)	Nitrogen (% dry mass)	Protein (% dry mass)	Phenolics (% dry mass)
Ceratophyllum demersum	8.7 ± 0.9a	70 ± 5a	57 ± 5a	40.9 ± 0.4a	2.4 ± 0.2a	2.6 ± 0.3a	5.4 ± 0.4a
N	5	9	9	5	5	3	4
Typha angustifolia	53.7 ± 2.7b	141 ± 9b	134 ± 9b	45.0 ± 0.5b	3.5 ± 0.2c	7.4 ± 0.5c	2.8 ± 0.3b
N	5	9	9	5	5	3	4
Habenaria repens	8.3 ± 1.5a	63 ± 3 a	56 ± 4a	42.4 ± 0.8a	2.9 ± 0.1ab	16.6 ± 1.9b	7.7 ± 0.1c
N	5	10	9	5	5	3	4
Saururus cernuus	5.4 ± 0.3a	126 ± 13b	114 ± 12b	46.5 ± 0.8b	3.1 ± 0.2bc	5.9 ± 0.1c	8.2 ± 0.8c
N	5	7	7	5	5	3	4

[a]Reported are mean values (±1 SE) and sample sizes for each analysis. Species that share a letter within a column are not significantly different from each other.

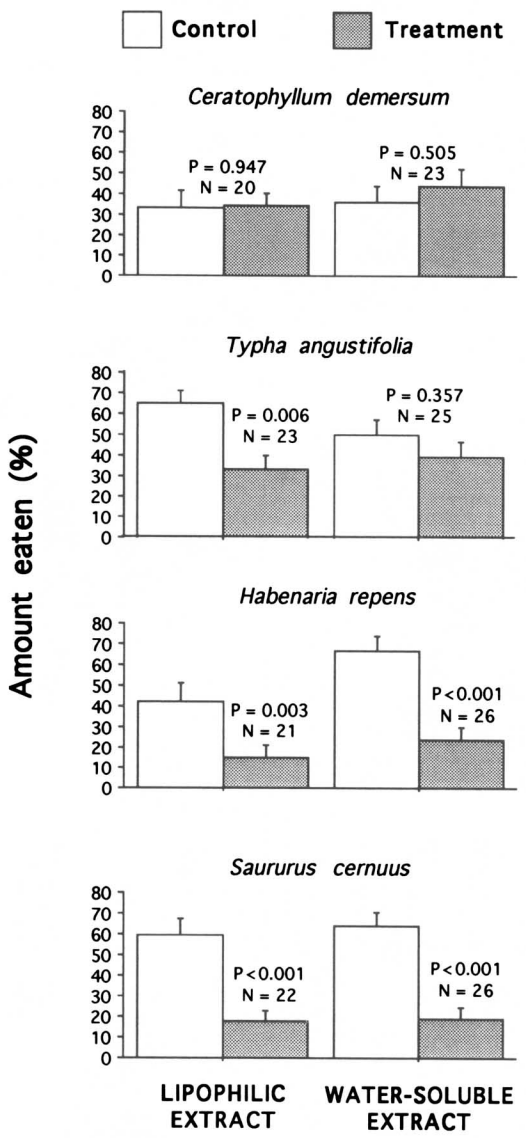

FIG. 2. Effects on crayfish feeding of lipophilic and water-soluble crude extracts from the four macrophytes. Paired control and treatment diets consisted of freeze-dried, powdered broccoli and lettuce without and with the addition of extract (at natural dry mass concentrations). Two-tailed P values were generated by paired t tests; N = numbers of replicate crayfish.

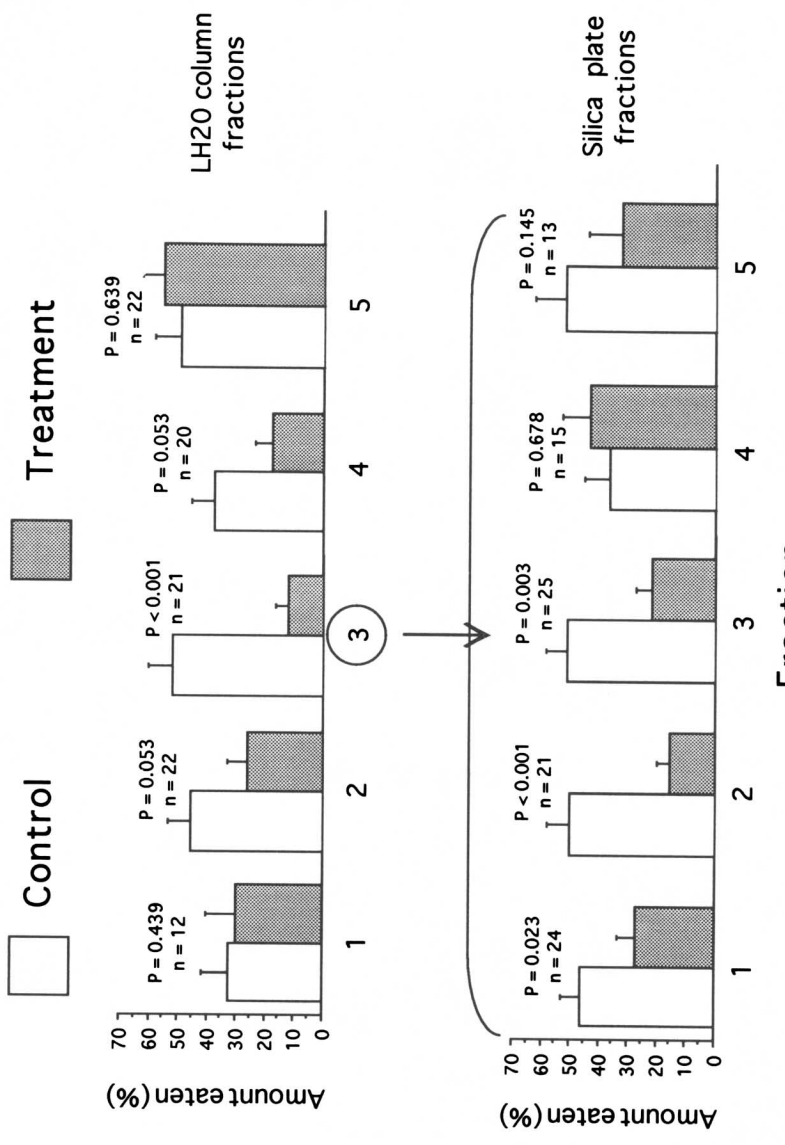

FIG. 3. Bioassay-guided fractionation of *Saururus* lipophilic extract. Five fractions from an LH20 (Sephadex) column were tested (top), and the most active fraction was further separated via preparative thin-layer chromatography into five more fractions (bottom). Two-tailed P values are from paired t tests; N = numbers of replicate crayfish.

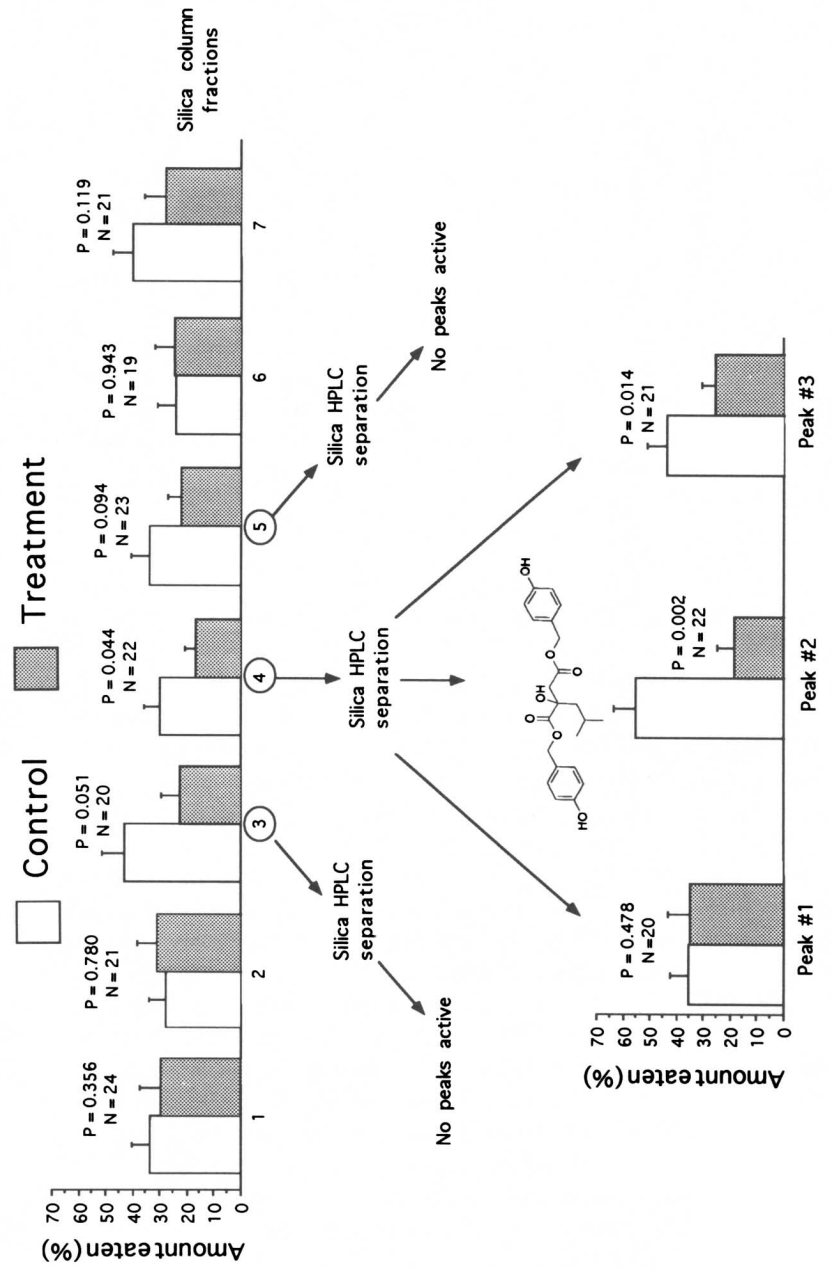

FIG. 4. Bioassay-guided fractionation of *Habenaria* lipophilic extract. Seven fractions from a silica flash column were tested (top), and the most active fractions were further separated via silica HPLC. The compounds were initially tested as twice the natural concentration (by dry mass) to counter apparent degradation and/or loss of activity. Only one peak significantly deterred crayfish feeding (bottom). The major constituent of this peak, habenariol, is depicted above the histogram.

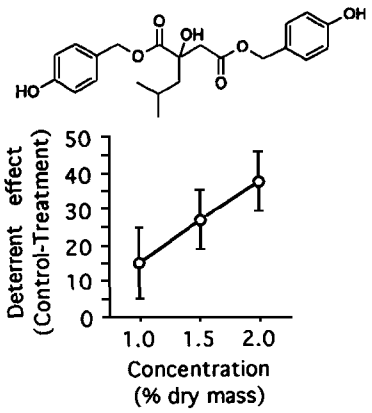

FIG. 5. Effect of habenariol on crayfish feeding when tested in agar-based diets at concentrations of 1.0%, 1.5%, and 2.0% dry mass. The deterrent effect is plotted as the difference in amount eaten between the treatment and control diets expressed as a percentage of the control diet.

spectral and chemical methods; these details are reported elsewhere (Wilson et al., 1998).

Crayfish assays testing three concentrations of pure habenariol showed a strong, linear relationship between concentration of the compound and effect on feeding (Figure 5). At the lowest concentration (1.0% dry mass), habenariol's deterrence was not statistically significant ($P_{1\text{-tail}} = 0.074$), whereas at 1.5% and 2.0% dry mass, it significantly reduced feeding by 54% ($P_{1\text{-tail}} = 0.002$) and 66% ($P_{1\text{-tail}} < 0.001$), respectively.

Crayfish assays testing lipophilic extract from *Habenaria* tissues subjected to freezing (to rupture cells) and subsequent thawing (to allow for enzymatic activation of chemical defenses) vs. an extract from still-frozen tissue indicated that thawing yielded a more potent chemical defense (Figure 6). The deterrent effect of natural concentrations of the extract from thawed plant tissue was almost three times greater than the effect of natural concentrations of the extract from frozen tissue ($P_{1\text{-tail}} = 0.030$). The concentration of habenariol in samples of thawed leaves was about twice the concentration occurring in frozen leaves (Figure 6; $P_{1\text{-tail}} = 0.005$). Fortuitously, this suggests that our initial assays of fractions at about two times their natural concentrations (Figure 4) would have contained concentrations of habenariol approximating the natural concentration that occurs following activation due to cell damage (see Paul and Van Alstyne, 1992 for a similar marine example).

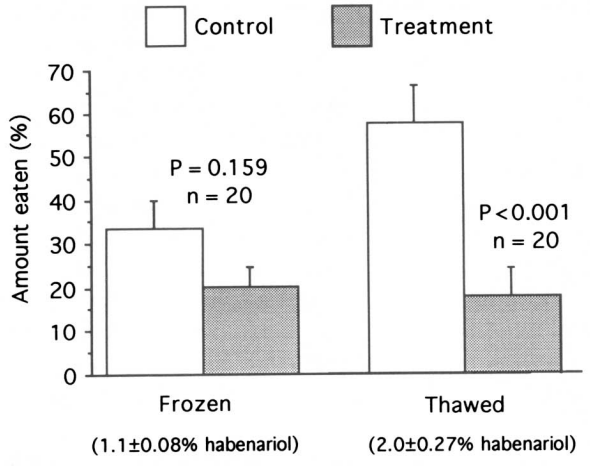

FIG. 6. Crayfish assays testing lipophilic crude extracts from *Habenaria* that were frozen and extracted immediately vs. frozen and thawed for 20 min before extracting. Two-tailed P values are from paired t tests; N = numbers of replicate crayfish. Reported beneath the bars are the concentrations (% dry mass) of habenariol in samples of frozen vs. thawed tissue (unpaired t test, $P_{2\text{-tail}} = 0.018$, $N = 4$ each).

DISCUSSION

Contrary to the traditional view that herbivory on freshwater macrophytes is rare (Shelford, 1918; Hutchinson, 1975; Wetzel, 1983; Lamberti and Moore, 1984), a growing body of evidence suggests that the evolutionary and ecological importance of herbivory is as great in freshwater habitats as in terrestrial or marine systems (Cyr and Pace, 1993; Lodge et al., 1998). Thus, selection may favor freshwater plants that have chemical and other types of antiherbivore defenses; however, few data exist to address this question. This paper is one of only a few studies in which the effects of macrophyte secondary metabolites on herbivore feeding behavior are directly tested and some of the specific deterrent metabolites are investigated. For three of the four aquatic plant species that we examined, chemical extracts significantly reduced feeding by a common aquatic omnivore (Figure 2). In particular, we found that *Ceratophyllum*, despite having the lowest nutritional value, was preferred by crayfish when offered as fresh tissue and that this species did not contain extracts that significantly deterred feeding. Crude lipophilic extract from *Typha* was significantly deterrent, but this highly nutritious plant was preferred when offered in an agar-based diet (Figure 1), suggesting that crayfish choose foods based on both defensive (e.g.,

chemical and physical) and nutritional traits and that they will readily consume foods with some chemical deterrents if those foods are nutritionally valuable. Finally, *Habenaria* and *Saururus* were largely avoided by crayfish in both fresh-plant and agar-based assays and did not appear to be structurally defended. Both the lipid-soluble and water-soluble extracts from these low preference species significantly deterred feeding (Figure 2). For *Habenaria*, we extended this process by purifying and identifying the structure of a novel bis-*p*-hydroxybenzyl-2-alkyl-2-hydroxysuccinoate (see the structure in Figure 4) that significantly deterred crayfish feeding at 1.5% and 2.0% of plant dry mass (Figures 5 and 6). In the only previous investigation to identify an active chemical defense in a freshwater plant, Newman et al. (1990, 1992, 1996) used information about the chemical defenses of terrestrial crucifers (e.g., Feeny, 1976) to definitively demonstrate defensive chemistry of an aquatic cruciferous plant, *Nasturtium officinale*.

Numerous studies of plant chemical defenses show correlations between the occurrence of secondary metabolites hypothesized to be defensive and the avoidance by herbivores of plants containing those metabolites (e.g., Steinberg, 1985; Chambers et al., 1991; Lodge, 1991). While informative, these correlations fail to provide direct evidence for the defensive roles of plant secondary compounds. Studies by Ostrofsky and Zettler (1986) and Chambers et al. (1991) demonstrate the conflicting patterns that correlative studies can produce. Ostrofsky and Zettler (1986) reported the presence of alkaloids in 15 species of freshwater macrophytes as evidence for chemical defenses, but no data were presented demonstrating that these alkaloids actually affected feeding by freshwater herbivores. Chambers et al. (1991) found no correlation between total alkaloid concentrations of 10 macrophyte species and crayfish feeding preferences. Thus, correlative studies can point out interesting patterns, but they cannot unambiguously demonstrate chemical mechanisms affecting herbivore feeding choices in aquatic habitats.

Given that investigations of chemical defenses in terrestrial plants have been conducted for many decades and that freshwater macrophytes are descendants of terrestrial plants, many specific metabolites known to defend terrestrial plants may also be produced by their freshwater relatives. However, simply reporting that a freshwater plant produces a secondary metabolite that is known to be an antifeedant in a related terrestrial plant fails to address the possibility that additional metabolites or components of plant chemistry could also be playing a defensive role. The general methodological approach of fractionating extracts, directly testing fractions, and further purifying the active fractions represents a more robust approach to identifying the mechanisms of chemical defense in freshwater macrophytes (or terrestrial plants). It is also an approach that can be employed by biologists who may have limited chemical training. Starting with the crude extract and progressively eliminating portions that do not exhibit

deterrent activity should reduce the chances of overlooking ecologically important metabolites that deter feeding in favor of a predetermined or more obvious (more concentrated, more structurally appealing, etc.) compound that may or may not affect feeding.

For two of the four macrophytes we examined, we found evidence for the presence of multiple deterrent components in the plants' chemical extracts. *Habenaria* and *Saururus* both contained lipophilic as well as water-soluble deterrent extracts (Figure 2). Furthermore, when we attempted to isolate the lipophilic metabolites in *Saururus* that were responsible for the deterrence we saw in the crude extract, three chemically distinct fractions significantly deterred crayfish feeding (Figure 3, bottom). Thin-layer chromatography, which we used to fractionate the *Saururus* extract, may not separate compounds that are very similar in polarity; thus, our three active fractions could have contained groups of compounds rather than a single pure metabolite. However, we can conclude that at least three different metabolites were deterrent, because the polarity, staining, and UV characteristics of the three fractions indicated no overlap of compounds among the three fractions.

Despite possessing many advantages, the general methodological approach we used can fail to detect chemical complexities such as the enzyme-activated defense found in watercress (Newman et al., 1990) unless initial collections are extracted both before and after allowing cells to be ruptured (also see Paul and Van Alstyne, 1992). For the freshwater orchid *Habenaria*, our discovery of habenariol and its activity was fortuitous rather than planned. The common practices of freezing or freeze-drying tissue prior to extraction and of placing whole fresh plants in solvent before breaking open the cells (all of which can retard or prevent enzymatic reactions) could have hindered production of the deterrent compound. The finding that thawing *Habenaria* tissue before extraction results in increased concentrations of habenariol and increased deterrency of the crude extract (Figure 6) suggests that cell lysis causes enzymatic activation of habenariol if the reactions are not retarded by solvents or low temperatures. A compound similar to habenariol is known from a terrestrial climbing orchid (Li et al., 1993), indicating that this type of compound occurs in other members of the family and that habenariol is unlikely to be an artifact created by our particular methodologies. However, this latter compound was isolated as a phenolic glycoside, rather than the free phenol derivative observed here. Given this comparison, it is conceivable that in *Habenaria*, the deterrent molecule is stored as a glycoside that would not be isolated by lipophilic extraction. On cell lysis, the glycoside could be hydrolyzed, liberating free habenariol. A process such as this, while currently unsubstantiated by experimental evidence, provides a reasonable explanation for the increasing concentrations of habenariol observed on prolonged extraction.

Newman et al. (1990) first demonstrated the chemical defense in watercress

by showing that cell damage via freezing of plant tissue resulted in the production of the toxic isothiocyanates that deterred herbivory by aquatic invertebrates. Other examples of rapidly activated chemical defenses can be found in terrestrial plants, and there are also a few examples in marine algae (e.g., Paul and Van Alstyne, 1992). The possibility that the phenomenon occurs widely among freshwater plants has yet to be investigated.

Our findings that three out of the four species we investigated contained extracts that significantly reduced crayfish feeding support the hypothesis that chemical defenses against herbivory could be common among freshwater macrophytes. Structural traits and nutritional quality appear to interact with chemical defenses to influence crayfish feeding choices. *Typha* provides a possible example of this in that it was minimally eaten in fresh plant assays (apparently due to its toughness) but rapidly consumed in the agar-based assays despite having a lipid-soluble extract that significantly deterred feeding. In the agar-based assays, crayfish preferred the more nutritious (Table 1), but chemically defended, *Typha* to the less nutritious, but chemically nondefended, *Ceratophyllum.* This suggests that these herbivores are making complex evaluations involving both the costs and benefits of a given food and that we often will not achieve an adequate understanding of feeding choices unless prey defenses are viewed in a larger context that includes nutritional rewards and other factors (Hay, 1996).

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IDENTIFICATION AND VARIATION OF VOLATILE COMPOUNDS IN STERNAL GLAND SECRETIONS OF MALE KOALAS (*Phascolarctos cinereus*)

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Abstract—The content of the sternal gland secretion from four male koalas (*Phascolarctos cinereus*) was analyzed using gas chromatography–mass spectrometry. Volatile fatty acids, aldehydes, ketones, mono- and sesquiterpenes were identified, together with various volatile nitriles and oximes, some of which had not previously been described from any mammalian skin gland. Benzyl cyanide and phenylacetaldehyde oxime were identified as major components of the secretions, together with smaller amounts of the novel 3-methylthiopropanal oxime. The relative abundance of the identified compounds was highly variable between individuals and the scent composition was found to be partly determined by the scent of surrounding conspecifics. Principal component analysis revealed a high similarity between related individuals.

Key Words—*Phascolarctos cinereus*, koala, GC-MS, sternal gland, essential oils, terpenes, benzyl cyanide, nitriles, phenylacetaldehyde oxime, principal component analysis.

INTRODUCTION

The koala (*Phascolarctos cinereus* Goldfuss) is probably one of the best known and most admired native Australian marsupials. However, in early accounts of the Australian fauna, the koala escaped attention and was first described in detail by Home (1808). Since then most attention has been focused on the selectivity the animal displays when feeding on *Eucalyptus* foliage (Fleay, 1937; Pratt,

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1937; Betts, 1978; Eberhard, 1978; Southwell, 1978; Hindell et al., 1985; Hindell and Lee, 1990). It is the only extant species of its family (Phascolarctidae) and is confined to mainland Australia. Although three subspecies have been described in New South Wales (*Phascolarctos cinereus cinereus*), Queensland (*Phascolarctos cinereus adustus*), and Victoria (*Phascolarctos cinereus victor*), these may only represent arbitrary selections from a cline.

Marsupials generally use urine and feces to mark their environment (Schultze-Westrum, 1965; Eisenberg and Golani, 1977; Müller-Schwarze, 1983; Russell, 1985; Salamon, 1996). The presence of scent glands and their use in scent marking has been studied for only a few marsupial species (Schultze-Westrum, 1965; Biggins, 1984; Stoddart and Bradley, 1991). The scent-marking behavior has been studied in several groups of captive *P. cinereus* (Smith, 1980; Thompson and Fadem, 1989; Mitchell, 1990), but no larger scale investigation has focused on the natural marking behavior in the field.

The main external scent gland is the large sternal gland located in the subcutaneous tissue of the chest of male koalas; it is not found in females (MacKenzie and Owen, 1919). The gland reaches its maximum size at about 4 years of age (Smith, 1980; Mitchell, 1990), and in some individuals the gland can be 8.5 cm long and 4.8 cm wide (Lee and Carrick, 1989). The size does not change over the year, although older males have been found to move more often between trees and therefore marking probably occurs more often and consequently more trees are marked (Smith, 1980; Mitchell, 1990). The color of the fur in this area is dirty brown to ginger in otherwise creamy white fur. MacKenzie and Owen (1919) noted: "The yellow secretion can be easily extruded on cutting which somewhat resembles milk." Particularly in older animals, the sternal area is often characterized by long thick guard hairs and an almost complete absence of under-fur. The local absence of under-fur certainly helps to place the glandular secretion onto the substrate, as otherwise the secretion may be adsorbed onto the fur. The underlying skin area seems to be slightly elevated and shows clearly visible pores through which secretion is expressed as clear yellow drops (MacKenzie and Owen, 1919).

Scent marking by the koala involving the sternal gland frequently results when the animal rubs his chest on trunks and branches of trees (Bolliger and Hardy, 1944; Sharpe, 1980; Smith, 1980) and occurs more often when koalas enter unfamiliar trees (Smith, 1980; Mitchell, 1990). The same pattern of exclusive use of trees appears to apply to females, which do not have a sternal gland. In this case the use of only a few trees for resting and feeding would mean that feces and urine could be concentrated round these trees, effectively marking them passively.

To date, no thorough chemical characterization of this prominent gland secretion has been undertaken. In 1981 Carman and Greenfield made the first

attempt to analyze the chemical components of the sternal gland using GC-MS, but their results were never published (R. M. Carman, personal communication). Here we describe a chemical characterization of the sternal gland secretion of male koalas using gas chromatography–mass spectrometry (GC-MS) and supply evidence that the scent secretion is more similar between related than between unrelated individuals.

METHODS AND MATERIALS

Sternal secretions were collected from four male koalas at the Bonorong Wildlife Park in Brighton, Tasmania. The animals were housed in two groups each consisting of two males and two females. All animals were fed with four locally grown *Eucalyptus* species (*Eucalyptus viminalis*, *E. amygdalina*, *E. globulus*, and *E. obliqua*). Swabs from the sternal gland area were taken from unanesthetized animals by rubbing a piece of glass microfiber filter paper (Whatman) three times over the sternal region. During this procedure the animals were resting in their trees or were held by their care-takers. Each filter paper was weighed before and after sampling and swabs were stored at −20°C after collection until analysis.

Each sample was analyzed by combined gas chromatography–mass spectrometry (GC-MS). The central part of the filter paper, which was mostly fully saturated with secretion, was cut into several strips measuring about 0.5 × 3 cm. These strips were directly inserted into the injection port of the GC. The injection port was resealed and heated to 150°C for 10 min. During this time the first 10-cm section of the column was cooled with a cold-trap containing a mixture of acetone and liquid nitrogen at about −80°C (cryo trap), in order to refocus volatiles from the filter paper on the front of the GC column.

GC-MS analyses were performed on a Hewlett-Packard model 5890 gas chromatograph connected via an open split interface to an HP 5970B mass selective detector (MSD). The transfer line was held at 290°C while the electron energy was 70 eV. A 25-m cross-linked methyl silicone column (HP1/internal diameter 0.32 mm, film thickness 0.17 μm) was used with helium as carrier gas operating at 40°C for 2 min, then 10°C/min to 290°C and held for 2 min. The scan range was m/z 35–300 with a scan rate of 2 scans per second.

Additional GC-MS analyses were carried out on a Hewlett-Packard model 5890 Series II directly coupled to a Kratos Concept ISQ high-resolution mass spectrometer. For chemical ionization, methane was used as the reagent gas, with an accelerating voltage of 4 kV and an electron energy of 200 eV (scan rate m/z 50–250 at 0.8 sec/decade) and a resolution of 2000. A 25-m cross-linked methyl silicone column (HP1/internal diameter 0.32 mm, film thickness 0.17 μm) was used with helium as a carrier gas with a head pressure of 0.5 bar.

The source temperature was held at 200°C while the interface was held at 250°C.

High-resolution GC-MS for accurate mass measurements was also performed on the HP model 5890 Series II directly coupled to the Kratos Concept ISQ. The accelerating voltage was 8 kV, with an electron energy of 70 eV (scan rate m/z 20–250 at 1.0 sec/decade) and a dynamic resolution of 5000. For this analysis a 30-m DBI column (internal diameter 0.25 mm, film thickness 0.25 μm) was used with a helium head pressure of 1.7 bars. Perfluorokerosene (PFK) was utilized as an internal calibrant.

Isobutyronitrile, 2-methyl-, and 3-methylbutyronitrile were obtained from Aldrich (Aldrich Chemical Company, Castle Hill, NSW, Australia). *syn*- and *anti*-Phenylacetaldehyde oximes were prepared from phenylacetaldehyde (Sigma Chemical Company, Castle Hill, NSW, Australia) by reacting it with excessive neutral hydroxylamine at room temperature for 24 hr (Dollfus, 1892). *syn*- and *anti*-3-Methylthiopropanal oximes were prepared by reacting 3-methylthiopropanal (Aldrich Chemical Company, Castle Hill, NSW, Australia) with hydroxylamine hydrochloride in ethanolic sodium hydroxide at 80°C for 30 min, diluting with water, and extracting into chloroform.

Principal Component Analysis (PCA). Comparisons of approximate relative abundance were obtained through peak-integration routines (Hewlett-Packard MS ChemStation) and standardization to the largest peak in the run, a branched alkan-2-one with 16 carbons, the carbon skeleton of which was not determined. To compare the relative abundance of selected compounds in the chest gland secretion of the four male koalas, we used principal component analysis (PCA). Principal component analysis (PCA) is a multivariate statistical method that reduces the dimensions of a single group of data by producing a smaller number of abstract variables. For the analysis we used GC-MS samples of each individual and calculated all factors on the basis of a correlation matrix (Jolliffe, 1986). SYSTAT 5.2 statistical program was used (SYSTAT, Inc., Evanston, Illinois).

RESULTS

The amount of secretion that could be collected from the four koalas varied substantially between individuals (Table 1). The oldest individual, Happy, displayed the largest gland size and produced the highest amount of secretion. Arnold, the youngest individual in the group, yielded only 0.52% of the quantity collected from Happy.

Individual GC-MS analyses of the swabs revealed the secretion to be a complex mixture containing predominantly volatile fatty acids, aldehydes,

TABLE 1. PHYSIOLOGICAL PARAMETERS AND GLAND CHARACTERISTICS OF FOUR MALE KOALAS FROM BONORONG PARK WILDLIFE CENTRE, BRIGHTON, AUSTRALIA[a]

Individual	Age (yr)	Weight (kg)	Gland diam. (cm)	Secretion (mg)	Cage
Happy	15	10	6	115	A
Boris	8	12.2	6	62	B
Buttons	3	11.5	5	3.8	A
Arnold	2.5	6.5	4.5	0.6	B

[a]The gland diameter describes only the diameter of the sparsely haired gland area, whereas the area of stained chest fur was much larger but could not be clearly defined.

ketones, monoterpenes, sesquiterpenes, and some nitriles and oximes, including a novel sulfur-containing oxime (Table 2, Figure 1).

Nitriles occurred in considerable amounts in some swabs and were identified by a combination of mass spectral library searches, high-resolution mass spectrometry, and ultimately comparison with synthetic samples. 2-Methylpropionitrile (isobutyronitrile), and 2-methyl-, and 3-methylbutyronitrile were identified in all swabs. These nitriles have not been reported previously in any olfactory gland secretion but have a strong potential to function as olfactory cues. The most abundant nitrile discovered in the scent of the koala was benzyl cyanide, which was a very significant component of the secretion and has so far only been described in the chest gland secretion of the African great bushbaby (*Galago crassicaudatus*) (Crewe et al., 1979).

syn- and *anti*-Phenylacetaldehyde oxime were identified from first principles by mass spectrometry and then confirmed by comparison with synthetic standards, and were among the largest components in some secretions [M^+ 135 (21%), *m/z* 118 (29), 117(100), 91(95), 90(62), 89(34), and 65(30) for the *syn* isomer and M^+ 135 (21%), *m/z* 118(27), 117(90), 91(100), 90(62), 89(29), and 65(28) for the *anti* isomer].

The novel sulfur-containing *syn*- and *anti*-3-methylthiopropanal oximes were identified in some secretions. These were identified by a combination of mass spectrometry from first principles and ultimately by comparison with a synthetic standard. Initial evidence for their identity was obtained from the similarity of their spectra with that of 3-methylthiopropanenitrile (National Institute of Standards and Technology (NIST) Mass Spectral Library).

Significant problems were encountered with the phenylacetaldehyde oximes and 3-methylthiopropanal oximes due to their facile dehydration to the corre-

TABLE 2. COMPOUNDS IDENTIFIED IN STERNAL GLAND SECRETION OF KOALA (*Phascolarctos cinereus*)

Ketones	Oximes
Acetone	*syn*- and *anti*-Phenylacetaldehyde oxime
Butenone	*syn*- and *anti*-3-Methylthiopropanal oxime
Butanone	
C_{14} alkan-2-ones	
C_{15} alkan-2-ones	
C_{16} alkan-2-ones	Nitriles
C_{17} alkan-2-ones	2-Methylpropionitrile (or isobutyronitrile)
	2-Methylbutyronitrile
Aldehydes	3-Methylbutyronitrile (or isovaleronitrile)
3-Methylbutanal	Benzyl cyanide
Hexanal	3-Methylthiopropanenitrile
Heptanal	
Octanal	Monoterpenes
Nonanal	α-Phellandrene
	β-Phellandrene
Carboxylic acids	*p*-Cymene
Acetic acid	1,8-Cineole
Propanoic acid	Limonene
Isobutyric acid	Piperitone
3-Methylbutanoic acid	Terpinen-4-ol
2-Methylbutanoic acid	
Pentanoic acid	Sesquiterpenes
Hexanoic acid	α-Gurjunene
2-Ethyl-hexanoic acid	Aromadendrene
Octanoic acid	Alloaromadendrene
	Bicyclogermacrene
Hydrocarbons	Viridiflorene
Octane	δ-Cadinene
Squalene	Epiglobulol
	Globulol
	Viridiflorol

sponding nitriles either in the transfer line between the GC and MS, or on the walls of the ion source (in addition to any loss of water caused by mass spectral fragmentation after ionization). Widely differing mass spectra were obtained on different injections, often with little or no nominal change in operating conditions. For 3-methylthiopropanal oxime, the least dehydrated mass spectum gave M^+ 119 (58%), *m/z* 102(68), 101(10), 91(7), 86(6), 75(21), 72(42), 61(100), 56(6), 47(11), and 41(8) for the first oxime isomer (source 200°C, transfer line 200°C), while the most dehydrated gave M^+ 119 (3%), *m/z* 102(4), 101(40),

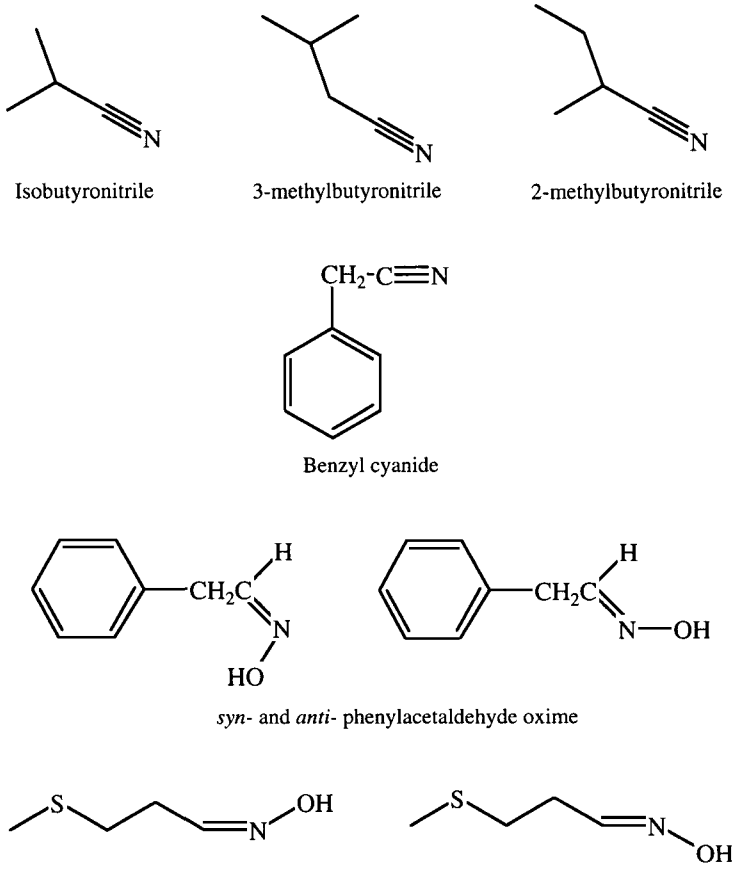

FIG. 1. Structure of identified nitriles and oximes in the sternal gland secretion of male koalas.

86(3), 75(2), 74(2), 73(3), 72(2), 61(100), 56(12), 54(20), 47(40), and 45(43) (source 190°C, transfer line 250°C) for the same isomer, with the latter spectrum being very similar to that of 3-methylthiopropanenitrile. The GC peaks maintained the correct retention time for the oximes, indicating this dehydration was not occurring during injection, although a small amount of 3-methylthiopropanenitrile was also observed as a separate GC peak in one koala swab and in the synthetic oxime sample [M^+ 101 (56%), *m/z* 73(2), 61(100), 59(3), 54(10), 47(10), and 45(11)]. The measured accurate mass for 3-methylthiopro-

panal oxime from the koala secretions was 119.0405 (C_4H_9NOS requires 119.0409).

The mass spectra obtained initially for the synthetic oximes were not a good match to the data from the koala secretion (particularly for the 3-methylthiopropanal oximes); however, eventually an excellent match between the standard and koala data (obtained several months apart) was observed, both in terms of GC retention index and MS ion intensities, for both pairs of oximes. The problem with variable dehydration was observed on both the HP 5970B and Kratos mass spectrometers.

This facile dehydration raised the question as to whether the significant benzyl cyanide observed in the secretions was present as such or was in fact an artifact caused by dehydration of the corresponding oximes during the desorption of the swab within the GC injection port. Analysis of the synthetic phenylacetaldehyde by desorption from a glass filter paper run under the same conditions as the koala swabs gave a nitrile peak 3% the size of the combined oximes, compared to 1.5% for a direct on-column injection of the same sample, 6% with normal splitless injection at 150°C and 53% with normal splitless injection at 250°C. The benzyl cyanide from the koala swabs was typically between 15 and 20% relative to the phenylacetaldehyde oximes; thus it seems likely that the nitrile was a genuine component of the secretions.

Various sesquiterpenes were also identified in the secretion. In the less volatile region, a complex range of apparent alkan-2-ones was also identified from the mass spectral data, ranging from 14 to 18 carbons, with up to three isomers for each carbon number. The most dominant of these was a C_{16} alkan-2-one, which was the largest peak in several swabs. This gave a mass spectrum with *m/z* 240(4%), 85(12), 71(42), 59(51), 58(99), and 43(100). The carbon skeletons of these alkanones were not determined.

A number of diterpene hydrocarbons ($C_{20}H_{32}$) and oxygen-containing diterpenes ($C_{20}H_{34}O$) were also detected in several runs, but no conclusive structures were assigned to these.

A comparison of the relative abundance of identified compounds showed significant variations among the four individuals sampled (Table 3). The scent secretions from Happy and Buttons, who were housed together, contained all compounds identified and were similar in their relative abundance levels. Only for benzyl cyanide and squalene did we observe a difference larger than 10%. Boris, the second oldest individual in the group, and Arnold, the youngest, showed a less diverse composition of the secretion, with two and 13 compounds, respectively, present in the secretions of the other koalas being undetectable. Furthermore, the relative abundance of nitriles to the C_{16} ketone was always $\leq 10\%$ in Boris, while the relative abundance was $\geq 5\%$ in Arnold.

Principal Component Analysis (PCA). To test whether the secretion of related individuals showed any similarities, we selected 11 compounds and

TABLE 3. RELATIVE ABUNDANCE OF COMPOUNDS IDENTIFIED IN CHEST GLAND SECRETION OF FOUR KOALAS AS PERCENTAGE OF PEAK AREA RELATIVE TO PEAK AREA OF UBIQUITOUS C_{16} KETONE

Compound	Happy	Buttons	Boris	Arnold
Acetone	*	*	*	*
Butenone	*	*	*	
Butanone	*	*	*	
Isobutyronitrile	*	*	*	**
3-Methylbutanal	*	*	*	
2-Methylbutanal	*	*	*	*
2-Methylbutyronitrile	**	**	*	***
Isovaleronitrile	**	**	*	***
Acetic acid	***	***	*	***
Hexanal	*	*	*	*
Octane	*	*	*	*
Propanoic acid	*	*		*
Isobutyric acid	*	**	*	**
Heptanal	**	**	*	***
3-Methylbutanoic acid	*	*	*	***
2-Methylbutanoic acid	***	***	*	***
Pentanoic acid	*	*		
Octanal	*	*	*	*
p-Cymene	*	*	*	*
1,8-Cineole	*	*	*	
Limonene	*	*	*	
Hexanoic acid	**	*	*	
Nonanal	***	***	***	***
Benzyl cyanide	***	*	**	***
2-Ethyl-hexanoic acid	*	*	*	
Octanoic acid	*	*	*	
Piperitone	*	*	*	*
syn-Phenylacetaldehyde oxime	***	***	***	**
anti-Phenylacetaldehyde oxime	***	***	***	**
α-Gurjunene	*	*	*	
Aromadendrene	**	***	***	***
Alloaromadendrene	*	*	*	*
Bicyclogermacrene	**	*	*	
δ-Cadinene	*	*	*	
Epiglobulol or ledol	*	*	*	*
Globulol	*	*	*	
Viridiflorol	*	*	*	*
C_{16} ketone	***	***	***	***
Squalene	*	***	*	**
syn-/anti-3-Methylthiopropanal	<1%	<1%	<1%	

[a] * = 1–5% of ketone peak area; ** = 5–10%; *** > 20%).

TABLE 4. COMPONENT LOADINGS FOR FIRST TWO PRINCIPAL COMPONENTS (PC) OF GC-MS PEAK AREA IN STERNAL GLAND SECRETION OF FOUR MALE KOALAS

Compound	Rotated component loadings	
	PC 1	PC 2
Isobutyronitrile	0.934	0.357
2-Methylbutyrontrile	0.905	0.397
Isovaleronitrile	0.929	0.368
p-Cymene	0.849	0.527
Benzyl cyanide	0.995	0.034
syn-Phenylacetaldehyde oxime	−0.240	−0.961
anti-Phenylacetaldehyde oxime	−0.249	−0.956
Aromadendrene	0.866	0.492
Alloaromadendrene	0.971	0.235
Epiglobulol	0.947	0.320
Viridiflorol	0.970	0.243
% variance	71.943	27.227

measured the percentage of their peak area relative to the peak area of the largest peak in TIC, a C_{16} ketone, which was present in all swabs. These relative percentages were compared using PCA in order to unravel similarities between related or commonly housed individuals.

Table 4 lists all compounds used in this analysis with their values for the component loading for principal component 1 (PC1) and 2 (PC2). The loading describes how well the component is correlated with the factor. In total the first and the second factors accounted for a total of 99.17% of the variance in proportional peak area.

Figure 2 displays the values of the four koalas for the first two principal components, showing a large similarity between the koalas Buttons and Boris, who are father and son. Happy, the oldest, and Arnold, who had not reached maturity, showed distinctly different values, indicating an age-dependent factor for the composition of the secretion.

DISCUSSION

In this study we were able to obtain chest gland secretion samples from four male koalas kept under controlled conditions. GC-MS analysis of these samples showed a variety of compounds present in the secretion, some of which have previously been identified in other mammalian scent secretions.

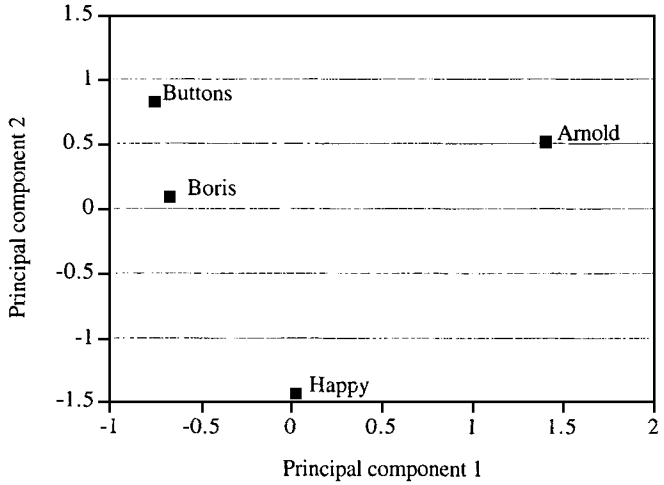

FIG. 2. Principal component analysis of the sternal gland secretion of four male koalas, plotting components 1 and 2 and showing the separation of individuals.

Benzyl cyanide was first described in a GC-MS study of chest gland secretion of the male African great bushbaby (*Galago crassicaudatus*) (Crewe et al., 1979). Similar to the situation in the koala, the great bushbaby also possesses a highly developed chest gland that is described as being frequently moist with secretion. Great bushbabies of both sexes employ this secretion exclusively for scent marking as they regularly move on so-called arboreal routes, which they mark with their secretion. In the *Galago* the abundance of benzyl cyanide is sex-dependent as the amounts identified in the secretion of males was higher than that of females (Crewe et al., 1979).

Benzyl cyanide, present as 8% of the total secretion in the great bushbaby, is a relatively volatile compound that has been shown to evaporate within a couple of hours. Therefore this nitrile may rather serve as a short-term messenger, indicating a small distribution range, and may function as a basis for territorial marking purposes (Katsir and Crewe, 1980).

In the case of the koala, Smith (1980) reported the use of the sternal gland for scent-marking of trees and suggests the secretion acts as a territorial marker at the base of a tree, indicating the presence of an animal in the tree above. Adult males mark the base of the trees they are climbing with sternal gland secretion, but it is not known how marking is distributed among preferred and nonpreferred trees in the wild. In the process of marking, the chest is flattened against the trunk and rubber up and down about six times (Smith, 1980). This

behavior is first observed in males 3 years of age and the marking frequency increases with age, reaching its peak when the males are 5 years old.

The use of chest marks as a territorial marker (Smith, 1980) and the absence of distinct arboreal routes in the koala indicates that benzyl cyanide might have a somewhat different function. The chest marking deposited by the koala on the base of his feeding tree needs to last for several days before the animal descends to mark the tree trunk again. Subsequently the gland secretion is expected to contain longer-lasting, less volatile components as the interval between the initial marking and subsequent marking of the same location is much longer than in the great bushbaby. Until detailed behavioral odor-presentation experiments have been performed, the function of this short-term messenger remains unclear.

The function of the volatile butyronitriles remains unclear, and so far these compounds have not been identified in any other mammalian scent secretion.

The compound 3-methylthiopropanenitrile is of particular interest, as organosulfur volatiles are generally associated with microbial fermentation, e.g., in the anal sac of carnivorous mammals or with urine and feces. GC-MS analyses have identified 150 of the 160 components identified in the skunk anal sac to contain sulfur (Andersen et al., 1982). Similarly sulfur-containing compounds have been identified in the anal sac secretion of hyanidae [5-thiomethylpentane-2,3-dione (Wheeler et al., 1975; Mills et al., 1980)] as well as mustelidae [various thietane and 1,2-dithiolane compounds (Crump, 1980a,b; Sokolev et al., 1980)]. Furthermore sulfur-containing compounds identified in the fecal pellets of adult male rabbits have been found to elicit heart-rate changes in other rabbits (Goodrich et al., 1981), while a sulfur-nitrogen heterocycle from the feces of the fox has been identified as eliciting a fear response in the Norway rat (Vernet-Maury, 1980). In all these cases, the presence of sulfur-containing compounds has been associated with microbial fermentation or degradation processes. To date no microbiological studies have been conducted on the chest gland area of the koala, and the presence of a specialized microflora can not be excluded. It is quite possible that the skin surface in this area or the sebaceous glands contain some microflora that could be responsible for some of the compounds identified in the secretion.

Nitrogen has been found to be of major importance for most animals, and a relative shortage or nitrogenous food for the very young seems to be an important factor limiting their numbers (White, 1978). This correlation has been suggested for the koala as well (Degabriele, 1981, 1983), indicating that they may select for low-fiber content in their *Eucalyptus* diet, which is usually correlated with high nitrogen levels in the leaves (Ullrey et al., 1981). Therefore, it seems rather unlikely that the koala uses the identified nitriles as a way to excrete excess nitrogen. This suggests that there might be an additional function in the nitrogenous compounds secreted through the gland, including their possible importance in olfactory communication in *Phascolarctos cinereus*.

The compounds *syn-* and *anti*-phenylacetaldehyde oxime have previously only been identified in the urine of male guinea pigs (Smith et al., 1977). Smith originally described these compounds as "novel testosterone-dependent mammalian metabolites" but also mentioned their potential function as semiochemicals. He showed that the presence of these oximes in the urine of adult male guinea pigs elicited a strong aversion from other male guinea pigs to the urine, compared with their attraction to the urine of females and immature individuals. The effect of these oximes on adult koalas was not tested.

Phenylacetaldehyde oximes had not been described as mammalian metabolic products prior to the study by Smith et al. (1977) but had been demonstrated to arise during the plant biosynthesis of *glucotropaeolin*, a mustard-oil glucoside (Tapper and Butler, 1967; Underhill, 1967). In both their studies, Underhill (1967) and Kindl and Underhill (1968) demonstrated that glucotropaeolin arises from the common amino acid phenylalanine through the intermediacy of phenylacetaldehyde oxime (Figure 3). Although direct comparison of plant metabolites to that of higher mammalian systems is difficult at best, precedent has been set for the conversion of phenylalanine to phenylacetaldehyde oxime in the male guinea pig by Smith et al. (1977). The significance of these oximes in the secretion, as well as the relevance of both compounds for the olfactory communication in the koala, is still unclear. Oximes as a class of compounds have only rarely been reported as natural products and only once before from a mammalian source, in the work of Smith et al. (1977) discussed above.

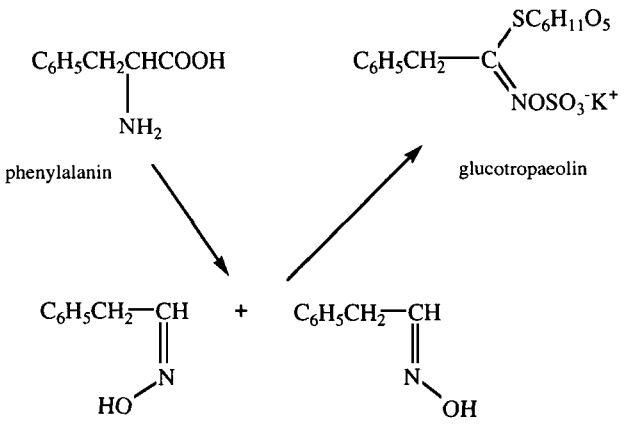

FIG. 3. Proposed biosynthetic pathway for the mustard-oil glucoside *glucotropaeolin* with phenylacetaldehyde oximes as intermediates (Underhill, 1967).

One of the unusual characteristics of the koala is the fact that it feeds almost exclusively on the foliage of various *Eucalyptus* species (Pratt, 1937; Eberhard, 1978; Hindell et al., 1985). This diet is generally of poor quality, containing high concentrations of indigestible compounds as well as potentially toxic phenols and terpenes. The water content is around 50% and reaches up to 60% in young leaves, which enables koalas to obtain their total water requirements from the leaves themselves (Eberhard et al., 1975). Apart from the valuable sugars, proteins, minerals, and lipids, *Eucalyptus* foliage contains a relatively high percentage of fiber that is difficult to digest and contains little energy (Cork et al., 1983; Cork and Sanson, 1990). Additionally some of the lipids are present as toxic essential oils and waxy compounds that need to be detoxified in the liver and are then excreted through the urine (Eberhard et al., 1975). Tannins and other phenolics are also significant components of the leaves estimated to present up to 40% of leaf dry matter (Fox and Macauley, 1977; Cork, 1984; Cork and Sanson, 1990). In the digestive tract these tannins are broken down, releasing their major constituent, phenols, which also have to be detoxified (Southwell, 1978; Cork and Sanson, 1990).

The secretion of all animals sampled contained some mono- and sesquiterpenes. These volatile terpenoids occur in high concentration in the *Eucalyptus* foliage, and the koala deals with those substances by voiding them through urine, feces, and through breathing and the skin, which is easily detectable as koalas always emanate a general *Eucalyptus*-like body odor (Macpherson, 1925). On average no more than 15% of the ingested oil has been reported to pass through the digestive tract without absorption (Eberhard et al., 1975). Up to 30% of the ingested oils may be detected in the feces, and it has been proposed that more than 50% of the essential oils are eliminated through urinary glucuronides (Eberhard et al., 1975; Baudinette et al., 1980). Recent studies have shown that marsupials also excrete significant quantities of terpenes via a series of nonconjugated, multiply oxidized forms (McLean et al., 1993; McLean and Foley, 1997).

The abundance of the different terpenoids identified did not represent a characteristic profile of the *Eucalyptus* species fed to the koalas (data not shown). Instead, some terpenes identified in the essential oils of the *Eucalyptus* species could not be identified in the chest gland secretion. However, whether the identified terpenoids originated from contamination through the *Eucalyptus* leaf diet or if they are endogenous to the koala remains difficult to determine because of the inability of *Phascolarctos* to thrive on any non-*Eucalyptus* oil-free diet.

The relative abundance of compounds in the secretion does show considerable variation among the four individuals (Table 3). Happy, the oldest individual (15 years), showed a complex array of substances in his sternal gland secretion with some compounds such as phenylacetaldehyde oxime, benzyl cyanide, and 2- and 3-methylbutyronitrile appearing as prominent peaks. The koala

Buttons (3 years), whose glandular area had not reached full size, produced a very similar secretion to that of his cage mate Happy. Only the relative content of squalene and the abundance of benzyl cyanide showed a pronounced difference. Although the size of his gland and the amount of secretion produced was much smaller than in the fully grown Happy, the secretion was similar in peak dominance and overall composition.

In contrast, the secretion of Boris, the second oldest male, who was housed with the juvenile Arnold, showed much less pronounced peaks of, for example, 2- and 3-methylbutyronitrile. This might indicate that the composition of the scent secretion is influenced by the scent composition of neighboring inividuals. The scent gland secretion of Arnold was characterized by the lack of a number of compounds, and generally low abundances otherwise. For his cage mate Boris, this could mean there was no necessity to produce all compounds in high amounts to signal his position as the older individual.

In summary, it appears that the composition of the sternal scent secretion in male koalas is in part determined by the scent of surrounding conspecifics. This production of scent on demand, depending on the presence of conspecifics would reduce the energy needed to produce the scent. As the koala is living on a low-energy diet with a basal metabolism of only 74% of the mean for other marsupials (Degabriele and Dawson, 1979) and conserving energy is one of the prime goals, this behavior would well ensure the most effective utilization of his resources.

Although the proportion of each component varies among individuals, PCA revealed a clear separation in the odor profile identifying related individuals such as Buttons and Boris (Figure 2). Furthermore a clear division was observed between the youngest and the oldest individual in the group, indicating an age-dependent factor that further influences the composition of the gland secretion. While it seems very likely that the koala uses this information to distinguish between individuals, a final proof that the koala is able to make a discrimination similar to the PCA will require a bioassay to test the response to synthetic mixtures of those components.

Odors are used by many species to recognize other individuals or classes of animals (Brown, 1979). However, the evidence of individual recognition of odors by koalas is equivocal. Mothers and their young apparently sniff each others' noses when reuniting after separation, but mothers accept the young of other females as their own, and young koalas climb on the back of anything furry, even male koalas or other species (Mitchell, 1989; Smith, 1979), suggesting that individuals are not recognized. However, males show different patterns of behavior when approaching or attacking males compared to their behavior with females. This difference might partly be related to different odors. From these observations no clear conclusions can be drawn on the role of scent in the social organization of koalas. Although koalas produce scent and inspect their

environment for scent, there is currently no direct evidence that they use scent to define space, recognize individuals, or recognize physiological states.

The results presented in this study indicate that the composition of koala scent secretion is determined by the scent of neighboring individuals as well as the genetic relationship between individuals. However, whether the koala uses these differences to distinguish between individuals remains unclear and further behavioral experiments will have to be conducted to elucidate the significance of the compounds identified.

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CONSEQUENCES OF ENRICHED ATMOSPHERIC CO_2 AND DEFOLIATION FOR FOLIAR CHEMISTRY AND GYPSY MOTH PERFORMANCE

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Abstract—Elevated concentrations of atmospheric CO_2 are likely to interact with other factors affecting plant physiology to alter plant chemical profiles and plant-herbivore interactions. We evaluated the independent and interactive effects of enriched CO_2 and artificial defoliation on foliar chemistry of quaking aspen (*Populus tremuloides*) and sugar maple (*Acer saccharum*), and the consequences of such changes for short-term performance of the gypsy moth (*Lymantria dispar*). We grew aspen and maple seedlings in ambient (~360 ppm) and enriched (650 ppm) CO_2 environments at the University of Wisconsin Biotron. Seven weeks after budbreak, trees in half of the rooms were subjected to 50% defoliation. Afterwards, foliage was collected for chemical analyses, and feeding trials were conducted with fourth-stadium gypsy moths. Enriched CO_2 altered foliar levels of water, nitrogen, carbohydrates, and phenolics, and responses generally differed between the two tree species. Defoliation induced chemical changes only in aspen. We found no significant interactions between CO_2 and defoliation for levels of carbon-based defenses (phenolic glycosides and tannins). CO_2 treatment altered the performance of larvae fed aspen, but not maple, whereas defoliation had little effect on performance of insects. In general, results from this experimental system do not support the hypothesis that induction of carbon-based chemical defenses, and attendant effects on insects, will be stronger in a CO_2-enriched world.

Key Words—*Acer saccharum*, carbohydrates, carbon dioxide, feeding trials, induced defenses, *Lymantria dispar*, phenolic glycosides, plant-insect interactions, *Populus tremuloides*, tannins.

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[1]Deceased.

INTRODUCTION

Allocation of fixed carbon to growth, reproduction, and defense is closely linked to internal source-sink dynamics in plants. These, in turn, are shaped by plant responses to variation in resource availability over ecological and evolutionary time scales (Bryant et al., 1983; Herms and Mattson, 1992; Lerdau et al., 1994; Mole, 1994).

Availability of one critical plant resource, carbon dioxide, has been increasingly globally since the late 18th century, and atmospheric concentrations continue to rise at about 0.4% per year (Houghton et al., 1996). One biological effect of this change is a shift in the chemical composition of plants. In general, foliar C:N ratios increase because of decreases in nitrogen and increases in carbon-based compounds such as starch and phenolics (Lincoln et al., 1993; Watt et al., 1995; Lindroth, 1996). Such changes in plant chemical composition can alter the feeding and growth performance of phytophagous insects (Lincoln et al., 1993; Watt et al., 1995; Lindroth, 1996). The magnitudes of change in both foliar chemistry and insect performance differ, however, among plant and insect species and in relation to the availability of other required resources (Kinney et al., 1997; Roth et al., 1997).

Herbivory itself can alter plant chemical composition either through changes in source-sink dynamics or through elicitation of active defensive responses (Tallamy and Raupp, 1991; Baldwin, 1994; Lerdau et al., 1994; Karban and Baldwin, 1997). Moreover, the magnitude of induced responses, and effects on herbivores, can be influenced by resource availability (Bryant et al., 1993; Yates and Peckol, 1993; Hunter and Schultz, 1995).

Recognizing the importance of both enriched CO_2 and herbivory to community dynamics and ecosystem function, researchers have begun to address how those factors *interact* to influence plant–herbivore associations. The few studies conducted to date, however, have emphasized interactive effects on plant physiology and growth (e.g., Fajer et al., 1991; Pearson and Brooks, 1996; Wilsey et al., 1997; Kruger et al., 1998). Exceedingly few have addressed the consequences of such interactions for plant chemical composition and herbivore performance. Such studies are vitally important for improving our understanding of trophic dynamics in a CO_2-enriched world.

Here we assess the independent and interactive effects of enriched atmospheric CO_2 and defoliation on the foliar chemistry of deciduous trees and the performance of a leaf-feeding insect. We predicted that growth under enriched CO_2 would enhance production of induced, carbon-based defenses in quaking aspen (*Populus tremuloides* Michx.) and sugar maple (*Acer saccharum* Marsh.). Furthermore, we predicted that enhanced defensive capacity would reduce the growth and feeding performance of gypsy moth (*Lymantria dispar* L.) larvae.

Several factors influenced selection of this experimental system. Firstly,

both aspen and maple produce carbon-based secondary metabolites, concentrations of which are responsive to atmospheric CO_2 levels (Lindroth et al., 1993; Roth and Lindroth, 1995; Kinney et al., 1997). Secondly, CO_2-mediated changes in foliage can alter performance of gypsy moth larvae (Lindroth et al., 1993; Roth and Lindroth, 1995; Kinney et al., 1997). Thirdly, these tree species are known to respond to defoliation by increasing foliar concentrations of phenolics, including phenolic glycosides in aspen (Clausen et al., 1989) and condensed and hydrolyzable tannins in maple (Baldwin and Schultz, 1983). Finally, aspen and maple are important constituents of forest ecosystems in the Great Lakes region, where gypsy moths are now becoming established.

METHODS AND MATERIALS

Experimental Design. This study was conducted in eight environmental control rooms at the University of Wisconsin Biotron. We employed a split-plot design, with two crossed factors (CO_2 and defoliation) as whole plots and tree species (aspen and maple) as the subplot. CO_2 treatments were designated as ambient ($\sim$360 ppm) and enriched (650 ppm). Defoliation treatments consisted of control (0% defoliation) and defoliated (50% defoliation) rooms. Two separate rooms for each CO_2 $\times$ defoliation combination (7–10 trees/species/room) afforded true, although little, replication at the whole-plot level. An alternative design, with defoliation treatments nested within CO_2 treatments, would have afforded greater statistical power to detect defoliation and CO_2 $\times$ defoliation interactions. We did not use such a design because of concerns about elicitation of defensive responses in undamaged trees due to "communication" with neighboring defoliated trees (Farmer and Ryan, 1990).

Controlled Growth Conditions. One-year-old aspen and 2-year-old maple seedlings were used in this study. Aspen were grown from seed the previous year and stored, bare-rooted, in a cold room over winter. [Aspen seeds (full sibs) were obtained from the University of Minnesota North Central Experiment Station (Grand Rapids, Minnesota)]. Maple seedlings were provided by the Wisconsin Department of Natural Resources (Wilson Nursery, Boscobel, Wisconsin). Trees were randomly assigned to CO_2 and defoliation treatments. Another set of trees, identically treated, was used for assessments of treatment effects on gas exchange and growth. Results from that parallel study are reported by Kruger et al. (1998).

Dormant seedlings were planted in 11-liter pots with a 1:2:2 mixture of forest topsoil, peat, and sand. An automatic drip irrigation system provided nutrients and/or water twice a day. The watering regime consisted of two days with nutrient solution followed by one day of water only to prevent accumulation of salts. The nutrient solution (half-strength Hoagland's solution) provided nitrate

at a concentration of 7.5 mM, sufficient for excellent tree growth (Kinney and Lindroth, 1997). We used a 15L:9D photocycle as representative of early summer in southern Wisconsin. At maximum light intensity, photon flux density (photosynthetically active radiation) averaged 501 ± 83 (SE) μmol/m^2/sec; intensity was reduced to 40% of maximum during the first and last 30 min of each photocycle. Light–dark temperatures and humidities were maintained at 25:20°C and 70:85%, respectively. Pots were rotated within rooms twice during the experiment.

Defoliation Treatment. The defoliation treatment was conducted seven weeks after average budbreak. Half of each leaf on each defoliated tree was removed by cutting, with hair texturizing scissors, along one side of the midrib. This technique produced a ragged leaf edge, mimicking feeding by large caterpillars.

Foliar Chemistry. Chemical analyses were conducted on leaf tissues to determine concentrations of nutritional factors (water, nitrogen, protein, and carbohydrates) and secondary metabolites (various phenolics). Sampling of each tree was accomplished by clipping several leaves cleanly at the petiole, then flash-freezing tissue in liquid nitrogen. Subsequently, samples were freeze-dried, ground (No. 40 mesh), and stored at −20°C. We sampled leaves from 7–10 trees per species per room on two occasions. The first sample was taken one day prior to the defoliation treatment; this collection allowed us to ascertain whether any chemical differences existed between control and "defoliated" trees before the treatment was applied. The second was six days following defoliation, during the period of insect feeding bioassays.

Leaf water content was determined gravimetrically. Tissue nitrogen concentrations were measured by conducting Kjeldahl digestions (25–50 mg tissue) (Parkinson and Allen, 1975), followed by nitrogen determinations using the micro-Nesslerization technique of Lang (1958). Glycine *p*-toluene-sulfonic acid was used as the standard.

Soluble protein was quantified by the procedure of Jones et al. (1989). Samples of 10–25 mg were extracted in 0.1 N sodium hydroxide containing 0.05% sodium dodecyl sulfate (SDS). SDS was added to prevent precipitation of protein upon addition of the acidic Bio-Rad dye reagent. Bovine serum albumin served as the standard. Values for protein concentrations obtained by this method are typically less than those derived from the common practice of multiplying total nitrogen concentrations by 6.25 (Jones et al., 1989; Lindroth, unpublished data), indicating that a portion of plant protein may not be soluble in this assay and/or that other foliar components contribute significantly to total nitrogen concentrations.

The carbohydrate procedure of Schoeneberger, Ludovici, and Faulkner (unpublished) was used to analyze starch and soluble sugars [hexose (fructose, glucose) and sucrose]. Leaf tissue (25 mg) was extracted in 80% ethanol. Sugars

and starch were then enzymatically converted to glucose and quantified indirectly via coupled enzyme reactions that reduce NADP to NADPH in amounts proportional to the glucose content in each sample.

The major secondary metabolites of aspen and maple are phenolics, including phenolic glycosides (salicylates) in aspen, hydrolyzable tannins (gallotannins and ellagitannins) in maple, and condensed tannins in both species. We measured levels of salicortin and tremulacin, the most abundant salicylates in aspen, via high performance thin-layer chromatography (HPTLC) (Lindroth et al., 1993). Leaf samples (50 mg) were extracted in methanol and duplicate aliquots (1 μl) were developed on HPTLC plates (silica gel 60, 10 $\times$ 20 cm). The plates were scanned at 274 nm using a Camag Scanner II (Camag Scientific, Inc., Wrightsville Beach, North Carolina), and chromatograms were analyzed using Camag TLC software (CATS 3.11). Salicortin and tremulacin purified by sequential flash chromatography and thin-layer chromatography served as standards.

Concentrations of gallotannins were measured by the procedure of Inoue and Hagerman (1988) as modified by Lindroth et al. (1993). Maple leaf tissue (50 mg) was extracted in 70% acetone containing 10 mM ascorbic acid. Extracts were then hydrolyzed in acid (1 M H_2SO_4) and the hydrolysate was assayed for total gallic acid. A similar analysis was completed on nonhydrolyzed extract to measure levels of free gallic acid. Gallotannin concentrations were then calculated as the difference between total and free gallic acid, and values are presented as gallic acid equivalents. Ellagitannins were quantified using the method of Wilson and Hagerman (1990) as modified by Lindroth et al. (1993). Tissue samples (15 mg) were hydrolyzed in acid (1 M H_2SO_4) and ellagic acid was measured according to the standard method. Ellagitannin concentrations are given in terms of ellagic acid equivalents.

For condensed tannin determinations, leaf tissue was extracted as described previously for gallotannins. Tannin concentrations were measured via the hydrolytic conversion of proanthocyanidins to anthocyanidins according to the method of Porter et al. (1986). Results are expressed in terms of quebracho tannin equivalents. Because tannins from different species react differently in the assay, interspecific differences should be interpreted cautiously.

Gypsy Moth Bioassays. Gypsy moth egg masses (New Jersey Standard Strain) were obtained from the Otis Methods Development Center (Otis Air National Guard Base, Massachusetts). Larvae were reared in an environmental chamber (Percival, Boone, Iowa) in the Department of Entomology's Gypsy Moth Quarantine Facility. Insects were maintained at 25°C with a photoperiod of 15L:9D. During stadia 1–3, larvae were fed artificial diet (ODell et al., 1985).

Bioassays were conducted with fourth (penultimate) instars to assess effects of CO_2 and defoliation treatments on insect consumption, growth, and food

processing efficiencies. We conducted assays with one to two larvae for each of 7–10 trees per species per room (total of 20–26 insects per CO_2 × defoliation × tree species combination). The trials commenced one day following defoliation of the trees. Newly molted caterpillars were placed individually into 15- × 1.5-cm plastic Petri dishes containing a single damaged or undamaged leaf. Leaves were excised from trees at the base of the petiole, and petioles were inserted into water picks to maintain leaf hydration. Leaves were replaced, as necessary, at least every two to three days. Upon molting into the fifth stadium, larvae were frozen. Larvae, frass, and uneaten leaf material were dried (65°C) and weighed. To estimate the initial dry weights of larvae used in the bioassays, we determined the proportional dry weight of 25 larvae from the same pool of insects as used in the bioassays. Calculated initial weights of larvae were not statistically different among experimental treatments. To estimate initial dry weights of leaves used in bioassays, we regressed fresh weight on dry weight for leaves used in the chemical analyses. The resulting regression equations were then used to estimate initial dry weights of bioassay leaves from their measured fresh weights. We calculated the following insect performance parameters: growth rate, final weight, consumption rate, total consumption, approximate digestibility {AD: [(food ingested − frass)/food ingested] × 100}, and efficiency of conversion of digested food {ECD: [biomass gained/(food ingested − frass)] × 100}.

Statistical Analyses. We used analysis of variance (ANOVA) (PROC GLM, SAS Institute, Inc., 1989) for statistical analyses of plant chemical and insect performance data. The general split-plot model with two factors at the whole plot level and one at the subplot level was:

$$Y_{ijkl} = \mu + C_i + D_j + (CD)_{ij} + E_{ijk} + S_l + (CS)_{il} + (DS)_{jl} + (CDS)_{ijl} + e_{ijkl}$$

where Y_{ijkl} represents the average response (phytochemical or insect) in CO_2 level i, defoliation treatment j, room k, and tree species l. Fixed effects consisted of CO_2 level (C_i), defoliation (D_j), CO_2 × defoliation interaction $(CD)_{ij}$, species (S_l), CO_2 × species interaction $(CS)_{il}$, defoliation × species interaction $(DS)_{jl}$, and CO_2 × defoliation × species interaction $(CDS)_{ijl}$. Random effects included whole-plot error (E_{ijk}) and subplot error (e_{ijkl}). F tests for C_i, D_j, and $(CD)_{ij}$ were computed using E_{ijk} as the error term ($F_{1,4}$); F tests for S_l, $(CS)_{il}$, $(DS)_{jl}$, and $(CDS)_{ijl}$ were computed using e_{ijkl} as the error term ($F_{1,4}$). In situations where not all the terms were relevant to the basic model (e.g., for secondary metabolites occurring in only one tree species), the model parameters were adjusted accordingly. Cell means for CO_2 × defoliation × species × room were computed using the SAS MEANS procedure prior to analysis of variance. The standard errors we report are for the treatment means, $\bar{Y}_{ijl}$, computed by the SAS MEANS procedure ($N = 2$ rooms).

Because of the low level of replication of CO_2 and defoliation treatments in this study, the probability of type II statistical errors was high. We therefore refer to P values in the range 0.05–0.10 as "marginally significant."

Growth and feeding of insect larvae are correlated with size. We used the regression procedure described by Kinney et al. (1997) to remove the effect of initial size on growth and consumption variables. Adjusted values for growth rate, final larval weight, consumption rate, and total consumption were then analyzed with the general linear model previously described.

RESULTS

Foliar Chemistry. We found no statistically significant differences in foliar chemical composition between control and defoliated trees one day prior to the defoliation event (data not shown). Thus, the following responses attributed to defoliation can be construed as true treatment effects rather than to preexisting variation between nondefoliated and defoliated trees.

Enriched CO_2 and defoliation decreased and increased, respectively, foliar water content (Figure 1). Overall changes were slight, however, and tended to be stronger in aspen than in maple, although species interaction effects were not significant. Nitrogen concentrations declined 12 and 24%, respectively, in aspen and maple under high CO_2 concentrations, but did not change in relation to defoliation (Figure 1). Overall nitrogen concentrations were higher in aspen than in maple. Responses of soluble protein differed from those of nitrogen. Protein levels declined under enriched CO_2, but the response was much greater in aspen than in maple (Figure 1). Defoliation led to a 24% decline in protein concentration of aspen under ambient CO_2, a 53% increase under enriched CO_2, and no significant change in maple (significant three-way interaction).

CO_2 concentration and defoliation also altered leaf carbohydrates, affecting levels in aspen more than those in maple. With respect to hexose concentrations, responses were dominated by the three-way interaction among CO_2, defoliation, and species (Figure 2). That is, enriched CO_2 led to a much greater increase of hexose in aspen than in maple, but this response was ameliorated in defoliated trees. In contrast, sucrose levels were unaffected by both CO_2 and defoliation treatments (Figure 2). Responses of foliar starch mostly paralleled those of hexose (Figure 2); levels were 2.7-fold higher in enriched-CO_2, nondefoliated aspen but only 1.6-fold higher in enriched-CO_2, defoliated aspen.

Foliar phenolic levels responded to the direct, but not interactive, effects of CO_2 and defoliation treatments. In aspen, levels of the phenolic glycoside tremulacin tended to increase (24–27%) in high-CO_2 foliage, whereas levels of both tremulacin and salicortin tended to increase (31–75%) in damaged foliage (Figure 3). In maple, concentrations of gallotannins and ellagitannins increased

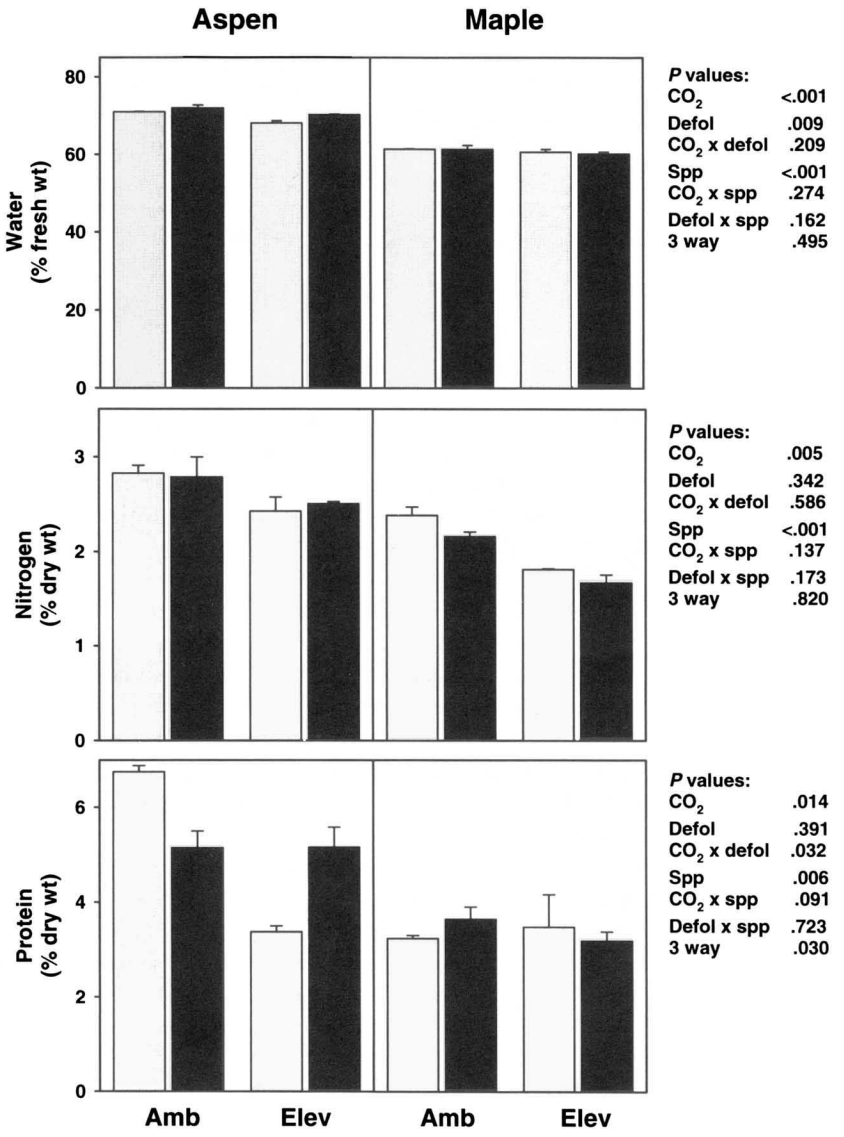

FIG. 1. Concentrations of water, nitrogen, and soluble protein in foliage of nondefoliated (light bars) and partially defoliated (dark bars) trees grown in ambient (Amb) or elevated (Elev) concentrations of CO_2. Vertical lines represent 1 SE. Defol = defoliation effect; Spp = species effect; 3-way = CO_2 × defoliation × species interaction.

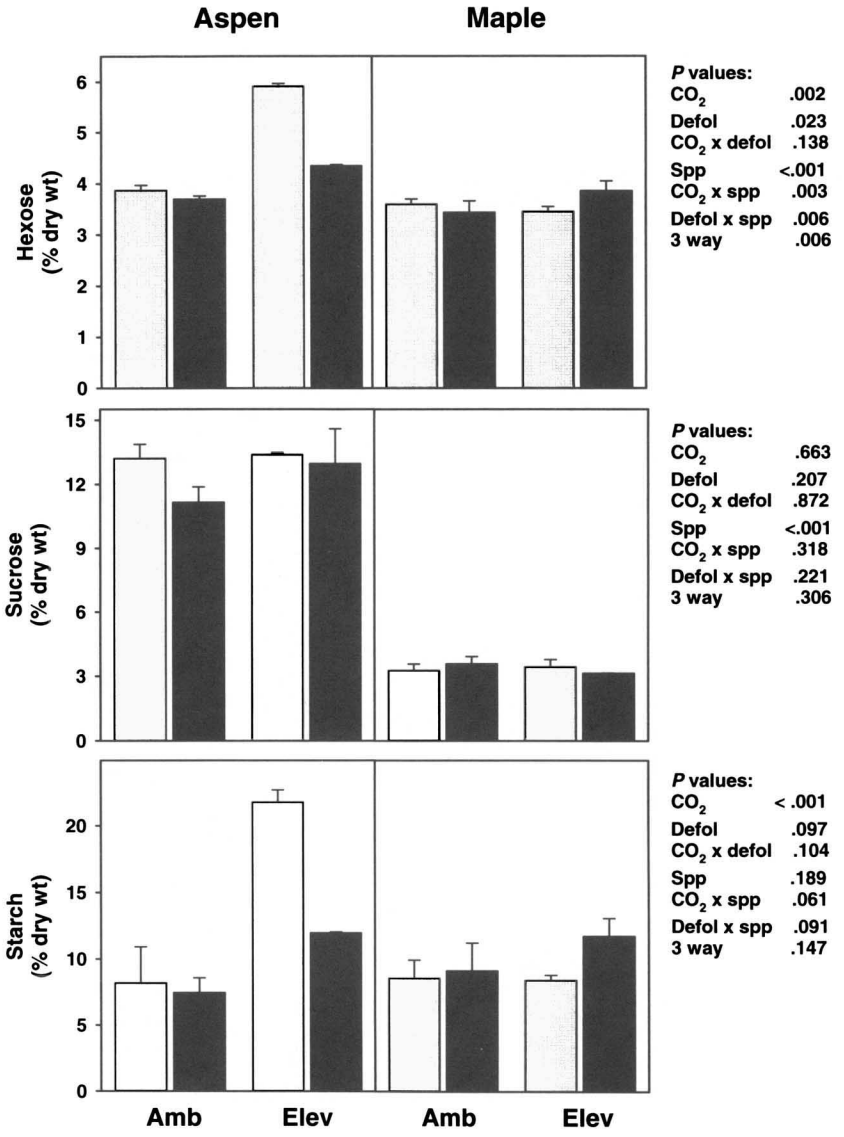

FIG. 2. Concentrations of carbohydrates in foliage of nondefoliated (light bars) and partially defoliated (dark bars) trees grown in ambient (Amb) or elevated (Elev) concentrations of CO_2. Format as described for Figure 1.

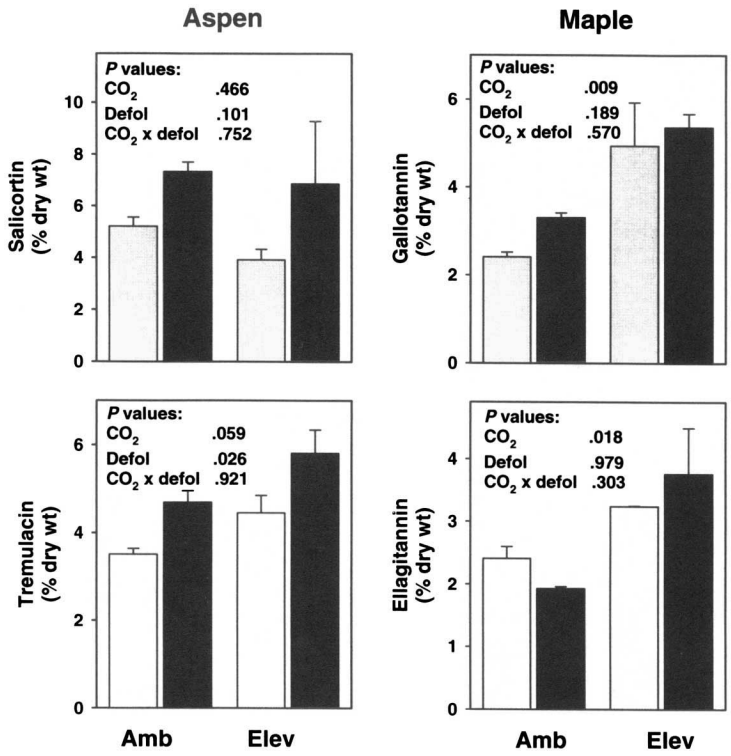

FIG. 3. Concentrations of phenolic glycosides (salicortin and tremulacin) in aspen foliage and hydrolyzable tannins (gallotannins and ellagitannins) in maple foliage of nondefoliated (light bars) and partially defoliated (dark bars) trees grown in ambient (Amb) or elevated (Elev) concentrations of CO_2. Format as described for Figure 1.

80 and 61%, respectively, under enriched CO_2, but were unaffected by defoliation (Figure 3). Similarly, levels of condensed tannins increased under enriched CO_2 in aspen (44%) and maple (59%), but were not affected by defoliation (Figure 4).

C:N ratios were calculated as the concentration of carbohydrates + phenolics relative to the concentration of nitrogen. C:N ratios increased substantially in response to high CO_2 in both aspen and maple, but were not significantly affected by defoliation in either species (Figure 4).

Insect Performance. In general, both CO_2 and defoliation treatments had little to moderate impact on insect performance. Duration of the fourth larval stadium was prolonged in insects reared on high-CO_2 foliage, especially in

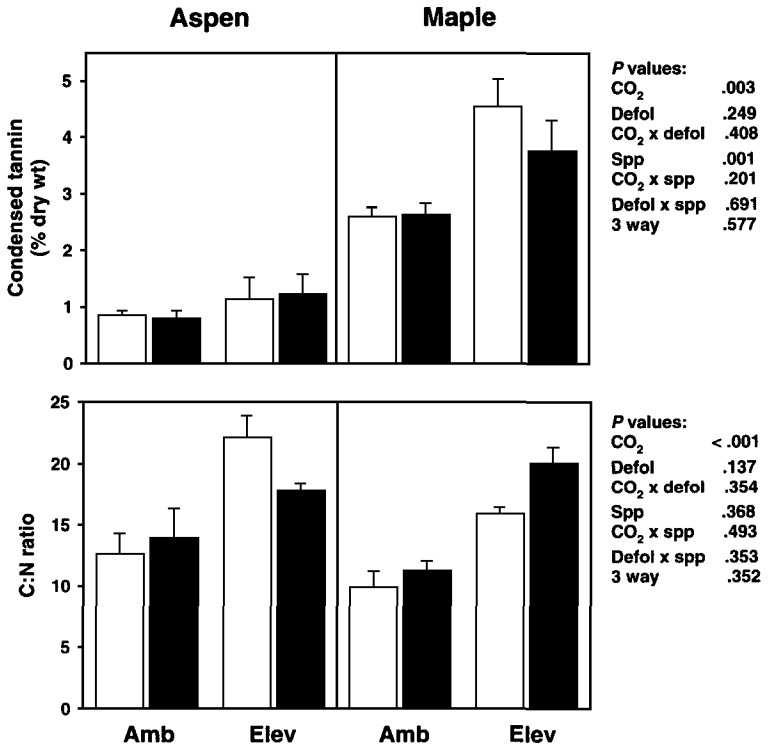

FIG. 4. Concentrations of condensed tannins and C:N ratios in foliage of nondefoliated (light bars) and partially defoliated (dark bars) trees grown in ambient (Amb) or elevated (Elev) concentrations of CO_2. Format as described for Figure 1.

larvae fed aspen (Figure 5). The changes, however, were small in magnitude. Defoliation did not significantly alter developmental rates overall, although the marginally significant three-way interaction suggests that the effects of defoliation may vary for different CO_2 levels and tree species. Enriched CO_2 reduced growth rates of insects fed aspen by 30%, but did not alter growth of insects fed maple (Figure 5). Reduced growth rates were offset by prolonged development times such that final larval weights of larvae fed ambient- and high-CO_2 aspen differed by only 15% (Figure 5). Leaf damage had no effect on growth rates or final larval weights. Prior defoliation did, however, tend to reduce consumption rates of larvae (Figure 6). Total leaf consumption was marginally increased and decreased, respectively, by CO_2 and defoliation treatments (Figure 6). Approximate digestibility declined slightly for insects fed damaged foliage,

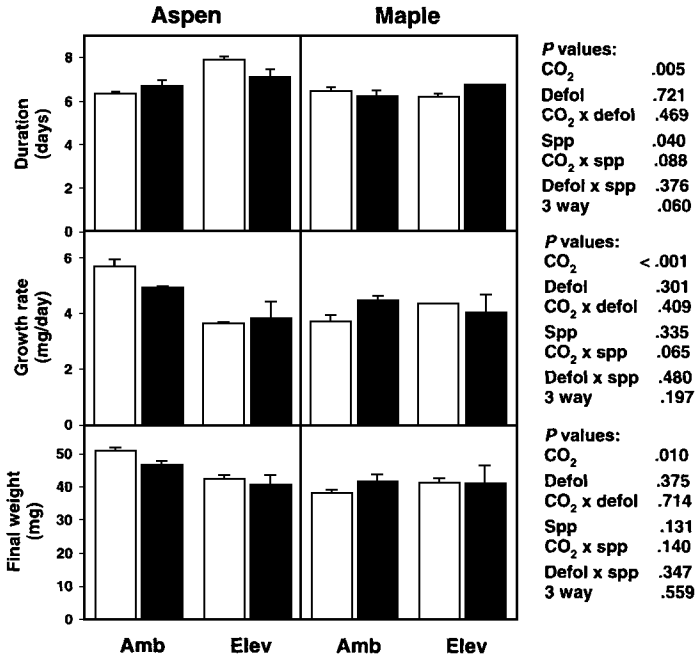

FIG. 5. Developmental time and growth performance of fourth-instar gypsy moths fed foliage from nondefoliated (light bars) and partially defoliated (dark bars) trees grown in ambient (Amb) or elevated (Elev) concentrations of CO_2. Format as described for Figure 1.

and values were much higher for insects fed aspen than for insects fed maple (Figure 6). The efficiency with which larvae converted digested food into biomass (ECD) was reduced in the high-CO_2 treatment for insects fed aspen, but not maple (Figure 6). Efficiency values tended to be higher for insects fed previously damaged foliage and were substantially higher for insects fed maple than for insects fed aspen.

DISCUSSION

Trees grown under enriched atmospheric CO_2 typically exhibit accelerated rates of photosynthesis, although negative acclimation (downregulation) occurs for some species and certain growing conditions after prolonged CO_2 exposure (Ceulemans and Mousseau, 1994; Curtis, 1996). Moreover, defoliation by leaf-chewing insects generally increases photosynthetic rates (Welter, 1989). Rea-

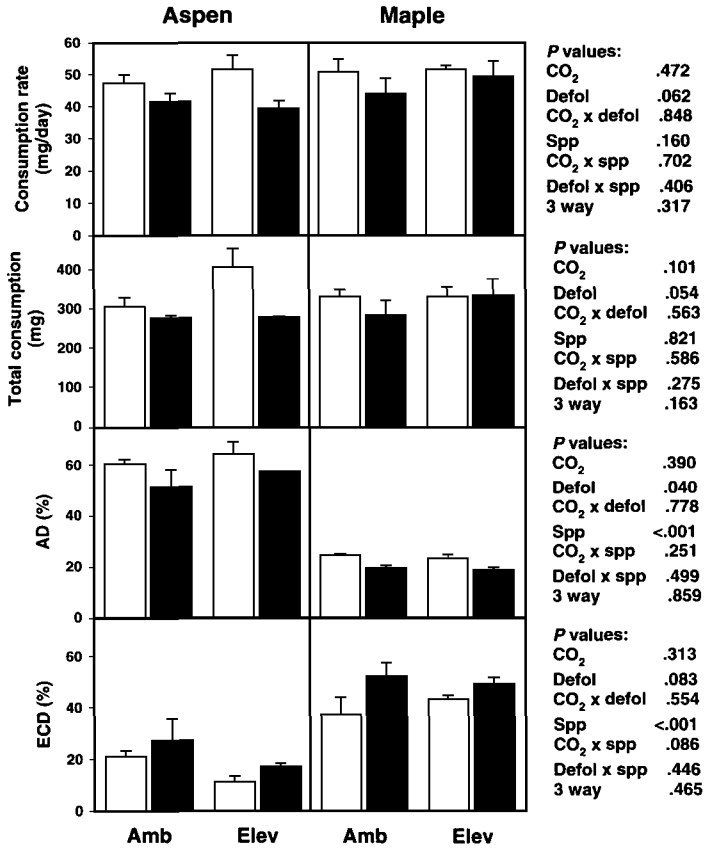

FIG. 6. Consumption and food processing parameters of fourth-instar gypsy moths fed foliage from nondefoliated (light bars) and partially defoliated (dark bars) trees grown in ambient (Amb) or elevated (Elev) concentrations of CO_2. Format as described for Figure 1.

sons for the latter response are multiple and varied, and include factors such as increased light penetration through the canopy and reduced feedback inhibition of photosynthesis. [Defoliation increases relative sink strength by increasing assimilate demand per unit of remaining leaf tissue (Welter, 1989)].

Whether enriched CO_2 and defoliation have additive effects on photosynthesis rates in trees is largely unknown. In a companion study to that described here, Kruger et al. (1998) assessed the effects of enriched CO_2 and defoliation on photosynthesis and growth in aspen and maple. They found that in aspen,

photosynthesis rates increased in high-CO_2 plants but were unaffected by defoliation. In maple, however, photosynthesis was not influenced by CO_2 level or defoliation. These results differ somewhat from those of Pearson and Brooks (1996), who showed that enriched CO_2 increased photosynthesis in *Rumex obtusifolius* and that herbivory by a chrysomelid beetle (*Gastrophysa viridula*) increased photosynthesis rates of plants in ambient, but not enriched CO_2. Kruger et al. (1998) also found that defoliation increased relative growth rates of maple trees in enriched CO_2 environments, and of aspen trees in both ambient and enriched CO_2 environments.

Foliar Chemistry. Here, we first discuss our results with respect to the direct effects of CO_2 and defoliation. Then we describe how those factors *interact* to affect foliar chemistry and compare our results with a similar open-top chamber study.

Phytochemical responses to CO_2 differed among compounds and plant species. Nitrogen (aspen and maple) and protein (aspen) levels declined in high-CO_2 plants, responses consistent with numerous other studies (McGuire et al., 1995; Watt et al., 1995; Lindroth, 1996; Roth and Lindroth, 1995). Enriched CO_2 led to increased carbohydrate (hexose and starch) accumulation in aspen, but not maple, responses linked to the differential effect of CO_2 on photosynthesis in the two species (Kruger et al., 1998).

Considering carbon-based seconary compounds, we found that levels of tremulacin, but not salicortin, increased in high-CO_2 aspen foliage. In six other studies (e.g., Lindroth et al., 1993; Roth and Lindroth 1995) of the effects of enriched CO_2 on aspen chemistry, our research group has usually found similar, marginal increases. Relatively weak responses by this group of compounds may be due to the fact that they are dynamic metabolites rather than stable end-products. Concentrations of the latter, such as tannins, may better reflect shifts in resource availability (Reichardt et al., 1991). Levels of condensed tannins in aspen, however, also showed small increases in response to CO_2. In contrast, gallotannin, ellagitannin, and condensed tannin concentrations all increased appreciably in high-CO_2 maple, results parallel to those of our other studies (e.g., Roth and Lindroth, 1995; Roth et al., 1997). The average 5% increase in leaf weight allocated to tannins in maple is particularly interesting given the fact that enriched CO_2 did not significantly accelerate photosynthesis in that species (Kruger et al., 1998). Slightly (although not significantly) elevated photosynthesis rates, sustained over many days, could account for some of the increase, as could a change in carbon metabolic processes (e.g., dark respiration) not measured by Kruger et al. (1998).

Phytochemical responses to defoliation also differed among compounds and tree species. Nitrogen concentrations were not affected by defoliation. Soluble protein levels in aspen, however, declined with defoliation under ambient CO_2, but increased with defoliation under enriched CO_2 (see discussion of interaction

below). Defoliation had little if any effect on carbohydrate levels in maple. Leaf damage reduced hexose and starch concentrations in aspen, and the magnitudes of change were affected by CO_2 treatment (interaction discussed below).

Carbon-based secondary metabolites exhibited varying responses to defoliation. In short, levels of dynamic metabolites (phenolic glycosides) increased, whereas those of static end-products (gallotannins, ellagitannins, condensed tannins) did not.

Overall, we observed few interactive effects of CO_2 and defoliation on foliar chemistry, and these were largely restricted to aspen. The strongest interactive effect occurred for soluble protein levels. The most abundant soluble protein in plant tissue is the carboxylating enzyme ribulose-1,5-bisphosphate (rubisco) (Jones et al., 1989). Reductions in rubisco concentrations in enriched-CO_2 aspen, especially in nondefoliated trees, is likely linked to carbohydrate metabolism and allocation. In high-CO_2, nondefoliated trees, levels of rubisco and other Calvin cycle enzymes may decline due to dilution from accumulating starch. In addition, declines may result from reallocation of nitrogen to other proteins (e.g., those required for electron transport or starch and sucrose synthesis) in photosynthetic tissue or to nonphotosynthetic tissue (Stitt, 1991; Ceulemans and Mousseau, 1994). (We did not, however, observe corresponding changes in foliar nitrogen levels.) In high-CO_2, defoliated aspen, protein levels did not decline. Because of a decrease in the carbohydrate source-sink ratio, the dilution effect did not occur and the demand for carboxylation activity remained high.

In research conducted following this study, Roth et al. (1998) evaluated the effects of enriched CO_2 and defoliation (by forest tent caterpillars, *Malacosoma disstria*) on aspen and maple in open-top chambers. Phytochemical results from that study and this one are nearly identical, with the exception that Roth et al. found that condensed tannin levels in aspen increased in response to defoliation as well as to CO_2, whereas ellagitannin levels in maple were unresponsive to CO_2.

A significant and similar finding in both studies was the lack of an interaction between CO_2 and defoliation with respect to allelochemical concentrations. Thus, at least for these species, CO_2 concentrations appear to affect levels of *constitutive*, but not *induced*, chemical defenses. We recognize that our studies have addressed only short-term induction responses (active defense mechanisms). CO_2 concentrations could conceivably play more important roles in mediating long-term induction responses in trees, as such responses may be more strongly affected by resource availability (Bryant et al., 1991). Finally, CO_2 is more likely to influence phytochemical induction in plants that respond more strongly to damage than do aspen and maple.

Insect Performance. Performance of gypsy moth larvae was affected by CO_2-mediated shifts in foliar chemical composition, although the magnitudes

of responses were generally small and differed between host species. Overall, CO_2 treatment did not significantly affect growth and consumption parameters of insects fed maple. These results are consistent with those of previous studies on CO_2, maple, and gypsy moths (Lindroth et al., 1993, Roth and Lindroth, 1995; Kinney et al., 1997), but differ from those of studies using forest tent caterpillars (*Malacosoma disstria*) (Lindroth et al. 1993; Roth et al. 1997) and whitemarked tussock moths (*Orgyia leucostigma*) (Agrell and Lindroth, unpublished data).

Gypsy moths reared on high-CO_2, nondefoliated aspen grew more slowly, even though eating more rapidly, due to marked declines in food conversion efficiencies. This is a common pattern of response for gypsy moths fed aspen (Lindroth et al., 1993, Roth and Lindroth, 1995) and can be explained on the basis of changes in foliar protein and phenolic glycoside levels. Gypsy moths increase consumption when fed diets low in protein (Sheppard and Friedman, 1992; Lindroth et al. 1997a). Moreover, phenolic glycosides are toxic to gypsy moths at moderate to high concentrations (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997). Thus, growth performance of insects fed high-CO_2, undamaged trees deteriorated because compensatory consumption increased their toxic load. In contrast, growth of insects fed high-CO_2, defoliated trees declined not because of changes in consumption, but directly because of high levels of phenolic glycosides in those plants. Prolonged stadium duration did not entirely offset the reductions in growth rates of larvae, resulting in slightly smaller insects at the conclusion of the short-term feeding trial. Given that such effects can be magnified over long-term feeding regimes, it is possible that the consequences of enriched CO_2 atmospheres for fitness of gypsy moths reared on aspen may be greater than indicated in this short-term study. Of central importance in studies of this kind, however, is assessment of how CO_2 affects concentrations of those phytochemical constituents that most strongly shape insect performance (Lindroth et al., 1997b).

Defoliation had relatively minor effects on insect performance. Both consumption rates and approximate digestibilities tended to decline for insects fed damaged foliage, but these changes were offset by improved conversion efficiencies. As a result, growth was not altered. These results differ somewhat from those of Roth et al. (1998), who evaluated the effects of CO_2 and defoliation (of aspen and maple) on performance of forest tent caterpillars. They found that damage to aspen did not alter insect performance. Defoliation of maple, however, reduced larval growth via decreases in consumption and food processing efficiencies.

We originally predicted that growth under enriched CO_2 atmospheres would enhance defoliation-induced accumulation of carbon-based defensive compounds, resulting in an additive, detrimental effect on the performance of gypsy moth larvae. Given that we found no such effect on foliar chemistry, it is not

surprising that we found little evidence of a CO_2 × defoliation effect on larval performance. This finding is consistent with those of Roth et al. (1998), who observed direct, but not interactive, effects of CO_2 and defoliation on forest tent caterpillars.

A growing body of literature suggests that the impact of enriched CO_2 on plant–insect interactions will be modulated by other abiotic factors, such as nutrient, water, and light availability (Johnson and Lincoln, 1991; Lawler et al., 1997; Kinney et al., 1997; Roth et al., 1998; Agrell and Lindroth, unpublished data). Relatively few studies have addressed how atmospheric CO_2 may interact with biotic factors to affect plant–insect interactions. To date, however, whether those biotic factors have been prior defoliation (this study; Roth et al., 1998), insect pathogens (Lindroth et al., 1997b), or parasitoids (Roth and Lindroth, 1995), true interactive effects have been mostly negligible. Ideally, future research will be expanded to include additional species, both short- and long-term induction responses, and more natural growing conditions. Emerging FACE (free air CO_2 enrichment) (Hendrey, 1993) technology at research sites located in many of the world's most important biotic communities may afford such opportunities.

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INHIBITORY EFFECT OF TERMITE FECAL PELLETS ON FUNGAL SPORE GERMINATION

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Abstract—The dampwood termite *Zootermopsis angusticollis* lines nest chambers and galleries with fecal pellets. The antifungal properties of feces were tested by recording germination rates of spores of the fungus *Metarhizium anisopliae* that had been incubated with various concentrations of fecal material. The presence of fecal pellet material significantly decreased the germination rates of spores relative to those of control spore solutions lacking fecal material. Spore germination rates were inversely proportional to the amount of fecal matter present in the spore–feces suspensions but were independent of incubation time. The fungistatic effect of the fecal material is virtually immediate and does not require prolonged contact with spores to inhibit germination. This mechanism of biochemical protection may reduce risks of fungal infection in termite nests.

Key Words—Termite, fungal pathogen, *Zootermopsis angusticollis*, *Metarhizium anisopliae*, disease, spore germination.

INTRODUCTION

Termites feed and nest in decayed wood environments laden with potential pathogens. These include bacteria, fungi, nematodes, viruses, spirochetes, and protozoa that can thrive within the humid and temperature-controlled conditions of the nest (Thaxter, 1914; Hendee, 1934; Toumanoff, 1966; Blackwell and Rossi, 1986; Grassé, 1986; Wood and Thomas, 1989; Khuong and Smart, 1994;

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Zoberi, 1995 and references therein). Pathogens, therefore, may be an important selective force favoring the evolution of behavioral, biochemical, and/or physiological adaptations to contend with disease (Wilson, 1971; Rosengaus and Traniello, 1993, 1994, 1997).

Nymphs of the dampwood termite *Zootermopsis angusticollis* Hagen react behaviorally to high spore concentrations of the fungus *Metarhizium anisopliae* (Metschnikoff) by increasing allogrooming and vibratory displays (Rosengaus et al., in press). *Z. angusticollis* also appear to develop an immune response to *M. anisopliae* and the bacterium *Pseudomonas aeruginosa* (Rosengaus et al., submitted). Although there is some evidence that termites also rely on biochemical mechanisms of disease control (Maschwitz and Tho, 1974; Olagbemiro et al., 1988; Rich, 1969; Batra et al., 1973; Batra and Batra, 1979; Grassé, 1982; Rosengaus et al., in preparation), detailed histological studies show that termites appear to lack glands specifically designed for the production of bacteriostatic and/or fungistatic substances (Noirot, 1969), unlike other social insects such as ants (Maschwitz et al., 1970; Wilson, 1971; Hölldobler and Engel-Siegel, 1984; Beattie et al., 1985, 1986; Attygalle et al., 1989; Hölldobler and Wilson, 1990). Instead, termites may have been biochemically preadapted to disease risks through body exudates and multifunctional glandular secretions. For example, alcohol extracts of *Reticulitermes lucifugus* and exudates of *Anacanthotermes ahngerianus* Jacobson and *Termes redemanni* Wasmann physogastric queens have bactericidal and fungistatic properties (Lavie, 1960a; Sannasi and Sundara Rajulu, 1967; Lutikova, 1990; Ljutikova and Judina, 1996). The labial and salivary gland secretions of several macrotermitine species, the defensive secretions of *Odontotermes gurdaspurensis*, *O. redemanni* (Wasmann), *O. praevalens* (John), and *Nasutitermes*, as well as sternal gland secretions of *Zootermopsis* nymphs can also inhibit microbial growth (Maschwitz and Tho, 1974; Olagbemiro et al., 1988; Rich, 1969; Batra et al., 1973; Batra and Batra, 1979; Grassé, 1982; Rosengaus et al., in preparation). Such multipurpose secretions appear to confer protection against potential pathogens in the absence of specialized glands to control microbial growth.

Dampwood termites such as *Z. angusticollis* form nests in decaying wood and cover nest chambers and gallery systems with fecal pellets and liquid feces, which appears to serve as "cement" to hold the pellets in place. Nest construction and maintenance seem to involve the extensive use of fecal matter as building material (Stuart, 1969; Grassé, 1984), and the inclusion of metabolites with antibiotic properties in the nest structure may help to reduce the risk of infection by naturally occurring pathogens. In this paper we investigate the fungistatic effect of solid, fresh fecal pellets of *Z. angusticollis* on the fungus *Metarhizium anisopliae*, a pathogen that has been isolated from termite nests (Lutikova, 1990; Zoberi, 1995).

METHODS AND MATERIALS

Spore Solution Preparation. Spores of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* were harvested from cadavers of *Z. angusticollis* Hagen that had been previously exposed to spores originally obtained from the American Type Culture Collection (batch 93-09, media 325; ATCC® 90448). A fungal stock suspension was prepared by scraping the spores, with a flamed metallic loop, from several dead termite hosts and transferring the spores to a 0.1% Tween 80 solution. Spore counts were then made using a hemacytometer at 400× magnification. Suspensions were serially diluted to give concentrations of 3.7×10^5, 3.7×10^4, and 3.7×10^3 spores/ml.

Preparation of Spore–Feces Suspension. Solid fresh fecal pellets (moist to the touch) were collected from a mature *Z. angusticollis* colony that originated from the Redwood Park in Oakland, California.

To prepare spore–fecal pellet suspensions, 500 μl of each of the spore concentrations were placed in a 1-ml Eppendorf tube. To each tube was added either 0.01, 0.1, or 0.25 g of fresh fecal pellets, resulting in 2%, 20%, and 50% spore–fecal pellet suspensions, respectively. Pellets were crushed with a flamed and cooled glass rod and centrifuged at 800*g* for 15 min at 23°C. Approximately 30 μl of the supernatant of each spore–fecal pellet suspension was immediately plated onto a microscopic slide that contained a thin layer of solidified potato dextrose agar (PDA, approximately 1 ml before it solidified).

To test whether the incubation time of spores and feces had an effect on spore germination rates, the same protocol was repeated, except that the spore–fecal pellet suspensions were allowed to stand for a 1- or 10-hr period at 13.5°C before we plated the supernatant onto the solidified PDA slides.

Overall, the three spore concentrations, three fecal pellet concentrations, and three incubation times give a total of 27 spore–fecal pellet treatment combinations. Of these, 23 treatments were replicated three times, while four had only two replicates.

The controls were two slides for each of the spore concentration–incubation time combinations (i.e., 2 × 9). Each control PDA slide was seeded with 30 μl of the relevant spore solution, with no fecal material added.

After seeding, the slides were placed in an incubator for 18 hr at 25°C. The slides were then examined under a microscope at 400× magnification. Conidia viability, measured as percentage germination, was determined by counting the number of spores with visible germ tubes as a fraction of the total number of spores in each field. Germination rates were determined after examining 10 consecutive fields of vision per slide, scanning the slide in a linear fashion from one end to the other. Subsequently, the mean percentage germination across the three replicates for each spore–fecal pellet suspension was

calculated. The mean germination rates of the control spore solutions per incubation time were based on 20 fields of vision. Thus, germination rates were based on a total of 950 fields of vision.

Statistical Analysis. Average percent germination rates among the various feces treatments within each incubation period were compared using ANOVA (SPSS, 1990). A linear regression model was built to establish the effects of the quantity of feces, spore concentration, and incubation time on germination rates, and the fit of the final model was assessed using residual analysis.

RESULTS

Fecal pellet material significantly reduced the germination rates of spores of *M. anisopliae*. The pattern of inhibition was consistent across all three spore concentrations (Figure 1A–C). Within each spore concentration, the inhibitory effect was independent of the time that spores and fecal matter were incubated together ($P > 0.05$). Moreover, germination was inversely proportional to the amount of fecal material added, after controlling for spore concentration and incubation time. The parameter estimates and 95% confidence intervals of the final regression model are shown in Table 1. Incubation time was retained in the model because of its biological importance in spite of its lack of significant association with germination rate. No collinearity or confounding effects were found between the variables, and the residuals of the model were normally distributed.

DISCUSSION

Chemical constituents of the fecal pellets of the dampwood termite *Z. angusticollis* appear to have an inhibitory effect on the germination rates of spores of the fungus *M. anisopliae*, a pathogen that has been isolated from termite colonies (Lutikova, 1990; Zoberi, 1995). These results support the findings of our pilot research on the antibiotic effects of fecal material, in which zones of fungal inhibition were measured planimetrically. *M. anisopliae*-seeded PDA plates that had five fecal pellets added to the surface of the culture medium had a greater area free of fungal growth (73.4%) than that of control-seeded plates with no feces added (18.3%). We now show that fecal pellets control fungal growth when spores and inhibitors come into contact in a spore suspension medium.

The use of feces during nest construction appears to be one mode of biochemical protection used by termites to combat disease. The origin and nature of the chemical(s) found in fecal material that lower the germination rates of

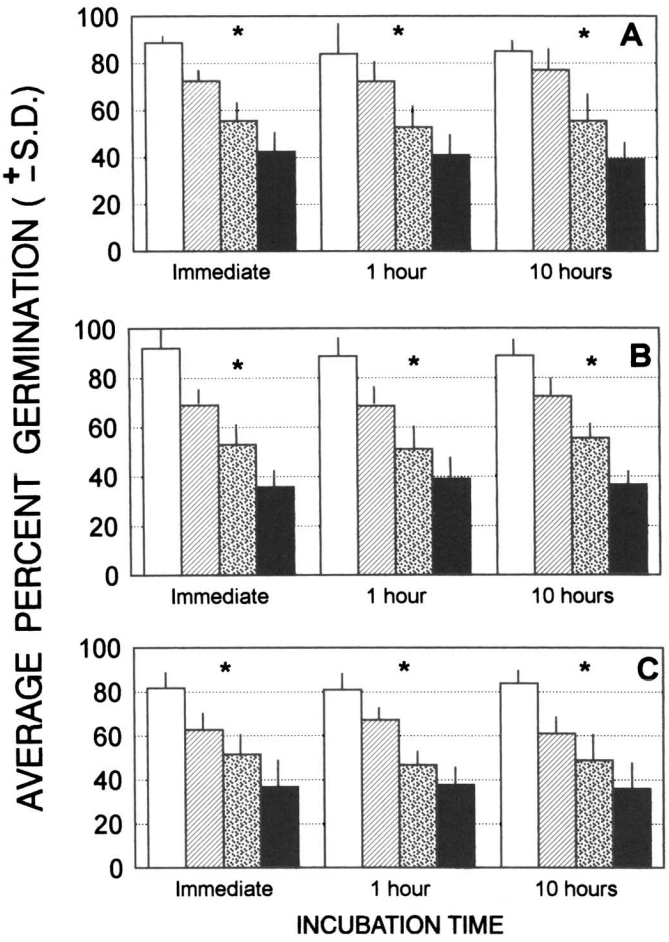

FIG. 1. Average percent germination (±SD) of *M. anisopliae* spores in concentrations of 3.7×10^5 (A), 3.7×10^4 (B), and 3.7×10^3 spores/ml (C) after incubation with various amounts of crushed fecal pellets (□ = control, based on 20 fields of vision; ▨ = 2% feces in spore solution, 30 fields of vision; ▩ = 20% feces in spore solution, 30 fields of vision; and ■ = 50% feces in spore solution, 30 fields of vision). Asterisks denote overall significant differences among the four treatments by an ANOVA test ($P < 0.001$). Pairwise comparisons were significant between any two treatments for each concentration and each incubation time ($P < 0.05$, Bonferroni correction) (SPSS, 1990).

TABLE 1. REGRESSION ANALYSIS OF EFFECT OF FECAL MATERIAL ON GERMINATION RATES[a]

Predictor	β	SE β	95% CI	P
Spore concentration	3.0	0.4	2.2–3.8	***[b]
Incubation time	−0.04	−0.1	−0.2–0.1	NS
Amount of feces in spore solution	−158.3	3.4	−165.0–151.6	***

[a] The estimates of the regression model β and SE β (standard error of β) are presented with the 95% confidence interval (CI) for each variable.

[b] *** indicates a significant effect of the predictor on germination rates, controlling for the other two variables ($P < 0.001$). The intercept for the regression model was 62.5 (with a confidence interval of 59.1–65.9 and a standard error of 1.7). To establish further the individual effects of the quantity of fecal material, a model with dummy variables was constructed. After controlling for spore concentration and incubation time, each quantity of feces was significantly and inversely associated in a linear fashion with germination rates ($P < 0.001$).

fungi have yet to be determined. The active inhibitor present in fecal pellets could be a metabolic by-product of digestion or may originate in the gut symbionts. Saliva and/or the passage of contaminated food with nonsymbiotic fungi through the termite's gut in the fungus-growing termite *Macrotermes bellicosus* (Smeathman) appear to inhibit greatly the germination of spores and growth of fungi other than its obligate symbiont *Termitomyces* spp. (Thomas, 1987; Wood and Thomas, 1989). Another possible source of chemicals with fungistatic properties could be the secondary compounds of the termite's host plants. For example, tannins ingested by some lepidopteran larvae appear to confer protection against disease agents (Hajeck and Renwick, 1993; Young et al., 1995). Both tannins and antibacterial substances found in foliage (Sikorowski and Lawrence, 1994 and references therein) may be sequestered during digestion, incorporated into the feces, and subsequently released into the nest surroundings via defecation. Termites could also exploit the fungistatic effects of humus or the production of bacteriocins by microorganisms found in the soil and/or nest to control potential pathogens (Clerk, 1969; Watson and Ford, 1972; Kermarrec et al., 1986a,b; Wood and Thomas, 1989; Keller and Zimmermann, 1989; Dykes, 1995; Ljutikova and Judina, 1996). Abiotic factors, such as pH, temperature, and CO_2 levels within the colony may further aid in the control of pathogen development (Burges and Fenton, 1953; Gonnet and Lavie, 1960; Watson and Ford, 1972; Batra and Batra, 1966, 1979; Wood and Thomas, 1989; Keller and Zimmermann, 1989).

In the social Hymenoptera, chemical antibiotic secretions are known to play a key role in adaptation to pathogen load. The majority of ant species, and

some bees and wasps, use antimicrobial secretions from a variety of sources such as the metapleural gland, Dufour's gland, venom gland, rectal fluid, salivary secretions, and body exudates. These glands produce a broad spectrum of antiseptic secretions that inhibit the growth of fungi, gram-positive and gram-negative bacteria, yeasts, and other microorganisms in the nest chambers (Lavie, 1960b,c; Maschwitz et al., 1970; Wilson, 1971; Michener, 1974; Vander Meer, 1983; Hölldobler and Engel-Siegel, 1984; Obin and Vander Meer, 1985; Beattie et al., 1986; Schmidt, 1986; Jouvenaz, 1986; Attygalle et al., 1989; Hölldobler and Wilson, 1990; Veal et al., 1992; Gambino, 1993). These secretions may be one reason why ants are significantly less susceptible to disease than termites (Rosengaus and Traniello, in preparation).

Unlike ants, termites do not appear to produce antibiotics in glands that have specifically evolved an antimicrobial function. This is intriguing for several reasons. First, cockroaches are ancestral to termites (Thorne and Carpenter, 1992) and the numerous cuticular glands present in cockroaches (Sreng, 1984, 1993) could have evolved into glands that synthesize and secrete antiseptics. It is thus conceivable that the microbially rich environment of prototermites could have favored the specialization of preexisting glands to produce secretions that reduced disease risk. Second, the phylogenetically unrelated ants and termites share similarities in many aspects of their biology, including nesting and foraging habits, that would appear to have been important to social evolution in both groups (Wilson, 1971), yet the biochemical mechanisms of disease resistance in ants and termites appear to differ. We believe that the evolution of antibiotic protection through glandular secretions in termites was compromised by the need to maintain the symbiotic fungi responsible in part for wood decomposition and increasing its nutritional value (Waller and La Fage, 1987). Termites must also protect the symbiotic, cellulose-digesting microbial community in their gut, which is sensitive to antibiotics (defaunated termites ultimately die due to starvation) (Eutik et al., 1978; Haverty and Howard, 1979; Mauldin and Rich, 1980). This constraint on the evolution of disease resistance could have favored other modes of defense, such as fungistatic feces, and physiological mechanisms of disease resistance, including the development of an immune response (Rosengaus et al., submitted).

We suggest that pathogen-related selection in termites favored the evolution of biochemical protection against pathogens by expanding the function of preexisting glands and secreta. Termites can control microbial growth through body exudates of unknown chemical identity (Batra and Batra, 1966, 1979; Sannasi and Sundara Rajulu, 1967; Lutikova, 1990; Ljutikova and Judina, 1996) or through glandular secretions that function in more than one context, including disease resistance.

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INFLUENCE OF RAGWEED (*Ambrosia trifida*) ON PLANT PARASITIC NEMATODES

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Abstract—The roots of ragweed *Ambrosia trifida* exude chemical compounds that have insecticidal activities. This paper reports a survey conducted in a soybean field infested with ragweed to determine if the ragweed affected the populations of plant parasitic nematodes. There was a strong indication that the ragweed decreased populations of plant parasitic nematodes in some cases.

Key Words—*Ambrosia trifida*, ragweed, soybean, plant parasitic nematodes.

INTRODUCTION

Nematodes are an important cause of problems limiting successful plant production. Environmental pollution as a result of using man-made pesticides to control plant parasitic nematodes has stimulated scientists to look for alternatives. The widespread Compositae weed, ragweed, *Ambrosia trifida*, is known to release chemical compounds from its roots (Lu and Parodi, 1993; Marco, 1991). Recently, these compounds have attracted attention due to their nematocidal effects (Lu and Parodi, 1993; Marco, 1991). Because of their possible environmental safety, root exudates may be promising alternatives to synthetic chemical pesticides for nematode control in the future.

Previous phytochemical studies have found that some constituents of the root exudates of ragweed are polyacetylenes, including their sulfur derivatives, and these compounds exhibit high biological activities against nematodes in

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laboratory experiments (Lu and Parodi, 1993; Marco, 1991; Handele, 1971). However, their efficiency was sometimes low when mixed with soil or under unlighted conditions (Akhtar and Mahmoud, 1993; Daulton and Curtis, 1963). The purpose of this survey was to determine whether ragweed influenced nematode populations under natural conditions.

METHODS AND MATERIALS

There is a wide range in the species and population numbers of nematodes occurring in agroecosystems. Our experimental field was limited to cropland where soybean grew intermixed with ragweed. The site is located 15 km northeast of Shenyang city in Liaoning province in northeast China. The survey was made in late June 1995 and early July 1996. Twenty soil samples were collected from the rhizosphere of soybean plants. We divided the samples into two categories: Category I, rhizospheric soil of soybean without ragweed nearby, and II, rhizospheric soil of soybean with ragweed growing nearby.

Five replicates for each of category I and category II samples were collected every year. The size of each replicate sample was about 20 cm × 20 cm × 15 cm deep. If the distance between the soybean plant and the ragweed plant was more than 30 cm, the soil was designated as category I. If this distance was less than 5 cm, it was designated as category II.

The soil samples were put into plastic bags to prevent drying and were transferred to the laboratory for separation and identification of nematodes. The method used to separate nematodes was the combination of a Baermann funnel with elutriation and sieving (Liu, 1995). We used 600 ml of soil for each experiment. Nematodes were collected from an aqueous suspension, the volume adjusted to 200 ml, and nematodes identified from 2 ml of this suspension with three repetitions. The number of nematodes in each genus identified was recorded, then extrapolated to the number in the 600-ml soil sample. If very few nematodes were found in the 2-ml subsample, the entire volume of 200 ml was examined.

RESULTS AND DISCUSSION

Thirteen genera of nematodes were identified: *Aphelenchoides*, *Aphelenchus*, *Atetylenchus*, *Coslenchus*, *Ditylenchus*, *Filenchus*, *Pararotylenchus*, *Paratylenchus*, *Pratylenchus*, *Psilenchus*, *Rotylenchus*, *Tylenchorhynchus*, and *Tylenchus*. *Pratylenchus* is a migratory endoparasitic nematode, *Aphelenchoides* and *Ditylenchus* are either endoparasitic or ectoparasitic nematodes depending on the host, and the others are ectoparasitic. Only *Ditylenchus*, *Pratylenchus*, and sometimes *Paratylenchus* and *Tylenchorhynchus* are considered as severe

TABLE 1. CATEGORY I: GENERA AND POPULATION DENSITIES OF NEMATODES IN SOIL SAMPLES FROM RHIZOSPHERE OF SOYBEAN WITHOUT *A. trifida* NEARBY IN 1995 (IN 600 ml SOIL)

Nematode genus	Number of nematodes							
	1	2	3	4	5	Total	Mean	Frequency[a]
Aphelenchoides	0	0	0	0	160	160	32.0	1/5
Aphelenchus	200	0	0	125	203	528	105.6	3/5
Atetylenchus	40	0	46	0	160	246	49.2	3/5
Coslenchus	0	0	0	10	0	10	2.0	1/5
Ditylenchus	120	0	0	10	80	210	42.0	3/5
Filenchus	40	0	0	163	40	243	48.6	3/5
Pararotylenchus	0	10	30	20	0	60	12.0	3/5
Paratylenchus	0	0	0	20	0	20	4.0	1/5
Tylenchus	0	0	70	78	0	148	29.6	2/5
Total	400	10	146	426	643	1625	325.0	

[a]Frequency refers to numbers of soil samples where nematodes were found among the total five samples. See also Tables 2–4.

plant-parasitic nematodes. Most species of *Aphelenchoides* and *Aphelenchus* feed on fungi.

As shown in Tables 1–4, 10 genera of nematodes were identified in 1995. Nine genera were present in category I and seven in category II. In 1996, there

TABLE 2. CATEGORY II: GENERA AND POPULATION DENSITIES OF NEMATODES FOUND IN SOIL SAMPLES FROM RHIZOSPHERE OF SOYBEAN AND ADJACENT *A. trifida* IN 1995 (IN 600 ml SOIL)

Nematode genus	Number of nematodes							
	1	2	3	4	5	Total	Mean	Frequency
Aphelenchus	0	60	260	30	90	440	88.0	4/5
Atetylenchus	0	10	0	0	0	10	2.0	1/5
Coslenchus	0	0	0	0	10	10	2.0	1/5
Ditylenchus	0	0	0	10	0	10	2.0	1/5
Pararotylenchus	0	0	20	0	10	30	6.0	2/5
Paratylenchus	0	0	40	0	0	40	8.0	1/5
Tylenchorhynchus	0	0	40	0	0	40	8.0	1/5
Total	0	70	360	40	110	580	116.0	

TABLE 3. CATEGORY I: GENERA AND POPULATION DENSITIES OF NEMATODES IN SOIL SAMPLES FROM RHIZOSPHERE OF SOYBEAN WITHOUT *A. trifida* NEARBY IN 1996 (IN 600 ml SOIL)

Nematode genus	Number of nematodes							
	1	2	3	4	5	Total	Mean	Frequency
Aphelenchoides	20	0	24	9	10	63	12.6	4/5
Aphelenchus	100	40	10	35	28	213	42.6	5/5
Atetylenchus	82	10	32	0	5	129	25.8	4/5
Ditylenchus	0	0	15	10	24	49	9.8	3/5
Filenchus	210	170	190	90	78	738	147.6	5/5
Pararotylenchus	90	96	240	118	152	696	139.2	5/5
Pratylenchus	0	0	0	0	11	11	2.2	1/5
Psilenchus	60	10	0	0	10	80	16.0	3/5
Rotylenchus	0	0	0	0	30	30	6.0	1/5
Tylenchus	30	80	115	46	30	301	60.2	5/5
Total	592	406	626	308	378	2310	462.0	

TABLE 4. CATEGORY II: GENERA AND POPULATION DENSITIES OF NEMATODES IN SOIL SAMPLES FROM RHIZOSPHERE OF SOYBEAN AND ADJACENT *A. trifida* IN 1996 (IN 600 ml SOIL)

Nematode genus	Number of nematodes							
	1	2	3	4	5	Total	Mean	Frequency
Aphelenchoides	10	0	0	8	0	18	3.6	2/5
Aphelenchus	5	15	4	4	20	48	9.6	5/5
Atetylenchus	24	15	4	0	5	48	9.6	4/5
Ditylenchus	49	9	0	0	0	58	11.6	2/5
Filenchus	160	105	35	28	35	363	72.6	5/5
Pararotylenchus	100	36	36	8	105	285	57.0	5/5
Pratylenchus	89	15	4	32	5	145	29.0	5/5
Psilenchus	0	20	0	0	0	20	4.0	1/5
Rotylenchus	30	35	0	8	0	73	14.6	3/5
Tylenchus	115	95	0	4	16	230	46.0	4/5
Total	582	345	83	92	186	1288	257.6	

were 10 genera in both category I and category II. For most genera, there are higher frequencies and larger numbers in category I than in category II.

Because only a few samples could be collected for valid comparison in the field, it was difficult to make a comparison of a large number of samples. Consequently, the great variation in nematode numbers in samples resulted in

large variances. In the following statistical analysis, we have considered a significance level at $P < 0.2$ only because we wanted to know if there were differences between the nematode densities of the two categories, even if the difference was slight. Tables 5 and 6 give the statistical test data in 1995 and 1996, respectively.

As the results in Table 5 show, there are seven genera that have higher population densities in category I than in category II. In category II, the numbers of *Aphelenchoides*, *Filenchus*, and *Tylenchus* nematodes are zero, significantly fewer than those in category I ($P < 0.05$). In *Atetylenchus* and *Ditylenchus* the nematode numbers are not different at $P < 0.05$. The differences are significant at $P < 0.2$. For *Aphelenchus*, *Coslenchus*, *Pararotylenchus*, and *Paratylenchus*, the differences between category I and category II are not significant at either level. In 1996 seven genera had low densities in category II, but only *Pararotylenchus* had significantly ($P < 0.05$) fewer nematodes in category II than in category I. At $P < 0.05$ there was no significant reduction of numbers of *Aphelenchoides*, *Aphelenchus*, and *Filenchus*, but if a probability of $P < 0.20$ is accepted, then they show a significant decrease. There were no significant differences in *Atetylenchus*, *Ditylenchus*, *Psilenchus*, *Rotylenchus*, and *Tylenchus*.

In summary, there are altogether eight genera showing low population densities in category II, and in seven of the genera, the numbers vary significantly between the two categories (Tables 5 and 6). Accordingly, most of the data reveal a tendency towards fewer nematode numbers in the rhizospheric soil of soybean and its adjacent ragweed (category II) than occur in the rhizospheric soil of soybean without ragweed nearby (category I).

TABLE 5. STATISTICAL DATA FOR 1995

Nematode genus	Category I		Category II		*t* test[a]	
	Mean	Variance (S^2)	Mean	Variance (S^2)	$P < 0.05$	$P < 0.2$
Aphelenchoides	32.0	5,120.0	0.0		*	
Aphelenchus	105.6	10,269.3	88.0	10,370.5	ns	ns
Atetylenchus	49.2	4,303.2	2.0	20.0	ns	*
Coslenchus	2.0	20.0	2.0	20.0	ns	ns
Ditylenchus	42.0	3,020.0	2.0	20.0	ns	*
Filenchus	48.6	4,489.8	0.0		*	
Pararotylenchus	12.0	170.0	6.0	88.0	ns	ns
Paratylenchus	4.0	80.0	8.0	320.0	ns	ns
Tylenchorhynchus	0.0		8.0	320.0	*	
Tylenchus	29.6	1,650.8	0.0		*	

[a] ns: not significant; *: significant.

TABLE 6. STATISTICAL DATA FOR 1996

Nematode genus	Category I		Category II		*t* test[a]	
	Mean	Variance (S^2)	Mean	Variance (S^2)	$P < 0.05$	$P < 0.2$
Aphelenchoides	12.6	90.8	3.6	24.8	ns	*
Aphelenchus	42.6	1158.8	9.6	55.3	ns	*
Atetylenchus	25.8	1136.2	9.6	95.3	ns	ns
Ditylenchus	9.8	105.2	11.6	452.3	ns	ns
Filenchus	147.6	3588.8	72.6	3376.3	ns	*
Pararotylenchus	139.2	3765.2	57.0	1859.0	*	
Pratylenchus	2.2	24.2	29.0	1251.5	ns	*
Psilenchus	16.0	630.0	4.0	80.0	ns	ns
Rotylenchus	6.0	180.0	14.6	280.8	ns	ns
Tylenchus	60.2	1355.2	46.0	2985.5	ns	ns

[a]ns: not significant; *: significant.

Since there were significantly fewer nematodes in category II (soybean and ragweed) than in category I (soybean only), it is probable that ragweed contributed to the lower numbers of nematodes. Roots of ragweed exude sesquiterpenes, stigmasterol, β-sitosterol, and some phenolic acids (Fischer and Quijano, 1985). In addition, thiarubrine A, thiophene A, thiarubrine B, and thiophene B represent the major sulfur-containing acetylenes that are present as constituents (Lu and Parodi, 1993; Ellis et al., 1993; Marco, 1991; Constabel and Towers, 1989). Thiarubrine A and thiophene A derived from thiarubrine A display antibiotic activity toward bacteria, fungi, nematodes, and Chinese hamster ovary cells (Towers, 1985; Wat et al., 1981). Consequently, we think these compounds may explain why the roots of ragweed reduced the numbers of some nematodes under field conditions in this study.

Nevertheless, there were five genera in which data were contrary to the foregoing conclusion. In the survey of 1995, the number of *Paratylenchus* and of *Tylenchorhynchus* found in category II is more than that in category I. *Ditylenchus*, *Rotylenchus*, and *Pratylenchus* also have higher population densities in category II than in category I in 1996. Among these five genera, nematode numbers of only two genera were significantly different between the two categories (*Tylenchorhynchus*, $P < 0.05$; *Pratylenchus*, $P < 0.2$) and differences were not significant in the other three genera. We are not sure of the reasons for the higher density in category II.

In the samples collected in 1996, the numbers of nematodes in three genera, *Pratylenchus*, *Ditylenchus*, and *Rotylenchus*, were against the trend. *Pratylenchus* are endoparasites, and *Ditylenchus* may be an endoparasite or ectoparasite

depending on the host plant. *Rotylenchus* are semiendoparasites and often bury the anterior part of their bodies in the roots. All these worms are mobile. It is likely that these habits result in weakening the influence of ragweed exudates.

In 1995, there were two genera, *Paratylenchus* and *Tylenchorhynchus*, in which ragweed did not appear to reduce the numbers of nematodes. We do not have an explanation for these results. Some observations show that the introduction of root secretions of the host plant into the soil can stimulate the transformation of the juveniles of *Paratylenchus* into adult worms (Decker, 1972). However, we do not know if soybean has such an effect or if such an influence can outweigh the influence of ragweed.

CONCLUSION

From our two-year survey, we conclude that ragweed depresses populations of several plant-parasitic nematodes. We identified 13 genera of nematodes in this survey, and depressed numbers were observed for 10 of those genera, with numbers in seven genera significantly depressed. In summary, we believe that ragweed root exudates play an important role in repelling at least some plant-parasitic nematodes.

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ORGANIC SULFUR COMPOUNDS FROM *Dictyopteris* spp. DETER FEEDING BY AN HERBIVOROUS AMPHIPOD (*Ampithoe longimana*) BUT NOT BY AN HERBIVOROUS SEA URCHIN (*Arbacia punctulata*)

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Abstract—Brown seaweeds in the genus *Dictyopteris* produce several C_{11} sulfur metabolites that appear biosynthetically related to the C_{11} compounds known to attract sperm to female gametes of many brown algae. All four of the C_{11} sulfur compounds that we tested strongly deterred feeding by the amphipod *Ampithoe longimana* but had no effect on feeding by the sea urchin *Arbacia punctulata*, even when tested at concentrations that were two to eight times greater than those that deterred amphipods. In numerous previous investigations, a variety of seaweed compounds have been shown to deter feeding by large mobile herbivores such as fishes and urchins but to be relatively ineffective against mesograzers, such as the amphipod of our study. Our results for the C_{11} sulfur compounds from *Dictyopteris* thus contrast sharply with patterns from previous studies and suggest that these metabolites may be defenses specifically targeted against small mesograzers such as amphipods. The occurrence of C_{11} metabolites in brown algal eggs could allow these defenses to be especially important in defending gametes, zygotes, or young sporelings from herbivorous mesograzers.

Key Words—Amphipods, C_{11} sulfur compounds, *Dictyopteris* spp., marine ecology, plant-herbivore interactions, sea urchins.

INTRODUCTION

Organic sulfur compounds are widespread in terrestrial and marine plants, but their presence, distribution, and function in seaweeds has not been systematically

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studied. Among the first seaweeds discovered to produce organic sulfur compounds were the Hawaiian brown algae *Dictyopteris plagiogramma* and *D. australis* (Phaeophyceae, Dictoytales) (Moore, 1976). From these species, eight compounds containing a C_{11} unit attached to a sulfur atom with an oxygen substituent at C-3 have been isolated and characterized (Figure 1). The compounds appear to be biogenetically related to simple, olefinic C_{11} hydrocarbons previously identified in the essential oils of the same, and other, brown algae, and it has been claimed that 1-undecen-3-ol, present in essential oils from *Dictyopteris* spp., may represent the common precursor to both classes of C_{11} compounds (Moore, 1976, 1977). Although the function and biosynthesis of the hydrocarbons as mating pheromones for gametes of brown algae have been studied extensively (Boland, 1995; Maier, 1995; Pohnert and Boland, 1996), little is known about the origin or ecological significance of the C_{11} sulfur compounds.

Seaweeds can be exposed to extremely high rates of grazing (Carpenter,

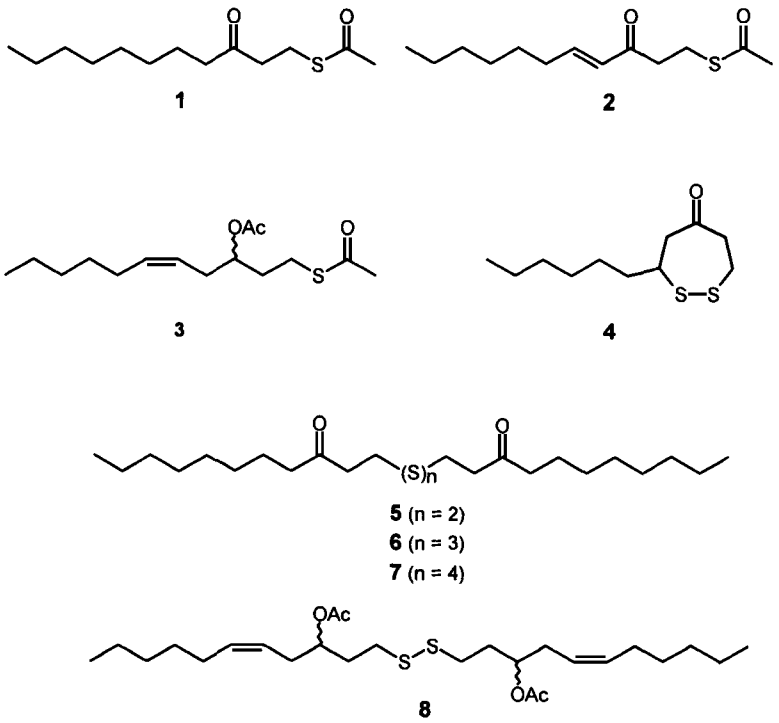

FIG. 1. C_{11} sulfur compounds previously isolated from Hawaiian collections of *Dictyopteris plagiogramma* and *D. australis* (Moore, 1976).

1986; Hay, 1991), and many species have developed structural, morphological, or chemical defenses that significantly lessen their susceptibility to herbivores (Duffy and Hay, 1990; Hay, 1996, 1997). Chemical defenses against herbivores have been especially well studied (see reviews by Hay and Fenical, 1988, 1996; Hay and Steinberg, 1992; Paul, 1992; Hay, 1996). Functionalized terpenoids, acetogenins, and cyclic peptides containing uncommon amino acid residues are especially widespread and common (Faulkner, 1994, and references therein). However, structurally simple C_{11} hydrocarbons from *Dictyopteris* can also significantly deter grazing by tropical reef fishes (Hay et al., 1988a). The obvious structural and biogenetic relationship between the C_{11} sulfur compounds and C_{11} hydrocarbons could indicate an important ecological role for both classes of compounds as herbivore deterrents, but this has not been evaluated for the C_{11} sulfur compounds.

To investigate this possibility, we determined the occurrence and potential ecological role of C_{11} sulfur compounds produced by *Dictyoperis membranacea* or other species within this genus. *D. membranacea* is the only species of this genus that occurs in Europe, but it is a cosmopolitan alga that occurs widely in warm temperate and tropical seas throughout the Atlantic, Caribbean, and parts of the Pacific (e.g., North Carolina to Brazil and Pacific Mexico) (Schneider and Searles, 1991). It belongs, like *D. plagiogramma*, *D. australis*, and *D. delicatula*, to the *Dictyopteris* subgroup that preferably produce C_{11} hydrocarbons instead of terpenoids (Boland and Müller, 1987). Despite a detailed analysis of the C_{11} hydrocarbons from this genus, there has been no report on the occurrence of C_{11} sulfur compounds in *D. membranacea*.

To evaluate the potential for the unstudied C_{11} sulfur compounds to function as defenses against herbivores, we tested their effects on feeding by the generalist sea urchin *Arbacia punctulata* and the generalist amphipod *Ampithoe longimana*. *Arbacia* is common and widespread on both sides of the Atlantic and is the most abundant sea urchin in coastal North Carolina, USA, where these assays were conducted. Feeding by *A. punctulata* has not been studied extensively, but it is known to readily consume various species of red and green seaweeds and to eat only small quantities of several species of brown seaweeds that have been tested (Hay et al., 1986). Brown algae of the genus *Dictyota* are avoided by this urchin, and various diterpene alcohols produced by *Dictyota* significantly deter feeding by *Arbacia* (Hay et al., 1987a; Cronin and Hay, 1996a,b). Feeding by omnivorous and herbivorous fishes is also deterred by these same metabolites (Hay, 1991), suggesting that *Arbacia* may serve as a general model for the response of generalist herbivores to algal secondary metabolites.

The herbivorous amphipod *Ampithoe longimana* is common in marine and estuarine habitats along the east coast of North America (Bousfield, 1973) and occurs on a wide variety of algae and seagrasses (Nelson, 1979; Duffy and Hay, 1991a, 1994). Like several marine mesograzers that dwell on the seaweeds they

consume (see reviews by Hay, 1992, 1996), *A. longimana* selectively consumes and lives on brown algae that deter fishes. By living on seaweeds that are chemically repugnant to omnivorous fishes, *A. longiamana* significantly reduces its susceptibility to fish predation (Hay et al., 1987a; Duffy and Hay, 1991a, 1994). Thus, the feeding behavior of *A. longimana* contrasts with the feeding behavior of generalist urchins and fishes in the western Atlantic in that *Ampithoe* tends to selectively consume seaweeds that are strongly avoided by the generalist urchins and fishes. Additionally, the brown algal chemical defenses that have been tested affect feeding by *A. longimana* much less than they affect feeding by *Arbacia*, herbivorous fishes, or even some other species of amphipods (Hay et al., 1987a, 1988b; Duffy and Hay, 1991a, 1994; Cronin and Hay, 1996a,b). Thus, *A. longimana* appears to be representative of small mesograzers that have evolved a high tolerance to seaweed chemical defenses, selectively consume and associate with chemically defended algae, and by doing so, minimize their exposure to consumers (see reviews by Hay, 1992, 1996).

The present study focuses on how four C_{11} sulfur compounds produced by brown algae in the genus *Dictyopteris* affect feeding by *Arbacia* versus *Ampithoe*. One of these compounds was the major C_{11} sulfur compound produced by the Mediterranean and western Atlantic populations of *Dictyopteris membranacea* that we investigated; the other three compounds are related metabolites known to be produced by other members of this genus. Based on the well studied response of *Arbacia punctulata* and *Ampithoe longimana* to the chemical defenses produced by seaweeds of the genus *Dictyota*, we expected *Arbacia* to represent a generalist herbivore that would be strongly deterred by these metabolites and *Ampithoe* to represent a generalist mesograzer that would be more resistant to the effects of brown algal chemical defenses. Our findings conflicted strongly with our expectations. The C_{11} sulfur compounds strongly deterred amphipod feeding at, or below, natural concentrations, but had no effects on feeding by urchins even when tested at concentrations two to eight times above those that deterred amphipods. Because C_{11} compounds are known to be produced by brown algal gametes that might be especially susceptible to small grazers such as amphipods, it seems possible that these metabolites play important roles in defending gametes, zygotes, or germlings against consumption by marine mesograzers. While chemical defense of juvenile or larval stages is known in benthic marine invertebrates (Lindquist and Hay, 1996), chemical defense of algal spores, sporelings, or juveniles has not been investigated.

METHODS AND MATERIALS

Ecological Methods. Mediterranean *Dictyopteris membranacea* used for natural product extraction was collected in June 1996 at Villefranche-sur-mer, Côte d'Azur, France (43°39′N, 7°23′E) and transported to the laboratory on

ice. In the western Atlantic, the same seaweed species as well as the amphipod *Ampithoe longimana* and the sea urchin *Arbacia punctulata* cooccurred on the rock jetty at Radio Island, North Carolina, USA (34°34′N, 76°40′ W). Collections from this site were made during September 1996. These species also cooccur on a variety of deeper reefs off the coast of the South-eastern United States.

Because hunger stress can alter herbivore feeding responses (Cronin and Hay, 1996a), both herbivores were fed with a mixed algal diet immediately before use in feeding bioassay. The amphipods were kept in 20-liter flow-through aquaria at the University of North Carolina's Institute of Marine Sciences and fed a mixture of freshly collected, local algae (*Sargassum filipendula*, *Padina gymnospora*, *Dictyota ciliolata*, *Dictyota menstrualis*, *Ulva* sp., *Enteromorpha* sp., and *Hypnea musciformis*). Sea urchins were held in flow-through seawater tanks and fed with fresh, palatable algae (*Gracilaria tikvahiae*, *Agardhiella ramosissima*, or *Codium fragile*).

The effects of the C_{11} sulfur compounds on feeding by the urchin and amphipod were assessed by comparing feeding on an artificial food that contained (i.e., treatment food) or did not contain (i.e., control food) the secondary metabolite being assayed. Artificial foods were made by mixing freeze-dried and finely powered algae (*Ulva fasciata* for urchins and a mixture of *Ulva fasciata*, *Enteromorpha linza*, and *Hypnea musciformis* for amphipods) into an agar base and forming this onto fiberglass window screening material. This provided support and an internal uniform grid that allowed us to quantify the amount eaten by counting the squares of the screen that had been cleared of algae (see Hay et al., 1994 for detailed methods). Before mixing the freeze-dried algal powder into the agar, the algal powder to be used for the treatment food was covered with diethyl ether containing the desired amount of the test metabolite, and the algal powder to be used for the control food was covered with diethyl ether alone. The solvent was then removed under a slow stream of nitrogen, and the dried powder was incorporated into the experimental foods. Each urchin (N = 30–40) or each container holding three amphipods (N = 30) was then offered equal amounts of both a control and treated food. Feeding was monitored periodically, and food was removed from individual replicates whenever one half or more of either food had been consumed, or at the end of the experimental period. Feeding was quantified by counting the number of window-screen squares that had been cleared of food. If there was no feeding in a replicate or if both foods were consumed completely between our monitoring intervals, then these replicates provided no information on relative palatabilities and were thus excluded from analyses. Such exclusions resulted in actual sample sizes of 24–30 for the amphipod assays and sample sizes of 21–34 for the urchin assays. After an assay, we extracted uneaten treatment foods and analyzed these with thin-layer chromatography to ensure that the test compound was still present

in the treated food at the end of the assay. Analyses of differences in feeding relied on paired-sample *t* tests and two-tailed *P* values. If differences in feeding were normally distributed, we employed paired-sample *t* tests. If differences were not normally distributed, we used the Wilcoxon signed-ranks test.

The natural concentration of the C_{11} sulfur compound that we isolated from our Mediterranean collection of *D. membranacea* was approximately 0.1% of plant dry mass; however, the few studies that have addressed intraspecific or intraplant variation in seaweed secondary metabolites have often found considerable variation (reviewed in Hay, 1996), suggesting that concentrations in some populations or plant parts could be higher or lower than this. Concentrations of the other C_{11} sulfur compounds that have been described from other species of *Dictyopteris* are not well known. Given this limited information on the natural concentration of these metabolites, we decided to initially test each metabolite at a concentration of 0.2% of food dry mass (i.e., about 2× the natural mean concentration that occurred in the Mediterranean population we investigated). If the metabolite was deterrent at this concentration, we repeated the test with a reduced concentration. If it was not deterrent at 0.2% of plant dry mass, we doubled this concentration and retested it. By this approach, we tested some compounds over concentrations ranging from 0.025 to 0.4% of plant dry mass to bracket the variations in concentration that might occur naturally between different populations or plant parts.

Chemical Methods. Analytical GLC was performed with a Carlo Erba GC 6000, Vega Series, gas chromatograph equipped with a flame ionization detector and a fused silica column (15 m × 0.32 mm) coated with SE 30. NMR analyses were conducted with a Bruker AC-400 and AC-250 spectrometer for ^1H and ^{13}C NMR spectra. ^1H and ^{13}C chemical shifts were referenced to $CDCl_3$. For high-resolution MS analysis we used a Finnigan ITD 800 and a Finnigan Mat 90 spectrometer (% eV). Infrared spectra were recorded on a Perkin-Elmer Series 1600 FTIR spectrophotometer.

Extraction of Algal Material. To isolate the dithiepanone **4**, algal material (500 g, *D. membranacea*, collected at Villefranche-sur-mer, France) was homogenized and stirred for 2 hr in methanol–chloroform (2.0 liters, 1:1, v/v) at room temperature. After filtration, the solvent was removed under reduced pressure (1.2 kPa) at room temperature. The residual brown oil was subjected to repeated column chromatography on silica gel 60 (60–100 μm, E. Merck, Darmstadt, Germany) employing first an *n*-pentane–diethyl ether gradient (95:5 to 60:40, v/v), followed by rechromatography with *n*-pentane–diethyl ether 90:10 (v/v) for elution. Evaporation of the solvent provided 42.0 mg 3-hexyl-[1,2]dithiepan-5-one **4** as a faint yellow oil. ^1H NMR, δ (ppm): 0.85 (t, 3H, C(13)], 1.2–1.4 [m, 8H, C(9–12)], 1.5 [m, 2H, C(8)], 2.7–2.9 [m, 7H, C(3–4) and C(6–7)]. ^{13}C NMR, δ (ppm): 14.08 [C(13)], 21.93, 27.11, 28.89, 31.64,

33.63 [C(8–12)], 35.19 [C(7)], 45.30 [C(4)], 50.32 [C(6)], 208.30 [C(5)]. MS (70 eV) [*m/z* (%)]: 233 (M^+ + H, 40), 215(12), 199(5), 167(100), 149(7), 131(15), 120(26), 95(9), 55(95), 39(24). High-resolution MS: $[M]^+_{calc}$ = 232.0963, $[M]^+_{obs}$ = 232.0959. $[\alpha]_D^{25} = -65°$ (CCl_4). This preparation was used in the feeding experiments. Thalli of *D. membranacea* collected at Radio Island, North Carolina, also were extracted as described above. The presence of **4** was confirmed by chromatography and mass spectrometry.

Synthesis of C_{11} Sulfur Compounds. While we obtained metabolite **4** from *Dictyopteris*, the other metabolites of our study were synthesized (see Figure 2). All moisture- and air-sensitive reactions were conducted under argon; solvents were dried according to standard methods. Silica gel, Si 60 (60–100 μm, E. Merck, Darmstadt, Germany) was used for chromatography. Thin layer chro-

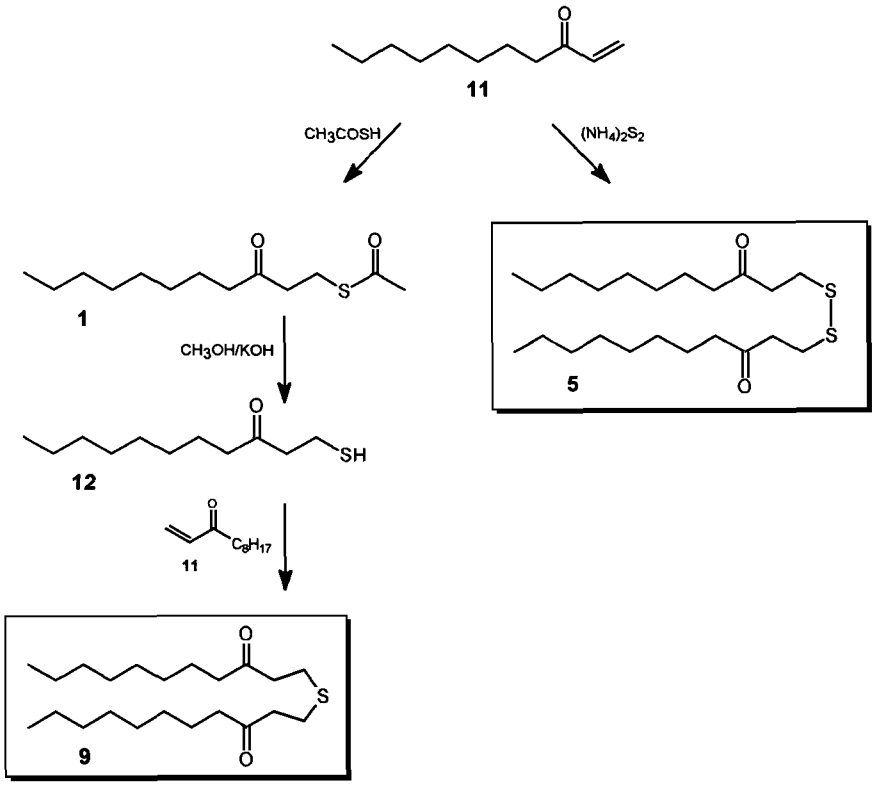

FIG. 2. Protocol for the synthesis of **1**, **5**, and the thioether **9**.

matography was performed with silica gel plates Polygram Sil G_{F254}, from Merck.

Thioacetic Acid S-(3-Oxo-undecyl) Ester ***1***. The thiol acetate was prepared by slow addition of thioacetic acid (3.04 g, 0.04 mol) to a solution of the enone **11** (3.36 g, 0.02 mol) in dichloromethane (500 ml) following the procedure of Asato and Moore (1973). Column chromatography on SiO_2 with *n*-pentane–diethyl ether 80:20 (v/v) afforded the thioacetate as a pale yellow oil. 4.72 g (31%). ^{1}H NMR δ (ppm): 0.8 [t, 3H, C(11)], 1.25 [m, 10H, C(6–10)], 1.5 [qui, 2H, C(5)], 2.3 [s, 3H, CH_3 acetyl], 2.35 [t, 2H, C(1)], 2.7 [t, 2H, C(2)], 3.05 [t, 2H, C(4)]. ^{13}C NMR δ (ppm): 14.1 [C(11)], 22.7, 23.1, 23.8, 29.1, 29.2, 29.4, 31.8 [C(1), C(5)–C(10)], 30.5 ($\underline{C}H_3CO$), 42.3 [C(2)], 42.9 [C(4)], 195.9 ($CH_3\underline{C}O$), 208.9 [C(3)]. GC-MS (70 eV) [*m/z* (%)]: 245 ($M^{+\cdot}$ + H, 2), 201 (<1), 185(2), 169(20), 141(8), 109(1), 95(6), 81(10), 71(32), 55(36), 43(100).

1-(3-Oxo-undecyldisulfanyl)-undecan-3-one ***5***. A solution of undec-1-en-3-one **1** (1.0 g, 6.0 mmol) and didecyl-dimethylammonium bromide as a phase-transfer-catalysis (Krein and Aizenshtat, 1993) in THF (10.0 ml) was gradually treated with stirring at room temperature with ammonium disulfide (0.30 g, 3.0 mmol). Extractive work-up and recrystallization from methanol yielded 0.424 g disulfide **5** (35%) as a colorless crystalline solid, mp: 69°C. ^{1}H NMR δ (ppm): 0.85 [t, 6H, C(11, 11′)], 1.25 [m, 20, C(6, 6′)–C(10, 10′)], 1.55 [m, 4H, C(5, 5′)], 2.4 [m, 4H, C(4, 4′)], 2.7 [dd, 4H C(2, 2′)], 2.85 [dd, 4H, C(1, 1′)]. ^{13}C NMR δ (ppm): 14.2 [C(11, 11′)], 22.7, 23.7, 26.0, 29.2, 29.3, 29.4, 31.8 [C(1, 1′), C(5, 5′)–C(10, 10′)], 41.9 [C(2, 2′)], 43.2 [C(4, 4′)], 209.1 [C(3, 3′)]. MS [*m/z* (%)]: 402 ($M^{+\cdot}$ 7), 352(21), 201(14), 169(28), 141(100), 81(15), 69(46), 57(50), 43(29).

1-(3-Oxo-undecylsulfanyl)-undecan-3-one ***9***. Because disulfides are highly reactive and can undergo several chemical reactions, we wanted to assure that feeding deterrence for bis-(3-oxoundecyl)disulfide **5** was not induced by decomposition or conversion products. We therefore also synthesized the bis-(3-oxoundecyl)monosulfide **9**, a monosulfide homologous to compound **5**. This allowed us to assess the potential feeding deterrent effects of both the disulfide and monosulfide. A solution of the thioester **1** (0.246 g, 1.0 mmol) in a 5% methanolic potassium hydroxide solution was hydrolyzed with stirring at room temperature to the corresponding thiol (Chapman and Owen, 1950). Then, the enone **11** (0.168 g, 1.0 mmol) was added, and stirring was continued for 30 min. Extractive work-up afforded **9** as a colorless crystalline solid. 0.281 g (76% overall), mp: 80°C. ^{1}H NMR δ (ppm): 0.83 [t, 6H, C(11, 11′)], 1.25 [m, 20, C(6, 6′)–C(10, 10′)], 1.50 [m, 4H, C(5, 5′)], 2.35 [m, 4H, C(4, 4′)], 2.7 [dd, 4H, (C(2, 2′)], 2.65 [dd, 4H, C(1, 1′)]. ^{13}C NMR δ (ppm): 14.1 [C(11, 11′)], 22.7, 23.7, 25.9, 29.1, 29.2, 29.4, 31.8 [C(1, 1′), C(5, 5′)–C(10, 10′)], 42.5

[C(2, 2')], 43.2 [C(4, 4')], 209.4 [C(3, 3')]. MS [*m/z* (%)]: 370 (M^+ 5), 352(95), 201(32), 169(53), 141(77), 95(20), 83(38), 71(68), 61(25), 57(100), 55(60).

RESULTS

Natural Product Extraction and Identification. GC-MS-analysis of extracts obtained from Mediterranean (Figure 3) and western Atlantic (i.e., North Carolina) samples of *D. membranacea* revealed several C_{11} hydrocarbons **13–16**, which are known as pheromones of brown algae (Boland, 1995; Maier, 1995), and a significant amount of a C_{11} sulfur compound. Mass spectra and retention characteristics of the sulfur compound were consistent with those of the cyclic thiepanone **4**. Extraction of 500 g (wet mass) of the alga yielded 42 mg of **4**, corresponding to about 0.1% of algal dry mass. Compounds **1** and **5**, which were previously isolated from *D. plagiogramma* and *D. australis* were not found in extracts from these collections.

Feeding Experiments. When the cyclic dithiepanone **4** from *Dictyopteris membranacea* was added to experimental foods, it strongly deterred the amphipod. Feeding was reduced by 66% ($P = 0.008$) at the natural concentration of 0.1% dry mass and by 60% ($P = 0.001$) at one half of its natural concentration (Figure 4). In contrast, the metabolite had no effect on feeding by the sea urchin, even when tested at 200% and 400% of its natural concentration ($P = 0.754$ and 0.566, respectively).

Although not analyzed across as wide a range of concentrations, the other C_{11} sulfur compounds we tested showed similar patterns of activity (Figures 4 and 5). All three compounds significantly deterred amphipod feeding by 53–72% at concentrations of 0.1–0.2% dry mass, while none significantly affected urchin feeding, even when tested at concentration of 0.4%. The monosulfide **9** and disulfide **5** did not strongly differ in their effects on herbivore feeding (Figure 5). Neither had any effect on urchin feeding and both deterred amphipod feeding; however, at concentration of 0.1%, the disulfide **5** diminished amphipod feeding by a significant 68% ($P = 0.003$), while the monosulfide **9** reduced feeding but not significantly so (47% less, $P = 0.289$). At 0.2% of food dry mass, both the disulfide **5** and monosulfide **9** significantly deterred the amphipod, reducing feeding by 72% ($P < 0.001$) and 56% ($P = 0.005$), respectively.

In a final experiment, amphipods were exposed to artificial food made by mixing freeze-dried and finely powdered material from freshly collected North Atlantic *D. membranacea* and plant material from a laboratory culture of the Mediterranean *D. membranacea*, which did not produce C_{11} hydrocarbons and sulfur compounds. In all cases, the artificial food without the C_{11} compounds

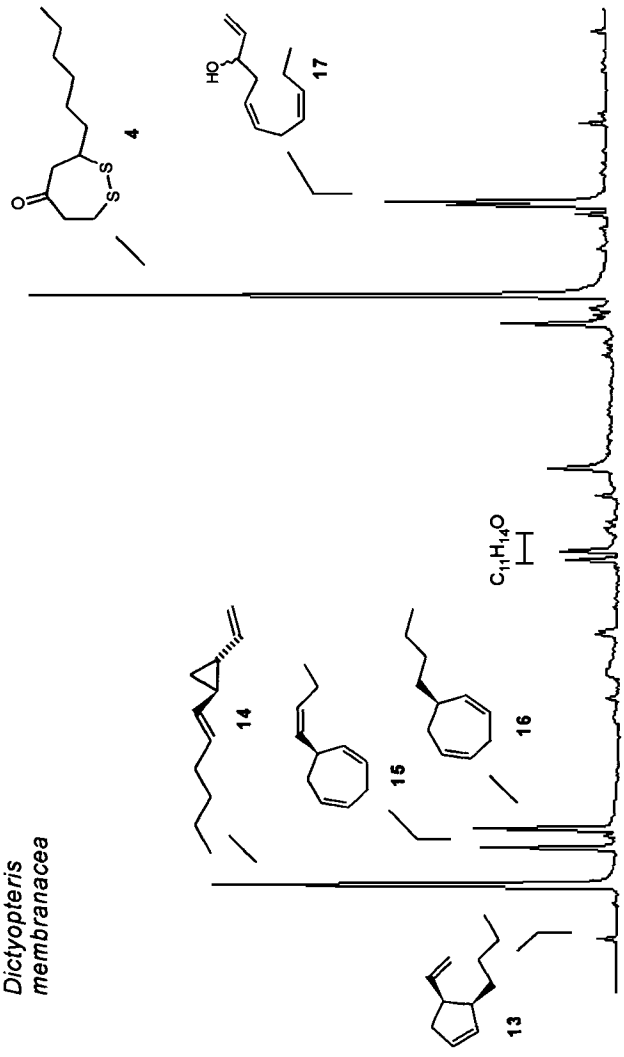

FIG. 3. GLC profile of volatile compounds from the nonpolar extract from *Dictyopteris membranacea* collected from the French Mediterranean. GLC analyses of the nonpolar extracts from *Dictyopteris membranacea* collections from North Carolina (western Atlantic) revealed a similar composition. Compounds were separated on a fused silica column, SE 30, 15 m × 0.25 mm, under programmed conditions (40°C for 5 min, then 10°C/min to 280°C). Identification of compounds: **13**, 3-butyl-4-vinyl-cyclopentene; **14**, hex-(1*E*)-enyl-2-vinyl-cyclopropane (= dictyopterene A); **15**, 6-but-(1Z)-enyl-cyclohepta-1,3-diene (= ectocarpene); **16**, 6-butyl-cyclohepta-1,4-diene (= dictyotene); **4**, 3-hexyl-[1,2]dithiepan-5-one; **17**, undeca-1,5,8-trien-3-ol and undeca-1,5-dien-3-ol (of. Moore, 1976, 1977; Kajiwara et al., 1982).

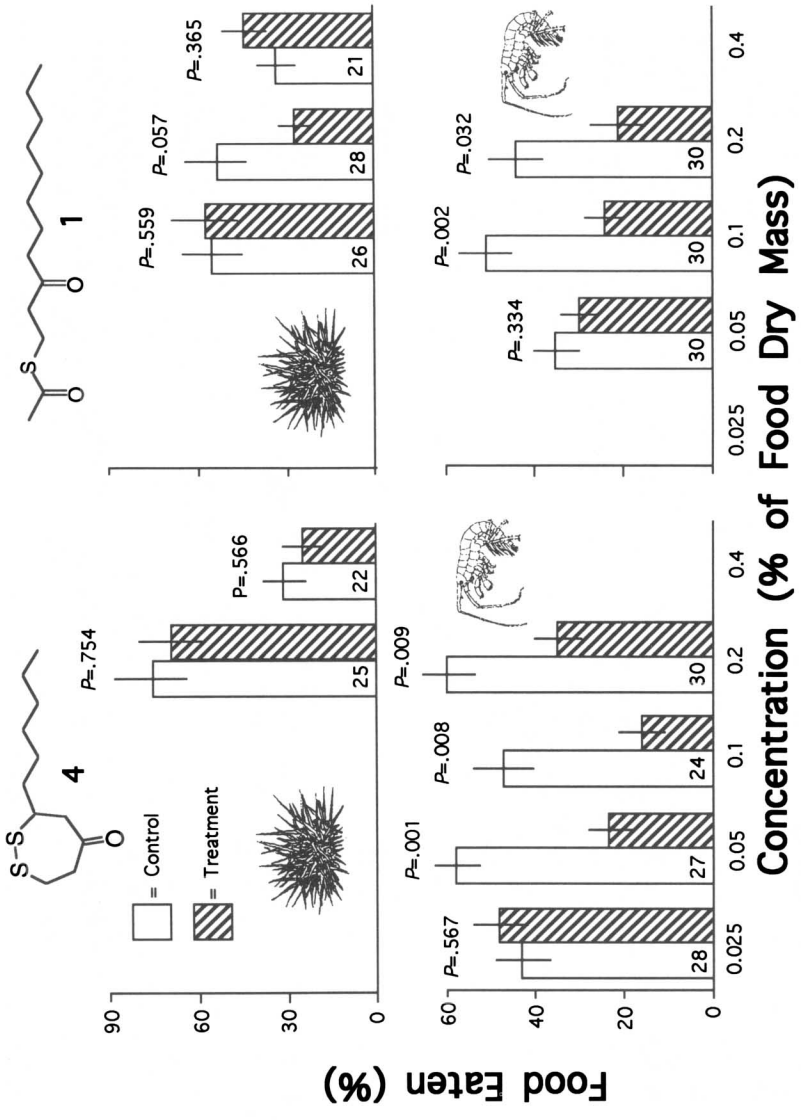

FIG. 4. Effects of different concentrations of 3-hexyl-[1,2]dithiepan-5-one **4** (the major metabolite produced by the populations of *Dictyopteris membranacea*) or of thioacetic acid *S*-(3-oxoundecyl)-ester **1** on feeding by the sea urchin *Arbacia punctulata* and the amphipod *Ampithoe longimana*. Natural concentration of 3-hexyl-[1,2]-dithiepan-5-one **4** in the populations we studied was 0.1% of plant dry mass. Histograms show mean percentage of food eaten ±SE. All *P* values are two-tailed and are from paired-sample *t* tests if differences were normally distributed and from the Wilcoxon paired-sample test if differences were not normally distributed. Sample sizes for each assay are shown in the base of each clear bar.

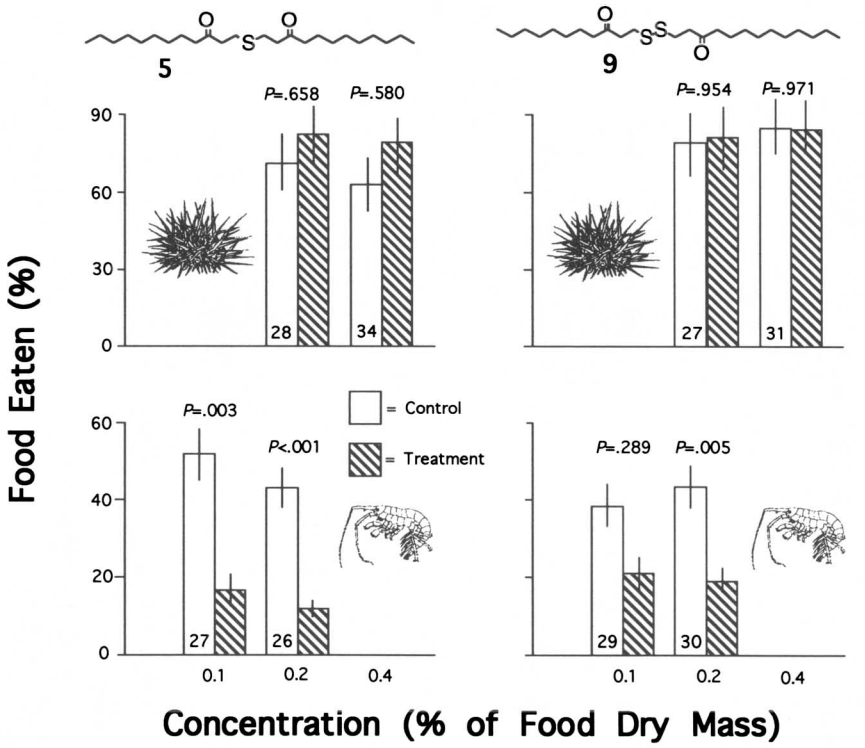

FIG. 5. Effects of different concentrations of 1-(3-oxo-undecyldisulfanyl)-undecan-3-one **5** or 1-(3-oxo-undecylsulfanyl)-undecan-3-one **9** on feeding by the sea urchin *Arbacia punctulata* and the amphipod *Ampithoe longimana*. Symbols and analysis are as in Figure 4.

was clearly preferred over the artificial food made from freshly collected *D. membranacea* ($P = 0.001$), corroborating the unique importance of the compounds for plant protection.

DISCUSSION

Our findings that the C_{11} sulfur compounds from *Dictyopteris* strongly deter amphipod feeding but have no effect on sea urchin feeding provide a strong contrast to many previous investigations. Our experiments suggest that sedentary mesograzers are relatively immune to seaweed chemical defenses that strongly deter other herbivores. Most previous investigations that focused on how sea-

weed chemical defenses affected feeding by large generalist urchins and fishes versus smaller more sedentary mesograzers (like tube-building or domicile-building amphipods and polychaetes) found that the more sedentary mesograzers were resistant to seaweed secondary metabolites that deterred feeding by larger herbivores (reviewed in Hay and Fenical, 1996; Hay, 1997). Previous investigations, some with the same species of amphipod and sea urchin that we studied here, (Hay et al., 1987a,b, 1988b, 1990a; Duffy and Hay, 1991a, 1994; Cronin and Hay 1996a,b) found that diterpene alcohols produced by brown seaweeds in the genus *Dictyota* significantly deterred feeding by both temperate and tropical fishes and sea urchins. The same compounds had no effect on feeding by the amphipod *A. longimana*, as well as by the tube building polychaete *Platynereis dumerilii* and the domicile building amphipod *Pseudamphithoides incurvaria*. In similar contrasts, both the halogenated monoterpene ochtodene from the red alga *Ochtodes secundiramea* and a mixture of aliphatic C_{11} hydrocarbons from the brown alga *Dictyopteris delicatula* deterred feeding by herbivorous reef fishes but failed to deter feeding by plant-dwelling amphipods (Paul et al., 1987; Hay et al., 1988a). Several other mesograzers such as plant-dwelling crabs and ascoglossans show similar patterns of immunity to seaweed chemical defenses that strongly deter larger herbivores (Hay et al., 1990b, Stachowicz and Hay, 1996). Patterns from all these examples are consistent with the hypothesis that seaweeds have evolved chemical defenses against large generalist herbivores that are known to have a tremendous impact on marine plant communities and that plant-associated mesograzers have, in turn, evolved a resistance to seaweed chemical defenses because living on chemically defended plants provides a safe site where they are less susceptible to predation (see Hay, 1992, 1997; Hay and Steinberg, 1992).

Although feeding by mesograzers is often considered to have little effect on populations of marine macrophytes (Bell, 1991), there are several examples of plant-dwelling mesograzers having strong negative effects on populations of marine macroalgae (Tegner and Dayton, 1987; Duffy and Hay, 1991b; Brawley, 1992). The strength and specificity with which the C_{11} sulfur compounds affected amphipod feeding suggest that selection for these metabolites could have been driven by damage due to mesograzer feeding and that they may function as defenses that are specifically targeted against these types of mesograzers. In addition to the published studies cited above, there are unpublished investigations of how feeding by *Ampithoe longimana* is affected by nine different secondary metabolites isolated from seaweeds in the Dictyotales (M. E. Hay, unpublished data). Although many of these metabolites deterred feeding by fishes and sea urchins, feeding by *A. longimana* was rarely affected by any of these secondary metabolites. To our knowledge, the C_{11} sulfur compounds represent the first group of seaweed secondary metabolites that appear to be specifically

targeted against *Ampithoe*-like mesograzers that are largely resistant to the effects of other defensive metabolites.

The ecological or evolutionary costs of producing chemical defenses against herbivores are poorly understood (Hay and Fenical, 1988; Hay and Steinberg, 1992), but it seems probable that costs could be minimized by producing compounds that serve multiple functions (Schmitt et al., 1995) or by slightly modifying existing biosynthetic pathways to produce compounds that serve new needs. The obvious relationship between the C_{11} sulfur compounds that deter feeding by the amphipod and the nonsulfur C_{11} compounds that serve to attract sperm to brown algal eggs (Boland, 1995; Maier, 1996) suggests that a slight modification of the pathway used to produce gamete attractants has provided a novel group of metabolites that strongly deter amphipod feeding. Thus, as outlined in Figure 6, (phospho)lipids may serve via fatty acid hydroperoxides and well established cleavage reactions (Pohnert and Boland, 1996; Wurzenberger and Grosch, 1985) as a common source for both compound classes. In accord with the different degree of unsaturation of the C_{11} hydrocarbons and the C_{11} sulfur compounds, the highly unsaturated eicosanoids like $C_{20:6}$, $C_{20:5}$, and $C_{20:4}$ are preferentially channeled into the hydrocarbon biosynthesis while the more saturated acids $C_{20:3}$ and $C_{20:2}$ serve as precursors for the family of the C_{11} sulfur compounds (Figure 1). Common to both pathways may be the cleavage of a 9-hydroperoxide intermediate providing 9-oxonona-(5*Z*,7*E*)-dienoic acid, a highly potent feeding deterrent (unpublished results) from the polar head of the precursor acid. The nonpolar segment is either converted into the family of C_{11} hydrocarbons or C_{11} alk-3-en-1-ols, depending on the precursor acid and the enzymes present in the plant (cf. Figure 6, **17**). The production of two molecules serving different ecological roles, e.g., gamete attraction and defense, from a single precursor represents a unique example of atomic economy in nature and, thus, clearly minimizes the costs of a constitutive chemical defense. The biosynthetic sequence leading to C_{11} hydrocarbons has been studied in detail (Pohnert and Boland, 1996; Stratmann et al., 1992, 1993), but the proposed biosynthetic route towards the C_{11} sulfur compounds still awaits experimental confirmation (Moore, 1976, 1977).

If the C_{11} sulfur compounds also occur in gametes, zygotes, and sporelings, or if the nonsulfur C_{11} compounds that are known to occur in gametes also deter amphipod feeding, then C_{11} compounds may play an important role in protecting juvenile stages from mesograzer consumption. Protection of juvenile stages may be especially critical because mesograzers could consume whole zygotes or germlings (i.e., cause death rather than partial predation) and could graze in cracks and crevices that provide juveniles with spatial escapes from larger herbivores. In addition, in seasonal habitats, dictyotalean brown algae may settle and grow to only a few cells during the fall and rely on these juvenile stages to reestablish the population the following spring (Richardson, 1979). Because

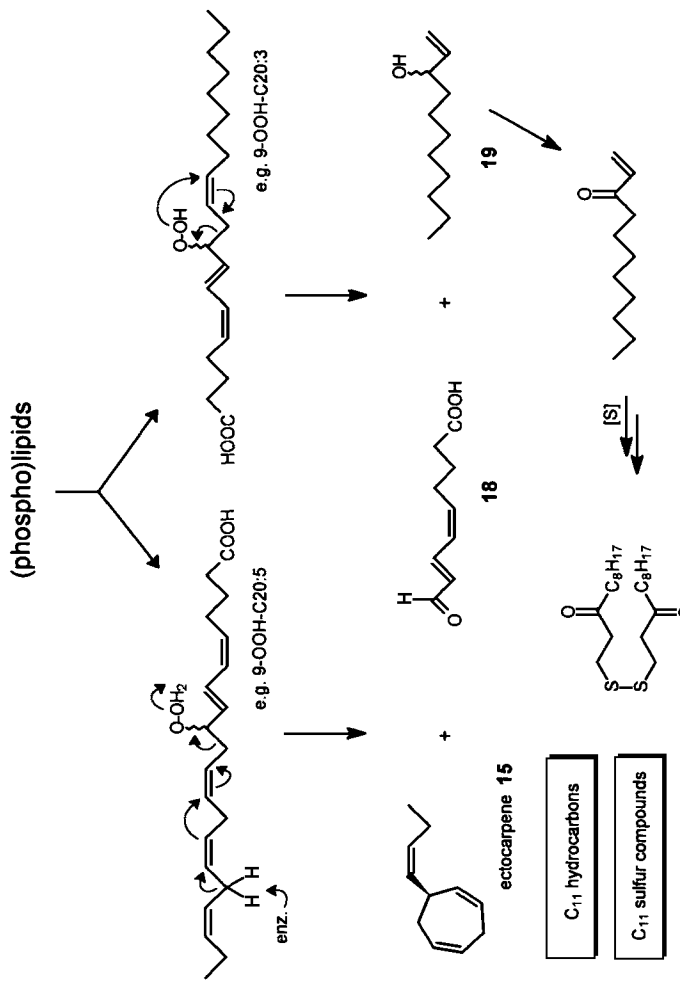

FIG. 6. Proposed pathway for the biosynthesis of C_{11} sulfur compounds. Cleavage of a 9-hydroperoxy fatty acid is assumed to give unsaturated C_{11} alk-1-en-3-ols together with 9-oxonona-(5*Z*,7*E*)-dienoic acid **18**. Oxidation of the allylic alcohols to the corresponding ketones followed by introduction of sulfur and dimerization may account for the biosynthesis of, e.g., **5**. C_{11} sulfur compounds such as **1**, **3**, and **8** require additional oxidoreductive modifications and acetylation. The pathway leading to C_{11} hydrocarbons and **18** has been experimentally confirmed (Pohnert and Boland, 1996).

amphipod abundances often increase dramatically during the cooler portions of the year when fish predators are less active (Nelson, 1979; Duffy and Hay, 1991a, 1994), chemical defense of these germlings from mesograzer predation could be especially important. Studies focused on the C_{11} compounds of zygotes and germlings, their effect on mesograzer feeding, and their concentration changes as a function of plant ontogeny might be especially rewarding. Recent studies have documented potent chemical defenses among the larvae of many marine invertebrates and have suggested that the presence of larval defenses may affect the evolution of larval size, color, and time of release; the dispersal mode and distance; and the general life-history patterns associated with different marine invertebrates (Lindquist and Hay, 1996). Studies of gamete, zygote, and sporeling chemical defenses might provide similar insights into the life-history and reproductive patterns of seaweeds.

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LONG-TERM EFFECTS ON FOOD CHOICE OF LAND SNAIL *Arianta arbustorum* MEDIATED BY PETASIN AND FURANOPETASIN, TWO SESQUITERPENES FROM *Petasites hybridus*

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Abstract—Sesquiterpenes (STs) from the Senecioneae have been found to be potent snail repellents. We investigated the range of activity of the STs petasin, isopetasin, furanopetasin, kablicin, and cacalol, which were isolated from *Petasites hybridus*, *P. kablikianus*, and *Adenostyles alpina*. We found the petasin content of leaves of *P. hybridus* to lie within the range of deterrence of the isolated compound. Furthermore, leaf extracts containing petasin proved to be deterrent, and leaf discs with low petasin content were preferred over discs with higher petasin content. The cacalol-containing fraction of a leaf extract of *A. alpina* was not deterrent to the snails. When the snails had experience with the relevant ST one week before a choice test, their sensitivity towards petasine and furanopetasine increased whereas for the other ST it remained at the same level. We speculate that this sensitivity increase could be the result of a rapid long-term associative learning process, but there is also the possibility that these STs are directly interfering with the feeding motor program of the snails, thereby eliciting a direct neurophysiological sensitization reaction which prevents them from further feeding.

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Key Words—Secondary compounds, sensitization, generalist herbivore, *Arianta arbustorum*, sesquiterpenes, *Petasites hybridus*, *Adenostyles alpina*, Asteraceae, *P. kablikianus*.

INTRODUCTION

When the botanist Stahl (1888) published his comprehensive study about plant compounds and mollusk herbivory, he was one of the first to argue that the function of the diversity of compounds found in plants was to deter herbivores. Although his ideas were disputed by Heikertinger (1914) and other zoologists (Schmid, 1929), they still persist, and most researchers nowadays would agree with this position (Rausher, 1992). Since then, however, most research in plant–herbivore interactions has been done with insects or mammals (Fraenkel, 1959; Ehrlich and Raven, 1964; Freeland and Janzen, 1974; Crawley, 1983; Strong et al., 1984). Mollusk herbivory is generally considered to be of ecological importance only during seedling recruitment (Hanely et al., 1995a,b). In suitable mollusk habitats, such as along creeks and riverbeds, however, mollusk biomass is high, and they may well be a major basal component of the food chain. Consequently, mollusk herbivory in these habitats should not only affect seedlings but also exert some pressure on fully grown plants. One of the very abundant plants along humid riverbeds, *Petasites hybridus* (L.), is widespread all over Europe and, due to its tall growing wide leaves, it is an important contributor to green plant biomass in its habitat. Although not usually heavily affected by herbivory, occasionally snails have stripped the plants completely by the end of the growing season (M. Klemm, personal communication).

As potentially protective secondary plant compounds, sesquiterpenes of the (furano-) eremophilane type (hereafter STs) have been found also in the leaves of *P. hybridus*, as they are typical to its tribe, the Senecioneae (Asteraceae) (Seaman, 1982). Two different chemovariants of the species, containing furanopetasin or petasin as their major compound, have been described (Stoll et al., 1956; Novotny et al., 1961, 1966). Since STs were found to deter snails (Hägele, 1992; Speiser et al., 1992; Hägele et al., 1996), we wanted further to establish their range of activity and possible effects on snail food choice over time. To help bridge the gap between the deterrent activity of an isolated compound in laboratory experiments and the effect of that compound on the behavior of an animal in its natural environment, we also tested plant extracts containing the ST together with other unknown plant compounds. Finally, we tested intact leaf tissue of *P. hybridus* to see whether snails are indeed distinguishing between leaf discs containing different concentrations of petasin.

Adenostyles alpina, another senecionean plant, was rarely found to suffer from snail herbivory (Hägele, 1992). Since the transformed ST cacalol was identified as being a major constituent of *A. alpina* leaves (J. Harmatha, unpub-

lished), we tested its range of activity and the cacalol containing plant extracts. Thereby, we aimed to discover whether STs are responsible for the limitation of herbivory on *P. hybridus* and the observed almost complete lack of mollusk herbivory on *A. alpina*.

METHODS AND MATERIALS

Choice experiments with pure substances and plant extracts were conducted with wheat wafers. Squares of about 25 mg dry weight were cut from commercially available wafers (Back-Oblaten, Küchle GmbH & Co.), consisting of wheat flour and starch. After cutting, the squares were weighed and placed on a glass plate where the test and control solutions were applied. After complete evaporation of the solvent (methanol), one treatment and one control square were placed into each experimental container. Then the snail was added and after 16–18 hr it was removed and the remains of the squares were recovered, dried, and weighed for estimation of consumption. Trials were started 3–4 hr before dark, lasted overnight (10 hr), and 3–4 hr into the light period of the next morning. During that period the animals would usually eat not more than half of the food presented. The masses eaten from the treatment and the control squares were compared by a paired *t* test. The tests with furanopetasin were conducted one year before the other tests and therefore followed a protocol that used leaf discs of lettuce (*Lactuca sativa*) as the test substrate (for details see Hägele et al., 1996). "Naive" snails always experienced the test substance for the first time, although they might have experienced another substance before. Care was taken in order to assure that snails tested with either petasin, isopetasin, or furanopetasin were not retested with another substance from this group.

Organs of *P. hybridus* were extracted and their petasin content was quantified by HPLC as described in Wildi (1997). Leaves and rhizomes of *A. alpina* were extracted as described in Novotny et al. (1972). The quantity of cacalol in relevant fractions was determined by HPLC (Hägele, 1996).

Clones of *P. hybridus* were regenerated from tissue cultures and kept in the garden when fully grown. On the morning of the experiment one leaf from each clone was harvested, put into a plastic bag and kept refrigerated until the experiment. The choice between leaves was provided by cutting discs from one half of the test leaves and pinning them to the ground of the experimental container. After the experimental period (as above), all leftovers were recovered, glued on a paper sheet, dried, and plant area was measured with a video-based image analysis system. The second half of the leaf was stored at −20°C until it was further processed for petasin quantification (Wildi, 1997).

The STs tested (Figure 1), were isolated from *P. hybridus* [furanopetasin (FP) and petasin (P)], from *P. kablikianus* [kablicin (KB) and isopetasin (IP)],

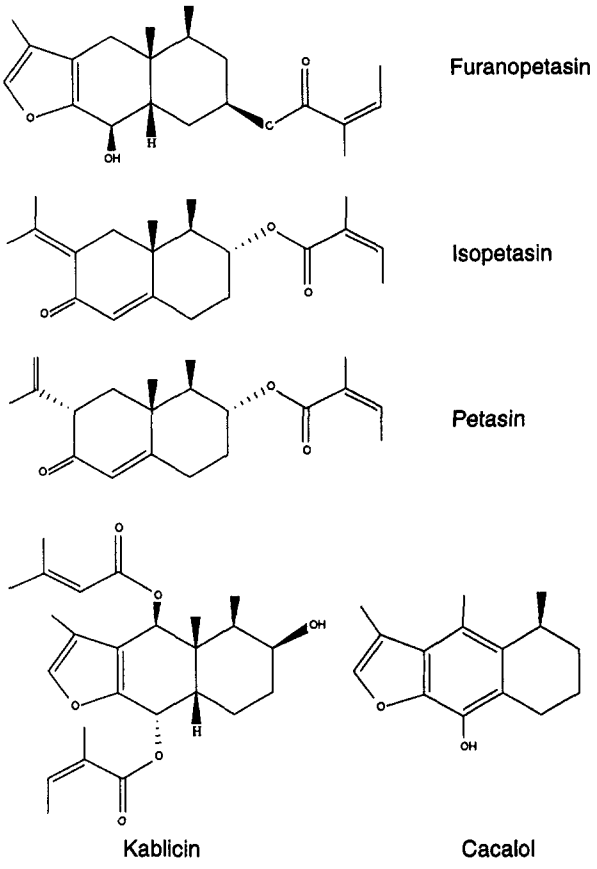

FIG. 1. Structural formulas of sesquiterpenes tested.

and from *Adenostyles alpina* [cacalol (CA)] (Novotny et al., 1972, 1987; J. Harmatha, unpublished results). All plant names correspond to the nomenclature of the *Flora Europaea* (Tutin et al., 1976).

All tests were made with adult specimen of *Arianta arbustorum* L. (Gastropoda, Helicidae). *A. arbustorum* is an omnivorous snail that includes green plants, decaying plant material, and fungi in its diet (Hägele, 1992). It is the commonest helicid snail in central Europe and is present in high densities in habitats where *P. hybridus* and *A. alpina* occur (Kerney et al., 1983; Hägele, 1992). All snails were collected as adults near Zastler (Germany, Black Forest) and kept individually on $CaCO_3$-enriched soil in plastic containers of 9 cm diameter in an incubator at 15°C and a 14L:10D regime. Snails were maintained

on leaves of fresh lettuce (*L. sativa*) and discs of carrot roots (*Daucus carota*). For experiments, snails were transferred to separate containers of the same size that had a moist plaster bottom and were lined with filter paper. Due to the high relative humidity in the containers, the wafers stuck to the filter paper so that there was no risk that the snails would displace them during feeding. Leaching of test compounds into the filter paper was unlikely, since all test compounds are of low polarity and therefore not water soluble.

RESULTS

The amount of P, IP, FP, and KB necessary to elicit food choice behavior in naive snails was 0.5% dry weight content (Figure 2A). The amount of CA in the test substrate necessary to elicit choice behavior was 0.05% dry weight content (Figure 2A). One week later the sensitivity of the same snails towards IP, KB, and CA was unchanged, whereas the amount of P that was now necessary to elicit choice behavior had decreased to 0.05%, and the amount of FP decreased to 0.1% (Figure 2B).

When we tested extracts from different tissues of *P. hybridus*, which all contained the P fraction, all extracts except the petiole extract were deterrent to the snails (Figure 3). The leaf extract of *A. alpina*, which contained CA (0.048% dry weight), was not deterrent to the snails. The CA-containing rhizome extract (0.375% dry weight), however, was strongly deterrent to the snails (Figure 3).

When we provided naive snails with a choice of leaf discs of *P. hybridus*, the discs that originated from clone C1, which had the lowest P content, were preferred over discs from clones C6 and C7 (Figure 4). No preference was apparent between discs from clones C6 and C7, which each had a different but relatively high P content (Figure 4). When we tested leaves from different individual plants of the same clones 11 days later, their leaf P content had changed drastically. C1 now had almost twice as much P in its leaves, whereas the level of P in C6 and C7 had fallen 6- to 10-fold to about the same low value. The experienced snails now ate more of the leaves with the low level of petasin (Figure 4), although only in the choice experiment of C1 versus C7 did they show a clear preference for C1 leaf discs (Figure 4).

DISCUSSION

The deterrence range for pure P on wheat wafers lies well within the range of leaf P content in the investigated *P. hybridus* leaves. We found P content in leaves to be 0.071–0.716% dry weight content, and deterrence to occur at 0.05% for experienced snails. Furthermore, the reaction of snails to leaf extracts con-

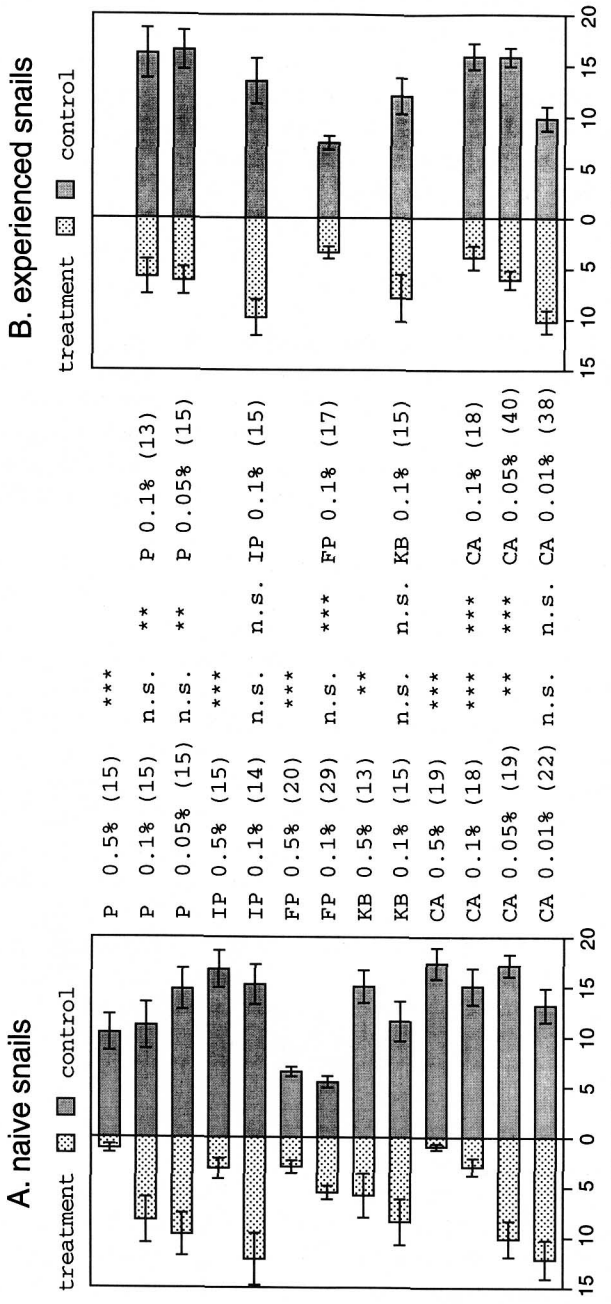

FIG. 2. Dry mass of treatment and control wafers eaten by snails when naive (A), or when tested again seven days after the first test (B). Means and standard errors are shown. Sesquiterpenes (STs) on treatment wafers were petasin (P), isopetasin (IP), kablicin (KB), and cacalol (CA). Furanopetasin (FP) was tested by applying its solution on lettuce leaf discs. Means of FP experiments are square centimeters of discs eaten. The amount of ST applied is given as percent of dry weight of the test substrate, and the number of snails tested is shown in parentheses. Significant differences ($P < 0.05$) between treatments and controls are indicated by asterisks (*, **, *** correspond to $P < 0.05$, 0.01, 0.001, respectively), n.s. indicates nonsignificant differences ($P > 0.05$).

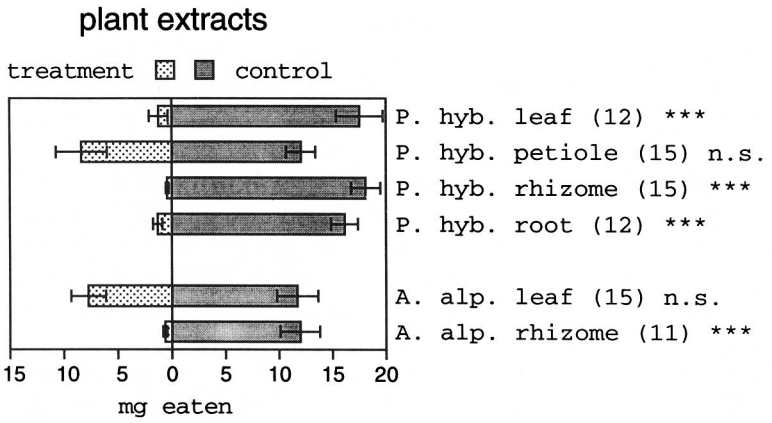

FIG. 3. Dry mass of treatment and control wafers eaten by snails, when treatments were extracts from different organs of *P. hybridus* and *A. alpina* containing petasin and cacalol, respectively. Means and standard errors are given. The concentration of petasin (in *P. hybridus* extracts) and cacalol (in *A. alpina* extracts) is given as percent of tissue dry weight, and the number of snails tested is shown in parentheses. Significant differences ($P < 0.05$) between treatments and controls are indicated by asterisks (***$P < 0.001$), n.s. indicates nonsignificant differences ($P > 0.05$).

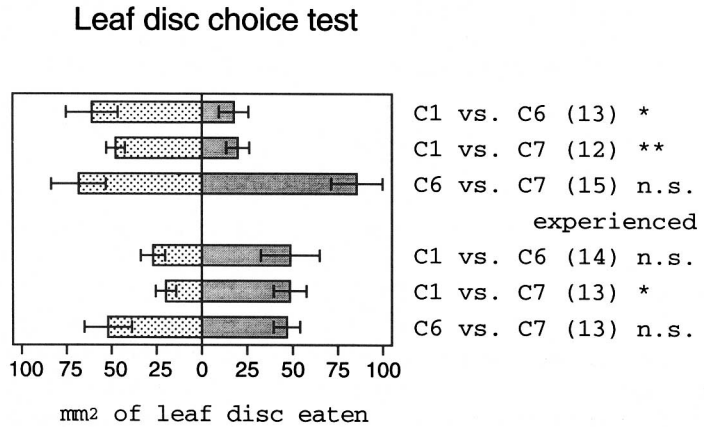

FIG. 4. Area of leaf discs from *P. hybridus* clones eaten by naive snails and the same (experienced) snails 11 days later. Means and standard errors are given. Petasin content of clones is given as percent of tissue dry weight, and the number of snails tested is shown in parentheses. Significant differences ($P < 0.05$) between leaf disc area eaten from different clones are indicated by asterisks (*, ** correspond to $P < 0.05$, 0.01, respectively), n.s. indicates nonsignificant differences ($P > 0.05$).

taining P fit the overall picture that P might indeed be the major snail deterrent in *P. hybridus*. The choice experiment with leaf discs of *P. hybridus* clones with different P content further showed that naive snails preferred the leaf with the lower P content if the difference was large (0.464%) and the P content of the alternative leaf was relatively low (Figure 4, upper part).

Whether FP is also the main effective snail deterrent in the FP chemovariant of *P. hybridus* remains an open question because we lack data about the range of FP leaf content in this chemovariant. However, since FP seems to have a deterrence similar to P (tests were made on lettuce leaves), and since a sensitivity change also occurred, we might well expect it to be as effective as P in its snail-deterring properties.

CA proved to be deterrent at 0.05% dry weight content in the wheat wafer experiment, but no sensitivity change occurred that would have lowered the deterrent concentration even further. CA content in *A. alpina* leaves was found to vary between populations from means of 0.008% to 0.022% dry weight content (Hägele 1996). Thus it seems unlikely that CA is the major snail deterrent compound in *A. alpina*. Furthermore, fractionation of *A. alpina* leaf extracts produced several snail-deterrent fractions other than the CA-containing one (Hägele et al., unpublished), so that the observed overall deterrence of the leaf might well be the result of additive or synergistic actions of several compounds (Adams and Bernays, 1978; Woodhead and Bernays, 1978). However, CA could still be of importance for deterring rhizome-feeding snails, since it is present in rhizomes in much higher concentrations (~0.38% dry weight) than in other tissues, and the CA-containing rhizome extract proved to be snail-deterrent (Figure 3). In addition, adenostylone and neoadenostylone, two related STs that have been shown to possess snail-deterrent activity (Hägele et al., 1996), are both also present in high quantities in *A. alpina* rhizomes (~0.46–0.25% dry weight; J. Harmatha, unpublished).

The slug *Limax maximus* is known to reject bitter foods after an initial taste contact (Carew and Sahley, 1986). Since sesquiterpene lactones and some other specific derivatives are known for their bitter taste, bitterness might well be the basis for the deterrent activity of the STs tested. The basis for the observed increase in sensitivity towards P and FP after an initial experience is still unknown. However, since the observed sensitivity effect in experienced snails is due to a failure to ingest larger amounts of the test substrate rather than to a failure to approach the test substrate (consumed mass of test substrate is significantly different from zero), we put forward two possibilities about the mechanisms in action.

Since P is known as a muscle-relaxing substance (Aebi et al., 1958) and is used pharmaceutically, it might well have postingestive effects in snails as well. If P is also muscle relaxing in snails, a partial immobilization would make them much more vulnerable to desiccation. Such an effect might be the basis of a learned aversion towards these substances.

Another possibility is that P and FP act directly at the neurological level of transmitter substances and receptors. Dopamine elicits the feeding motor program in *L. maximus* in a dose-dependent manner (Wieland and Gelperin, 1983), and P, IP, and FP inhibited binding of [^{3}H]spiperone (a ligand to the dopamine receptor) to the vertebrate dopamine-D_2 receptor (Berger et al., 1998). It thus might well be that the observed sensitivity change towards P and FP is the result of sensitization that involves direct long-term facilitation of the neurological pathways due to a partial blocking of dopamine receptors. However, at this stage it is not clear why the treatment with IP did not show the same sensitivity characteristics as with P and FP, since it was also able to block the dopamine receptor (Berger et al., 1998). So the exact mode of action of P and FP still has to be established, especially whether they also affect serotinergic neurons, which have been shown to participate in adult snail feeding sensitization (Zakharov and Balaban, 1987).

In the nutritionally more complex context, when experienced snails were feeding from whole leaf tissue, their reaction to different concentrations of P in the leaf tissue was less clear. Only in the choice C1 vs. C7 could we see a clear preference for the leaf disc containing less P, whereas in the choice C1 vs. C6, which was a similar choice regarding P content (Figure 4), we could not detect any differences. This reminds us that the action of deterrents on feeding is context dependent (Schoonhoven et al., 1992) and other factors such as different phagostimulants or learned associations might have influenced the actual food choice. It is therefore possible that from the previous experiment the snails learned to associate characteristics of plant C1 with low P content and characteristics of C6 with high P content. Subsequently, at their second experience with leaves from the same plants (clones), the snails only adjusted their behavior towards the now changed P content after initially choosing the "wrong" (C1) disc, which now had a higher P content than before. A single trial has been reported as sufficient for *L. maximus* to acquire a learned aversion (Sahley et al., 1981, 1990), and Delaney and Gelperin (1986) reported that learned postingestive food aversions could last for several months.

The dynamics of ST change are remarkable. Leaves from the same clone of *P. hybridus* varied up to 10-fold in their P content from week to week. Together with the observed change in sensitivity after experience with P, there is potentially a complicated interaction between P leaf content and snail herbivory. In the beginning of the growing season, when young leaf tissue has to be especially protected from herbivory, a high P content would deter snails from feeding on it. Through possible sensitization and associative learning, this effect could then be reinforced and maintained over the season even when the leaf P content starts to decline. A specific feature of the P-snail system might be that it involves not only a substance that is possibly bitter tasting and therefore learned to be avoided, but perhaps one of the rare cases where a plant compound has a direct neurophysiological influence on the feeding behavior itself.

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AGE-RELATED CHANGES IN ODOR PREFERENCES BY HOUSE MICE LIVING IN SEMINATURAL ENCLOSURES

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Abstract—Changes in odor preferences with age were assessed in house mice inhabiting a series of eight 0.1-ha outdoor enclosures. The most recent capture in a live trap was assumed to provide the predominant odor in that trap for one week. Data were analyzed for two separate years for females and males and for three age classes within each sex. Initial analyses confirmed the use of the odor baiting technique; significantly more mice than expected entered traps that had captured another mouse in the previous week relative to traps that had not caught a mouse for at least a week. For female mice two predictions were tested. (1) Young female mice avoid traps with odors from adult males. The findings support this prediction. (2) As they mature, female mice will shift their odor preference and enter significantly more traps odorized by adult males. The findings also support this prediction. Two predictions were tested for male mice. (3) Young male mice will avoid odors from adult males. The data do not support this prediction. (4) As they mature, males will shift their preference and enter more traps odorized by female mice. Some, but not all of the data support this hypothesis.

Key Words—House mice, odor cues, chemosignals, pheromones, live traps, social behavior, age, odor preference.

INTRODUCTION

Social communication among house mice (*Mus domesticus*) involves considerable olfactory communication via urinary chemosignals (Bronson, 1983; Drickamer, 1986). Mice use odors cues to recognize sex, age, reproductive condition, kin, specific individuals, dominance status, and individuals from local versus

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strange populations (Bowers and Alexander, 1967; Cox, 1984; Kareem and Barnard, 1986; Winn and Vestal, 1986; Drickamer, 1989; 1995, 1997; Hurst, 1990a–c; Mossman and Drickamer, 1996).

These olfactory cues include urinary chemosignals that accelerate or delay the onset of puberty in young female mice (Vandenbergh, 1969; Drickamer, 1977; Vandenbergh and Coppola, 1987). Among the consequences of earlier puberty for female mice are higher mortality and lower overall reproductive output relative to females that attain puberty at normal ages or are delayed in attaining puberty (Drickamer, 1988). Prepubertal female house mice appear, from laboratory studies, to regulate their reproductive development to some degree (Coppola and O'Connell, 1988; Drickamer, 1992). They do this by avoiding male odor cues that would accelerate puberty until they are peripubertal. As they mature, they shift their odor preference to favor odors from adult males. Hurst and Nevison (1994), also working in the laboratory, claim that young female house mice do not regulate their exposure to the puberty-influencing chemosignals. Recently, Solomon and Rumbaugh (1997) found that young female pine voles (*Microtus pinetorum*) shifted their odor preference with age. Female voles, tested in the laboratory, showed an increased preference for male pine vole odor as they matured.

Our first objective was to examine changes in female house mouse odor preference with age under field conditions. If what has been found for pine voles and some house mice under laboratory conditions is true, we predict that as young female house mice mature, they should shift their odor preference from an avoidance of male odors when they are prepubertal to a preference for male odors as adults. We tested this prediction by using odors in traps in seminatural field enclosures with free-living house mice.

Our second objective was to examine possible shifts in odor preferences in male house mice as they mature. Male house mice living in commensal situations are generally terrotorial (Crowcroft and Rowe, 1963; Crowcroft, 1966; Bronson, 1979), but in a field setting they appear to have overlapping home ranges (Brown, 1966; DeLong, 1967; Mikesic and Drickamer, 1992). Because they would be at a potential disadvantage in an aggressive encounter, we might expect that young male mice would avoid the odors of adult males until they were of sufficient size to defend themselves. Thus, we predict that in a field setting, juvenile and possibly subadult male mice would avoid traps containing odors from adult males. This pattern might shift as the mice matured.

Subadult mice that are nearing or have attained puberty, and adult mice seek out females for mating opportunities. Past studies provide data to indicate that as adults, male mice prefer to enter traps that previously contained females, particularly estrous females (Drickamer, 1995). In the present study, we predicted that as adults, males would prefer females, and confirm the earlier finding.

METHODS AND MATERIALS

Enclosures. Eight 0.1-ha enclosures located in an abandoned pasture were used for these tests (see Drickamer and Mikesic, 1990, for a complete description of the enclosures and their construction). Each enclosure measures 25 × 40 m and has galvanized wire mesh and aluminum flashing walls 1 m high. Each enclosure contains four symmetrically placed "mouse cities"; 2-m-diameter circular structures made of 1-m-high aluminum fixed to wooden posts. Each mouse city has a plywood roof, two entrance holes on opposite sides just above ground level, and nesting cavities located within the center column that holds up the roof. There are 12 in-ground nest sites in each enclosure, consisting of a cinder block sandwiched between two patio stones, with entrance holes at ground level. These nest locations are positioned in four rows of three sites, evenly distributed in each enclosure. Food (Teklad Mouse Chow) was supplied in all mouse cities and at each in-ground nest location on a weekly basis and water was supplied within each mouse city each week.

A 1.5-m strip was mowed around the interior perimeter of each enclosure once each month. In all other areas of each enclosure, the vegetation was allowed to grow uncut, attaining heights of 1–1.5 m. The mowed strips serve as pathways for human travel in the enclosure and prevent vegetation near the walls from reaching heights that could enable mice to escape. Each enclosure contains a 6 × 9 trapping grid with a 5-m interval between trap stations. Each station contains one folding Sherman livetrap housed within a 2-liter waxed milk or juice carton. The trapping grid thus has 28 stations in the interior and 26 stations along the perimeter walls.

Procedures. Data collected during 1990 and 1993 were used for the present tests. In late March of each year, populations were introduced into each of the eight enclosures. These starter populations consisted of second- and third-generation mice derived from two different stocks of wild house mice (*Mus domesticus*). One stock was bred from mice trapped at the Southern Illinois University Swine Center and the other stock was derived from mice captured near Fort Collins, Colorado. Since the results of our analyses did not differ for these two mouse stocks, the data have been combined for all analyses presented here.

Populations were initiated by placing 8–16 males and 16–32 females into each enclosure, divided equally among the four mouse cities located within an enclosure. These populations were allowed to grow freely for 30 weeks, after which the mice were removed by using a combination of live- and kill-traps. Starter mice were 90–160 days of age when populations were initiated.

Trapping Procedure. The live traps were opened and baited at about 18:00 hr on two nonconsecutive nights each week. They were checked and closed starting at 06:00 hr the next morning. As in earlier studies where the odor-

baiting techique has been used (Drickamer, 1995, 1997), we made the assumption that the most recent occupant of a trap provided the most salient odor for up to one week and that other previous occupants supplied a general background or contextual odor. Mice selecting a trap to enter could be entering because they are attracted to that odor or because they find that odor represents a potential threat and they need to investigate. Traps were not cleaned or scraped out during the course of either 30-week field season. A total of 12 traps out of 864 used over the two years for this study were replaced during the course of the field season due to malfunctions or damage. This number is sufficiently small that we doubt that the results were influenced by the fact that it could take a few weeks for such replacement traps to contain odors. Cotton squares (Nestlets, Ancare Corp.) were placed in each trap so that the mice could make nests. The Nestlets could hold some mouse odors, and they were replaced only when they became wet due to rain.

Each mouse placed in the enclosures was given a unique toe-clip and ear-punch number. Each time a mouse was captured we noted the location, number, body mass (nearest 0.5 g with a Pesola spring scale), and reproductive condition. Mice were always released at their site of capture. Mice born in the enclosures were given a number the first time they were caught and the same measurements made on the adult starter mice were made on the recruits at each subsequent capture. For our study, female mice were considered to be juveniles if they weighed ≤ 10.5 g, subadults if they weighed 11–15 g, and adults if they weighed ≥ 15.5 g. Male mice were considered to be juveniles if they weighed ≤ 11 g, subadults when they weighed 12–16 g, and adults if they weighed ≥ 16.5 g. These categories were used with respect to mice responding to odors in the traps.

Analyses. Traps were considered odorized for up to one week (two trapping sessions) after they had caught a mouse. At that point, if the trap had not been occupied again, it was considered to contain only general contextual odors and was not counted as an odorized trap.

Because there is a lag period between the initiation of populations in the enclosures and the time when juvenile recruits start to be trapped, we limited the period for the analyses reported here to the time from June 6 to the end of the field season for 1990 and from May 28 to the end of the field season for 1993. We have analyzed only the responses of these recruit mice as they matured; starter mice could serve to odorize traps, but we have not tabulated or analyzed their responses to test our predictions.

For our analyses of responses to trap odors, we used only the initial capture of a mouse when it was in a particular age class. We conducted separate analyses for each sex within each year and for each of the three age classes of responder types. These procedures ensure that the data points used in each analysis are

independent. For each year, sex, and age class, we first analyzed the data to determine whether mice responded differentially to odorized traps (those with odor up to one week old) compared to other traps that had not caught a mouse within the previous week.

The second analysis in each sex/year/age class grouping was to determine whether there were any shifts in odor preferences as the mice matured. For these analyses the odorized traps that the mice could enter were divided into four categories: odorized by prepubertal females (weighing ≤ 13 g, where the vagina was generally still closed); odorized by adult females (weighing ≥ 13.5 g, involving females with perforate vaginas and those that were reproductively active—pregnant and/or lactating); odorized by prepubertal males (weighing ≤ 14.5 g and generally with testes not scrotal); and odorized by adult males (weighing ≥ 15 g, generally with scrotal testes). Self-captures, that is, those situations in which a mouse entered a trap that it had odorized within the previous week (total occurrence $<3\%$ of all captures) were dropped from the analyses.

Chi-square tests (Zar, 1996) were used for all analyses. To generate the expected values for these chi-square tests of odorized vs. nonodorized traps, we assumed that the mice should respond in direct proportion to the available numbers of odorized and nonodorized traps. These numbers were computed for each trap night and summed for the entire period covered by our analyses. To obtain expected values for the analyses of differential response to particular age and sex class odor types, we used a similar procedure. Since we know what has been caught in the previous week at each trap, we summed the possible traps that had been odorized by each of the four age and sex classes. These values were added across the period used for the analysis. The values obtained from this process served as the basis for determining the proportions of the total captures that should be expected at each odor type.

RESULTS

The initial analyses of odor preference for both female and male mice in both 1990 and 1993 revealed that at all three age categories, mice were significantly more responsive to traps that were odorized than they were to traps that had not caught a mouse for at least one week (Table 1). For 1990, females at all three ages responded differentially to the odor types in the traps (Table 2). Juvenile females entered traps odorized by prepubertal females significantly more often than expected; they avoided traps odorized by adult females and, to a lesser degree, those odorized by adult males. Subadult females entered traps odorized by adult males more than expected and avoided traps odorized by prepubertal males. Finally, adult females avoided prepubertal males and showed a strong, significant preference for traps odorized by adult males.

TABLE 1. DIFFERENTIAL ODOR PREFERENCES OF MICE OF THREE AGES IN 1990 AND 1993: ODORIZED VS. NONODORIZED TRAPS

	Age class of responding females		
	Juvenile	Subadult	Adult
Females in 1990			
No odor in trap	89(42.2)[a]	55(43.3)	47(46.5)
Expected	173.2	104.3	82.9
Odorized trap	122(57.8)	72(56.7)	54(53.5)
Expected	37.8	22.7	18.1
χ^2 (df = 1)	228.993	130.177	87.009
P	<0.001	<0.001	<0.001
Females in 1993			
No odor in trap	89(45.2)[a]	52(51.0)	36(50.7)
Expected	164.1	85.0	69.1
Odorized trap	108(54.8)	50(49.0)	35(49.3)
Expected	32.9	17.0	11.9
χ^2 (df = 1)	205.588	76.541	61.033
P	<0.001	<0.001	<0.001
	Age class of responding males		
	Juvenile	Subadult	Adult
Males in 1990			
No odor in trap	72(48.3)[a]	51(44.7)	37(48.1)
Expected	122.3	93.6	63.2
Odorized trap	77(51.7)	63(55.3)	40(51.9)
Expected	26.7	20.4	13.8
χ^2 (df = 1)	117.447	108.347	60.765
P	<0.001	<0.001	<0.001
Males in 1993			
No odor in trap	98(37.7)[a]	48(42.9)	39(51.3)
Expected	171.6	93.3	63.3
Odorized trap	108(62.3)	64(57.1)	37(48.7)
Expected	34.4	18.7	12.7
χ^2 (df = 1)	188.847	131.503	55.823
P	<0.001	<0.001	<0.001

[a]The numbers given represent the animals trapped with the trap odorized or not. Values in parentheses are the percentages of mice caught at each type of trap within that vertical column.

For 1993, females at all three ages responded differentially to the odor types in the traps (Table 3). Juvenile females exhibited significant tendencies to enter traps odorized by prepubertal females and males more frequently than expected, and they avoided traps odorized by adult females and adult males. As

TABLE 2. ANALYSIS OF ODOR PREFERENCES EXHIBITED BY FEMALE MICE OF THREE AGES DURING 1990

	Age class of responding females		
Odor source	Juvenile	Subadult	Adult
Prepubertal female	45(36.9)[a]	22(31.9)	15(27.8)
Expected	31.3	18.5	13.9
Adult female	31(25.4)	22(31.9)	15(27.8)
Expected	41.3	24.4	18.3
Prepubertal male	33(27.0)	8(11.1)	0(0.0)
Expected	31.2	18.4	13.8
Adult male	13(10.7)	18(25.0)	24(44.4)
Expected	18.2	10.8	8.1
χ^2 (df = 1)	10.113	11.559	38.037
P	<0.005	<0.001	<0.001

[a]The numbers given represent the animals trapped with each of the types of odors in the traps. Values in parentheses are the percentages of mice caught at each type of trap within that vertical column.

TABLE 3. ANALYSIS OF ODOR PREFERENCES EXHIBITED BY FEMALE MICE OF THREE AGES DURING 1993

	Age class of responding females		
Odor source	Juvenile	Subadult	Adult
Prepubertal female	29(26.9)[a]	6(12.0)	3(8.6)
Expected	16.1	7.4	5.2
Adult female	35(32.4)	15(30.0)	9(25.7)
Expected	47.5	22.0	15.4
Prepubertal male	27(25.0)	14(28.0)	7(20.0)
Expected	19.6	9.1	6.4
Adult male	17(15.7)	15(30.0)	16(45.7)
Expected	24.8	11.5	8.0
χ^2 (df = 1)	18.888	6.251	11.545
P	<0.001	0.001 < P < 0.025	<0.001

[a]The numbers given represent the animals trapped with each of the types of odors in the traps. Values in parentheses are the percentages of mice caught at each type of trap within that vertical column.

TABLE 4. ANALYSIS OF ODOR PREFERENCES EXHIBITED BY MALE MICE OF THREE AGES DURING 1990

Odor source	Age class of responding males		
	Juvenile	Subadult	Adult
Prepubertal female	23(29.9)[a]	19(30.2)	9(22.5)
Expected	19.8	16.2	10.3
Adult female	24(31.2)	19(30.2)	19(47.5)
Expected	26.0	21.3	13.5
Prepubertal male	17(22.1)	16(25.4)	6(15.0)
Expected	19.7	16.1	10.2
Adult male	13(16.9)	9(14.3)	6(15.0)
Expected	11.5	9.4	6.0
χ^2 ($df = 1$)	1.789	0.757	4.113
P	>0.20	>0.50	$0.025 < P < 0.05$

[a] The numbers given represent the count of animals trapped with each of the types of odors in the traps. Values in parentheses are the percentages of mice caught at each type of trap within that vertical column.

subadults, these females still exhibited some avoidance of traps odorized by adult females and exhibited small preferences for traps odorized by both prepubertal and adult males. As adults, the females shifted their preference to exhibit a strong bias toward traps previously occupied by adult males.

For 1990, males exhibited some differential response to the odor types only as adults (Table 4). That significant effect was largely attributable to the avoidance of traps odorized by prepubertal males, and a significant attraction to traps odorized by adult females.

In contrast, the analyses for males in 1993 revealed significant effects for juveniles and subadults, but not for adults (Table 5). For this year, juvenile males were caught significantly more often than expected at traps odorized by prepubertal females and prepubertal males, and they were caught significantly less than expected at traps odorized by adult males. Subadult males were also caught more often than expected in traps odorized by prepubertal mice of both sexes, and exhibited some avoidance of traps odorized by adults of both sexes.

DISCUSSION

The initial analyses (Table 1) confirm the use of the odorized trap technique as a measure of mouse responsiveness to odors that are no more than one week old in traps. All of the analyses indicated a strong differential bias toward en-

TABLE 5. ANALYSIS OF ODOR PREFERENCES EXHIBITED BY MALE MICE OF THREE AGES DURING 1993

	Age class of responding males		
Odor source	Juvenile	Subadult	Adult
Prepubertal female	21(19.4)[a]	13(20.3)	8(21.6)
Expected	16.1	9.5	5.5
Adult female	41(38.0)	20(31.2)	15(40.5)
Expected	47.5	28.4	16.3
Prepubertal male	33(32.4)	20(31.2)	6(16.2)
Expected	19.6	11.6	6.7
Adult male	11(10.2)	11(17.2)	8(21.6)
Expected	24.8	14.7	8.5
χ^2 (df = 1)	12.200	10.609	1.319
P	<0.001	<0.005	>0.50

[a]The numbers given represent the animals trapped with each of the types of odors in the traps. Values in parentheses are the percentages of mice caught at each type of trap within that vertical column.

tering traps that had been occupied by another mouse up to one week prior to the capture relative to entering traps with odors that were more than one week old.

Under field conditions, female house mice shift their odor preferences as they mature. As juveniles, they avoid the odors of adult mice and prefer, instead, to enter traps that have been odorized by prepubertal mice of either sex. These findings support our prediction with respect to the behavior of females and the results of laboratory experiments on pine voles (Solomon and Rumbaugh, 1997) and house mice (Coppola and O'Connell, 1988; Drickamer, 1989). If, in fact, as was previously shown, female mice can suffer higher mortality and lower reproductive output as a consequence of attaining puberty at an early age (Drickamer, 1988), then it makes sense for them to avoid cues, such as male mouse odor, that would accelerate sexual development. As expected, as female mice mature and begin seeking mates, they shift their preference in favor of entering traps odorized by males.

The finding for young females, and to a lesser degree, subadult females, that they avoid traps odorized by adult females may appear to be in contrast to the previous laboratory finding that females of such ages prefer the odor of grouped females. The odor in this case, however, is not a concentrated odor from grouped females, but rather the odor of a single female. We suggest that the current findings may reflect several aspects of the social biology of young

female mice. Adult females, regardless of their age, could represent potential competitors and, given the size disadvantage for a maturing female house mouse, odors of such females would thus be avoided. It could also be that odors from the mother and possibly from related females (e.g., older siblings from previous litters born to the same dam) are not avoided, but the traps odorized by strange females are avoided. Further testing, where genetic relationships are known, would be necessary to resolve this issue.

Because the data for the two separate years analyzed provide contrasting results with regard to the responses of male mice as they mature to odorized traps, it is difficult to draw any overall conclusions. That the results are equivocal is partially in agreement with earlier field data (Drickamer, 1995), but contradict previous laboratory findings for house mice (Drickamer, 1988). The earlier field data from the enclosure, but using different years, revealed that in only one of three tests did young male mice respond by displaying a significant avoidance of traps odorized by adult males. The laboratory data, from which our prediction for age-related changes in odor preferences among males was derived, showed that young males avoided adult male odors. In the field, the male odors present in traps could include those from the father, from older male siblings from the previous pair, with one parent in common with the responder mouse, as well as odors from unrelated males. When the laboratory tests were conducted (Drickamer, 1988), the male odors used were all from strange males, unrelated to the test males. Thus, choices available in the field were not present when the laboratory tests were conducted. The pattern of trap captures under field conditions may reflect both a preference to enter traps odorized by related males and avoidance of traps odorized by strange males. Again, as with the female mice, further data on genetic relatedness among the mice in each enclosure are needed to test this hypothesis further.

Finally for one of the years, 1990, there was a significant shift toward a preference for traps odorized by adult females as the male mice matured. This pattern was not observed in 1993. Data from other years involving these mouse enclosures suggest that male mice do in fact seek out female mice, particularly those in estrus as possible mates (Drickamer, 1995). We do not have an explanation concerning the fact that for 1993 this pattern was not repeated.

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RESPONSES OF CAMBARID CRAYFISH TO PREDATOR ODOR

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Abstract—The responses of individuals of four sympatric species of cambarid crayfish to the introduction of the odor of a common predator, the snapping turtle *Chelydra serpentina*, were recorded in the laboratory. Adult *Orconectes virilis* spent significantly more time in a lowered posture and reduced the frequency of nonlocomotory movements following introduction of snapping turtle odor but showed no change in behavior upon introduction of the odor of painted turtle (*Chrysemys picta*). Recently released young *O. virilis* did not respond to snapping turtle odor initially but did so after turtle odor and conspecific alarm odor had been paired. Individuals of *O. propinquus* did not respond to snapping turtle odor. Initial tests with *O. rusticus* did not yield any response to snapping turtle odor but after experience with paired turtle and alarm odor, individuals showed a decrease in nonlocomotory movements when just snapping turtle odor was introduced. Individuals of *Cambarus robustus* spent less time in the lowered posture, less time in their burrow, and more frequently executed nonlocomotory movements, in response to snapping turtle odor. The differences in responses to the odor of a common predator are correlated with ecological differences among the crayfish species.

Key Words—Crayfish, predator odors, chemical cues, behavior, snapping turtle, learning, *Orconectes*, *Cambarus*.

INTRODUCTION

Behavioral responses to cues from potential predators are common in a variety of animals and are to be expected if these responses decrease the probability of predation (Mathis and Smith, 1993a). In aquatic systems, one of the most commonly utilized classes of predator avoidance cues is chemical (Chivers et al., 1996; Kiesecker et al. 1996; Smith, 1992). Differences among related species

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with regards to their responses to predator cues are of particular interest when such differences can alter competitive interactions (Werner and Anholt, 1993) and ultimately alter the composition of biological communities.

Past studies on the responses of crayfish to chemical cues from predators have reported that some species do not respond to predator odors, while other species do show responses. Young *Pacifastacus leniusculus* responded to chemical cues from fish predators (Blake and Hart, 1993) as did the parastracid *Paranephrops zealandicus* (Shave et al., 1994). However, Willman et al. (1994) reported no changes in the behavior of *Orconectes propinquus* or *O. virilis* when individuals were exposed to odors from largemouth bass (*Micropterus salmoides*), while individuals of *O. rusticus* increased the time spent out of shelters in the presence of the fish predator cue.

The authors of these studies used somewhat different methods and thus the differences in responsiveness could be related to either methodological differences or to ecological or phylogenetic differences among the species studied. Because a learned association between predator odor and alarm odor has been demonstrated for individuals of fish and insects (Chivers et al., 1996; Mathis and Smith, 1993b), differential experience must also be considered in explaining species differences.

The following tests were conducted to resolve the question of variation in responsiveness to predator cues among species of crayfish by using the same methodology on adult individuals of four species of crayfish occurring in the same area and all subject to predation by a common predator, the snapping turtle, *Chelydra serpentina*. Given the importance of predator–crayfish interactions in determining species replacements (Butler and Stein, 1985; DiDonato and Lodge, 1993; Garvey et al., 1994; Mather and Stein, 1993; Quinn and Janssen, 1989; Stein, 1977) and, in particular, the continuing replacement of native species by *O. rusticus* (DiDonato and Lodge, 1993; Garvey et al., 1994; Hill and Lodge, 1994; Olsen et al., 1991), it seemed particularly important to use a uniform method to examine the responses of several species of sympatric crayfish to the introduction of the odor of a predator they have in common, the snapping turtle. The role of experience in the establishment of a response by individuals of *O. rusticus* and young *O. virilis* was also examined. The specificity of the response of adult *O. virilis* was also tested by using the odor of another species of turtle.

METHODS AND MATERIALS

Experiments with Adult Crayfish. Individuals of the four most common crayfish species (Family Cambaridae) found in Michigan were tested in the laboratory. Adult *Orconectes virilis* and *O. propinquus* were collected from the

Maple River in Emmet County, Michigan, and were tested at the University of Michigan Biological Station, Pellston, Michigan. Berried female *O. virilis* collected from ponds in Saline, Michigan, were tested at the University of Michigan in Ann Arbor, Michigan. The embryos in the eggs on the pleopods of these females were well developed, and in most cases young hatched a few days after the females were tested. *O. rusticus* were obtained from a commercial supplier in Wisconsin and tested in Ann Arbor. *Cambarus robustus* were collected from Fleming Creek near Ann Arbor and tested in the laboratory at Eastern Michigan University, Ypsilanti, Michigan.

For each species, crayfish were placed in individual 10-gallon aquaria. These aquaria were visually isolated from one another, well aerated, contained 12.5 liters of continually aerated well water, and half a clay pot for a burrow. Crayfish were allowed three days to acclimate to the aquaria before testing, and each crayfish was tested just once. Behavior patterns (see below) were detected by an observer and the temporal duration of patterns recorded for 5 min on a portable computer with an event program. For every experiment, behavioral responses were recorded for the same individual during two time periods: (1) 5-min control period following the introduction of 20 ml of clean well water, and then (2) a 5-min period following the introduction of 20 ml of test water. All solutions were introduced quietly via syringe into one corner of the aquarium. The amount of stimulus water used was chosen based upon similar studies on other crustaceans (Hazlett, 1996, 1997).

The behavior patterns recorded were: (1) time spent in the burrow, (2) time spent locomoting by movement of the ambulatory legs, (3) time spent moving any appendage in activities other than locomotion (i.e., grooming, feeding movements of the ambulatory legs, movements of the chelipeds, movements of the antennae), with the exception of flicking of the antennules, and (4) time spent in each of three postures: raised, intermediate, lowered (Hazlett, 1994). The postures were characterized as follows: in the raised posture, the body was elevated off the substrate, the chelipeds held off the substrate parallel to the substrate or even higher, and the abdomen and telson were extended. In the intermediate posture, the body was held just off the substrate, the tips of the chelipeds lightly touched the substrate, and the telson was perpendicular to the substrate. In the lowered posture, the body was in contact with the substrate, the chelipeds drawn in towards the body, and the telson curled under the abdomen.

Twenty individuals were tested for each experiment. The only exception was the set of tests involving female *O. virilis* that were bearing young, only 12 of which were tested. For these animals the only behavior pattern recorded was the number of aeration movements of the egg-laden pleopods during control and test periods. All tests were run between 09:00 and 12:00 hr.

Responses to predator odor were tested for all four species of crayfish. The

source of predator odor was the snapping turtle, *Chelydra serpentina*, an important predator of all the species tested (Hobbs, 1993). For all tests, the 3.2-kg turtle was not fed for three days prior to being placed in 27 liters of clean, aerated well water and left for 24 hr. Predator odor was always freshly drawn from the snapping turtle's aquarium because the effectiveness of this odor decreases in a few hours (Hazlett, unpublished observations).

To test the specificity of the response of adult *O. virilis*, individuals were tested for responses to the odor of another turtle, the painted turtle *Chrysemys picta*. The painted turtle odor was generated by holding a 315-g turtle in 2.7 liters of water for 24 hr; this was the same weight-to-volume ratio as for the snapping turtle tests.

Because the initial tests with *O. rusticus* indicated no response to snapping turtle odor (see below) and because work with other aquatic species indicated a role of experience in recognition of predator odor (Chivers et al., 1996; Mathis and Smith, 1993b), two additional sets of tests were conducted with *O. rusticus*. To test for a response to alarm odor, individuals were exposed to 20 ml of alarm odor generated by crushing a 30-mm cephalothorax length *O. rusticus*, placing it in 400 ml of distilled water, and filtering through coarse filter paper. Individuals were then placed in a communal tank with 27 liters of water, and 400 ml of alarm odor and 400 ml of turtle odor were introduced to the tank simultaneously. This paired introduction was done three time: at 36, 24, and 6 hr prior to placing the crayfish in individual observation aquaria. Observations were made following control and snapping turtle odor introductions 18 hr later.

Pairing of odors was not done with *O. propinquus*, the other species that showed no response to predator odor, because it does not respond to alarm odor (Hazlett, 1994). Without a response to the unconditioned stimulus (alarm odor), it would make little sense to pair another stimulus with the unconditioned stimulus.

For each experiment, paired *t* tests were used to compare the number of seconds spent in the various activities and postures during test and control periods because observations were made on the same individuals under both conditions. Because the crayfish had to be in one of the three postures, the time spent in two postures dictates the time spent in the third. Therefore only two of the postures were analyzed statistically (intermediate and lowered) because less than 10% of the time overall was spent in the raised posture.

Experiments with Young O. virilis. Following testing, female *O. virilis* bearing young were placed in a 30-gallon aquarium and over the following week a number of young were released. About two weeks later young were removed, placed individually in small ceramic cups containing 10 ml of well water, and observed under 6× power with a dissecting microscope. The microscope was needed to see cleaning and feeding movements of the walking legs of the young which were 8–9 mm in total length at the time of testing. The ceramic cups had

a flat bottom and were opaque and thus visually isolated the young crayfish. After a 3-min acclimation period, individuals were observed for 3 min following the introduction of 0.5 ml of well water and then for 3 min following the introduction of 0.5 ml of snapping turtle odor. The number of seconds spend executing nonlocomotory movements (mostly cleaning and feeding movements of the ambulatory legs), locomoting, and on the sides of the observation cup were recorded on a computer with an event program. Individuals could execute two of the behaviors simultaneously or be executing none of the three behavior patterns at any point during observations. Ten individuals were observed and were then placed in a separate holding aquarium following testing. Paired t tests compared the time spent in the different behaviors during test and control periods. The young of the other crayfish species were not available for testing.

Because the young *O. virilis* did not respond in the same fashion to snapping turtle odor as adults of this species, two other experiments were conducted. First a set of 10 individuals was tested for a response to conspecific alarm odor (Hazlett, 1994). A small adult *O. virilis* (32 mm carapace length) was crushed in 150 ml of distilled water and the liquid filtered through course filter paper. Responses to the introduction of 0.5 ml control water and alarm odor were recorded, and these individuals were then placed in the holding aquarium. These young were then exposed simultaneously to snapping turtle odor and alarm odor by adding 100 ml of each to the holding aquarium 24 and 6 hr prior to testing. The young were then removed and carefully rinsed four times in clean well water by transfers to new containers (to remove odors from the holding container). Ten individuals were then tested with control and snapping turtle odor in the same manner as described above.

RESULTS

Adult individuals of *Orconectes virilis* showed two changes in behavior when the predator odor was introduced (Table 1). The crayfish spent less time in the intermediate posture and more time in the lowered posture, and the amount of time during which there was nonlocomotory movement decreased (Figure 1A). There were no other significant changes in behavior, although there was a trend towards less locomotion. In many cases, the crayfish appeared to freeze wherever it was in the tank and not move anything. Berried females significantly reduced the number of aeration movements of the pleopods when predator odor was introduced [control (mean $\pm$ SE) 212 $\pm$ 20.7; test, 147 $\pm$ 14.3; $t = 4.85$, $P = 0.001$]. Most of that reduction appeared to be in the first minute or so after odor introduction.

When the odor of painted turtle was introduced adult individuals of *O. virilis* showed no change in behavior compared to control periods (Figure

TABLE 1. ASSOCIATED PROBABILITIES AND *t* TEST VALUES FROM COMPARISONS OF NUMBER OF SECONDS SPENT IN POSTURES AND ACTIVITIES SHOWN BY INDIVIDUALS OF FOUR SPECIES OF CRAYFISH ($N = 20$ FOR EACH) DURING 5-MINUTE OBSERVATION PERIODS FOLLOWING INTRODUCTION OF CONTROL WATER AND PREDATOR WATER

	O. virilis		*O. propinquus*		*O. rusticus*		*C. robustus*	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Posture								
Intermediate	2.1	0.048	1.2	0.22	1.4	0.16	1.7	0.097
Lowered	2.3	0.030	1.1	0.27	1.3	0.19	2.4	0.026
Movement								
Locomotory	1.7	0.096	1.0	0.31	0.1	0.91	1.2	0.23
Nonlocomotory	2.8	0.010	0.9	0.34	0.1	0.89	3.7	<0.001
In Burrow	0.3	0.74	1.6	0.12	0.9	0.34	2.2	0.037

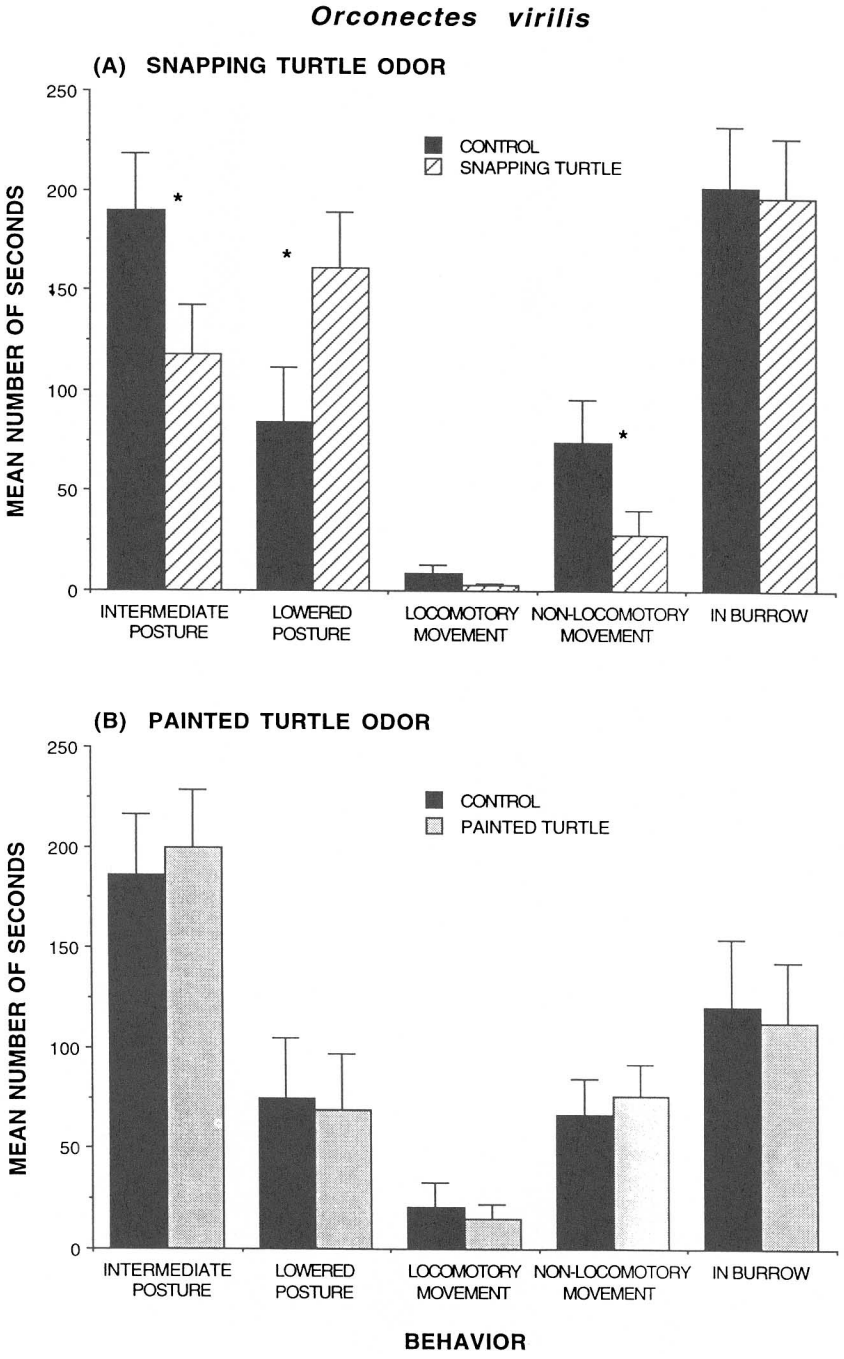
1B). All comparisons between values for the test and control periods were associated with $P > 0.30$.

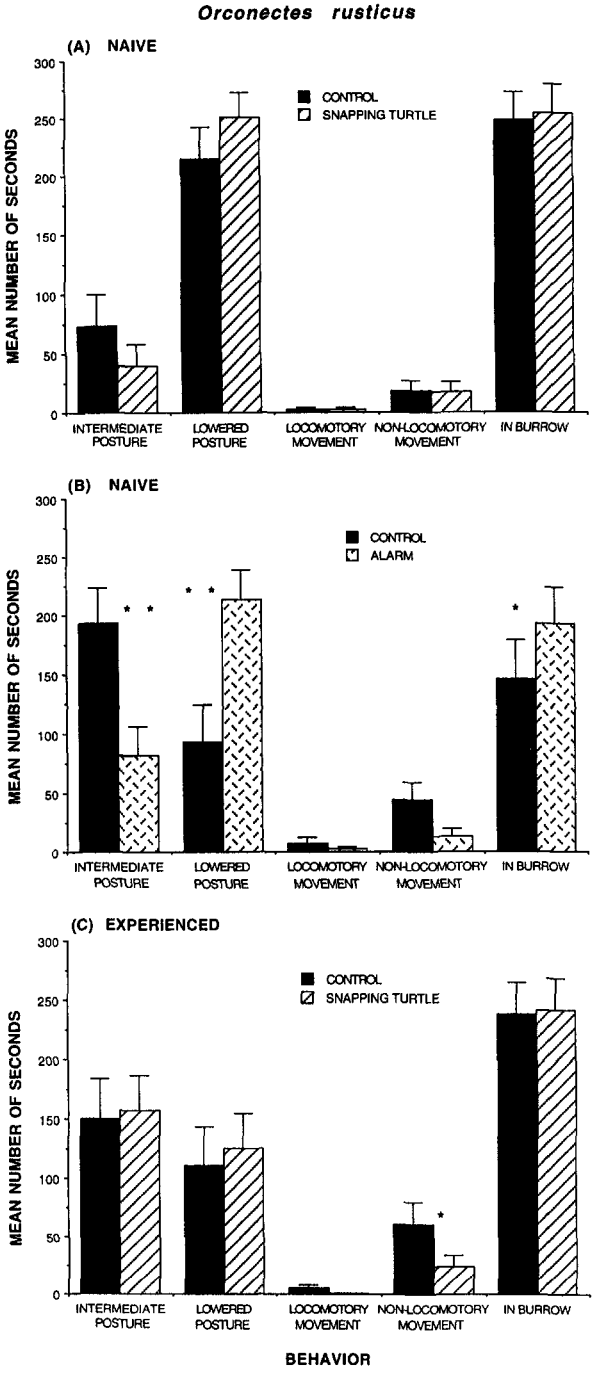
Individuals of *Orconectes propinquus* showed no change in behavior when predator odor was introduced (Table 1). There were no significant differences in either posture or activity between the test and control periods.

Similarly, individuals of *Orconectes rusticus* showed no change in behaviors compared to control periods when predator odor was introduced (Table 1). There were no significant differences in either posture or activity (Figure 2A), even though there was a tendency towards more time in the lowered posture and less in the itnermediate posture. Introduction of conspecific alarm odor resulted in a significant increase in time spent in the burrow, an increase in time in the lowered posture, and a decrease in time in the intermediate posture (Figure 2B). After experience with paired alarm and snapping turtle odor, individuals of *O. rusticus* showed a decrease in the amount of nonlocomotory movement when just snapping turtle odor was introduced (Figure 2C).

In contrast to the patterns shown by the above species, individuals of *Cambarus robustus* responded to the introduction of predator odor by spending less time in the lowered posture, less time in the burrow, and more time executing nonlocomotory movements (Table 1, Figure 3).

FIG. 1. Mean (+SE) number of seconds spent in postures and activities by adult individuals of *Orconectes virilis* during 5-min observation periods following introduction of: (A) snapping turtle odor and (B) painted turtle odor. Asterisks indicate a significant difference between test and control values.


Orconectes virilis
(A) SNAPPING TURTLE ODOR
MEAN NUMBER OF SECONDS
250
200
150
100
50
0
CONTROL
SNAPPING TURTLE
*
*
*
INTERMEDIATE POSTURE
LOWERED POSTURE
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
IN BURROW
(B) PAINTED TURTLE ODOR
MEAN NUMBER OF SECONDS
250
200
150
100
50
0
CONTROL
PAINTED TURTLE
INTERMEDIATE POSTURE
LOWERED POSTURE
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
IN BURROW
BEHAVIOR


Orconectes rusticus
(A) NAIVE
CONTROL
SNAPPING TURTLE
MEAN NUMBER OF SECONDS
300
250
200
150
100
50
0
INTERMEDIATE POSTURE
LOWERED POSTURE
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
IN BURROW
(B) NAIVE
CONTROL
ALARM
MEAN NUMBER OF SECONDS
INTERMEDIATE POSTURE
LOWERED POSTURE
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
IN BURROW
(C) EXPERIENCED
CONTROL
SNAPPING TURTLE
MEAN NUMBER OF SECONDS
INTERMEDIATE POSTURE
LOWERED POSTURE
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
IN BURROW
BEHAVIOR

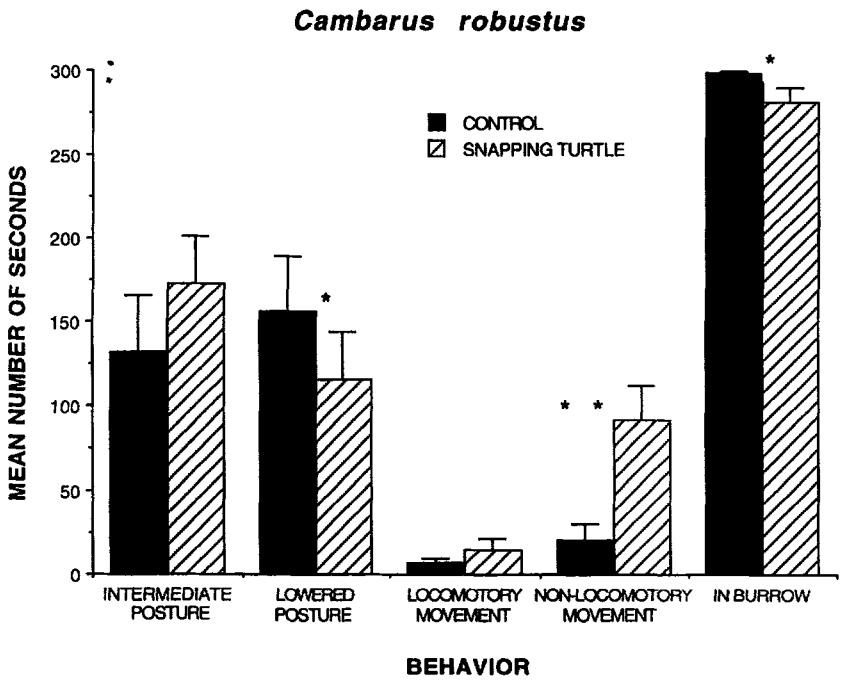
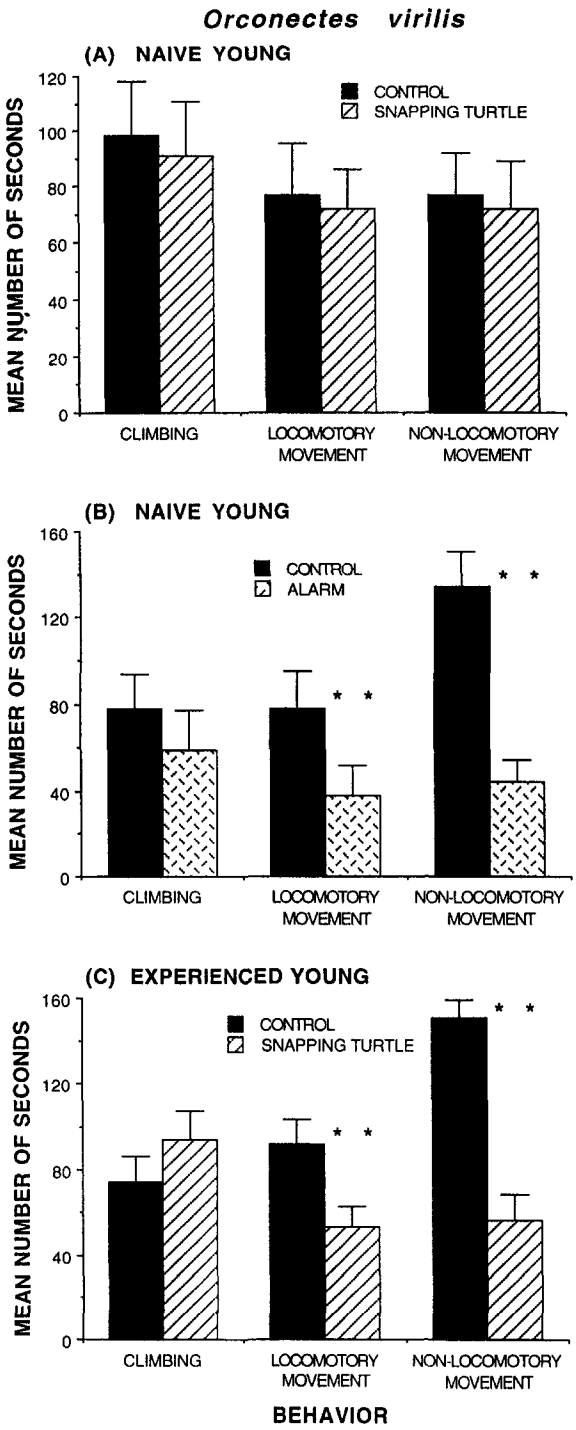


FIG. 3. Mean (+SE) number of seconds spent in postures and activities by adult individuals of *Cambarus robustus* during 5-min observation periods following introduction of control and snapping turtle odor. Asterisks indicate a significant difference between test and control values.

When first tested, young individuals of *O. virilis* showed no changes in behaviors when snapping turtle odor was introduced (Figure 4A). Young did respond to alarm odor with a significant decrease in the time spent executing nonlocomotory movements ($t = 5.56$, $P < 0.001$) and locomotory movements ($t = 4.09$, $P = 0.003$) (Figure 4B). Following experience with alarm and predator odor simultaneously, young individuals showed a significant decrease in both locomotory movement ($t = 4.17$, $P = 0.002$) and nonlocomotory movement ($t = 8.66$, $P < 0.001$) following introduction of snapping turtle odor (Figure 4C).

FIG. 2. Mean (+SE) number of seconds in postures and activities by adult individuals of *Orconectes rusticus* during 5-min observation periods following introduction of: (A) snapping turtle odor to naive crayfish, (B) conspecific alarm odor to naive crayfish, and (C) snapping turtle odor to crayfish that had experienced simultaneous introduction of alarm and snapping turtle odors. Asterisks indicate a significant difference between test and control values.


Orconectes virilis
(A) NAIVE YOUNG
CONTROL
SNAPPING TURTLE
MEAN NUMBER OF SECONDS
120
100
80
60
40
20
0
CLIMBING
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
(B) NAIVE YOUNG
CONTROL
ALARM
MEAN NUMBER OF SECONDS
160
120
80
40
0
* *
* *
CLIMBING
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
(C) EXPERIENCED YOUNG
CONTROL
SNAPPING TURTLE
MEAN NUMBER OF SECONDS
160
120
80
40
0
* *
* *
CLIMBING
LOCOMOTORY MOVEMENT
NON-LOCOMOTORY MOVEMENT
BEHAVIOR

DISCUSSION

When tested under the same protocol, it is clear that there is variation in the responses of different species of crayfish to the odor of a common predator. Individuals of *Orconectes virilis* and experienced individuals of *O. rusticus* responded to snapping turtle odor by a marked decrease in nonlocomotory movements, primarily feeding and cleaning movements of the walking legs. A decrease in movement is a very common response to the detection of a predator (Werner and Anholt, 1993); individuals of the crayfish *Pacifastacus leniusculus* decreased movement after they detected fish predator odor (Blake and Hart, 1993). In the laboratory, snapping turtles respond strongly to movement by potential prey (Hazlett, unpublished observations). The lack of a change in locomotory movements by adult *O. virilis* may be simply a result of the low level of locomotion by these crayfish during the control periods. The lack of a response to painted turtle odor by *O. virilis* suggests that the crayfish can distinguish between the species of turtles chemically. At least for these adult crayfish, the painted turtle may not represent a serious threat of predation, even though this species of turtle does include crayfish in its diet (Hobbs, 1993).

The lack of a response by *O. virilis* to odor from a fish predator reported by Willman et al. (1994) may be because the authors did not record the time spent executing nonlocomotory movements or the time spent in different postures, the behaviors that changed the most in this study. Young *O. virilis* respond to the odor of fish predators by a decrease in nonlocomotory movements (Hazlett, unpublished observations).

Individuals of *Cambarus robustus* responded to predator odor in somewhat the opposite fashion by spending less time in the burrow, similar to the change reported for *O. rusticus* by Willman et al., (1994), and by increasing the locomotory movements shown following introduction of predator odor. The latter response would make sense for crayfish that more frequently live in a stream situation where detection of predator odor would indicate where the predator was (upstream) and directional movement away from the predator decreased predation risk. *C. robustus* is found primarily in rivers and fast-moving streams, while *O. virilis* and *O. rusticus* can be found in both streams and lakes (Crocker and Barr, 1968).

The most interesting result in this study was the lack of response by individuals of *O. propinquus*. It is always possible that these crayfish responded

FIG. 4. Mean (+SE) number of seconds spent in activities by young individuals of *Orconectes virilis* during 3-min observation periods following introduction of (A) snapping turtle odor to naive animals, (B) alarm odor from crushed conspecifics to naive animals, and (C) snapping turtle odor to animals that had simultaneously experienced alarm and predator odors. Asterisks indicate a significant difference between test and control values.

with a change in some behaviors not recorded in this study [or in that of Willman et al. (1994)] or the chemical cue primes the crayfish for detection of a second type of predator cue. The lack of a response to a chemical cue from a predator by individuals of *O. propinquus* is consistent with the lack of response to other types of chemical cues by individuals of this species, such as disturbance pheromones (Hazlett, 1990), alarm pheromones (Hazlett, 1994) and a less clear response to sex pheromones than *O. virilis* (Tierney and Dunham, 1982). I suggested elsewhere (Hazlett, 1994) that perhaps the more diurnal activity patterns of *O. propinquus* have resulted in less reliance upon chemical cues in general. Most species of crayfish are distinctly nocturnal, and this may be correlated with the use of chemical cues to various extents.

An alternative explanation for the lack of a response by *O. propinquus* is that it is considered an invasive species in the Midwest (Capelli and Munjal, 1982), and there has not been an evolutionary history of interactions with snapping turtles in that region. However, the same snapping turtle species occurs in the native ranges of this crayfish and, thus, there is an evolutionary history of interaction. A variation on this theme is that the particular individuals tested might not have had experience with the simultaneous detection of a particular predator odor and alarm substances. Experience has been shown to be necessary for predator recognition in fish (Mathis and Smith, 1993b), dameslflies (Chivers et al., 1996), and young *O. virilis* and adult *O. rusticus* (this study). In the case of the *O. propinquus* used in this study, they were obtained from the same stream as the *O. virilis* and presumably were exposed to the same experiential situations as the individuals of *O. virilis* that did respond to the predator odor. Snapping turtles readily feed upon individuals of *O. propinquus* (Keller and Hazlett, 1996). However, if in fact individuals of *O. propinquus* do not respond to alarm odors from conspecifics (Hazlett, 1994), this would mean there was no unconditioned chemical stimulus with which to associate predator odors and a learned association would not be possible for this species.

In conclusion, this study showed that there are significant differences in the responses of sympatric species of crayfish to the odor of an important predator. These results emphasize the importance of using a uniform methodology in comparative studies before any ecological explanations for differences among species can be put forth.

Acknowledgments—We wish to thank the staff at the University of Michigan Biological Station for their assistance and the staff at the Saline Fisheries Station, Saline, Michigan, for providing the berried *O. virilis*. Thanks are extended to Catherine Bach, Dan Rittschof, and Troy Keller for their comments on the manuscript.

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ROSEFURAN: THE SEX PHEROMONE OF AN ACARID MITE, *Caloglyphus* sp.

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Abstract—Rosefuran was identified as a female sex pheromone from an unnamed species of acarid mite, *Caloglyphus* sp., whose phoretic hypopi had been collected from the elongated yellowish chafer, *Heptophylla picea*. Behavior of sexually excited males was demonstrated by exposing them to doses of 1–100 ng of synthetic rosefuran. The pheromone was present in females and also in males and was detected in trace amounts in nymphal stages. Pheromone concentrations were estimated to be 87 ± 14.2 ng per female and 10.4 ± 2.5 ng per male. The quantitative difference between sexes may allow males to discriminate females for purposes of mating.

Key Words—Mite, Acaridae, sex pheromone, rosefuran, *Caloglyphus* sp.

INTRODUCTION

Sex pheromones have been identified from five species of acarid mites (Acarina: Acaridae), as follows: β-acaridial [(2*E*)-(4-methyl-3-pentenylidene)-butanedial] from *Caloglyphus polyphyllae* (Leal et al., 1989a); 2-hydroxy-6-methylbenzaldehyde (2,6-HMBD) from *Aleuroglyphus ovatus* (Kuwahara et al., 1992) and *Acarus immobilis* (Sato et al., 1993); undecane from *Caloglyphus rodriguezi* (Mori et al., 1995), and (2*R*,3*R*)-epoxyneral [(2*R*,3*R*)-2,3-epoxy-3,7-dimethyl-6-octenal] from an unidentified *Caloglyphus* sp. (tentatively named *Caloglyphus* sp. MJ) (Mori and Kuwahara, 1995; Mori et al., 1996), which was obtained

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from the cockchafer, *Melolontha japonica* (Coleoptera: Scarabaeidae) as phoretic hypopi. The female sex pheromones function not only as attractants for males, but also as stimulants to mount and adhere to females in *C. polyphyllae* (Leal et al., 1989a) and *Caloglyphus* sp. MJ (Mori et al., 1996). In the other three species, however, their functions have not yet been examined fully.

All of these female sex pheromones also have been detected in males. This differs from many cases of lepidopteran insects in which the active sex pheromone is produced by females. Furthermore, even nymphs were found to contain significant amounts of female sex pheromones in *A. immobilis* (Sato et al., 1993), *C. polyphyllae* (unpublished data), and an unidentified *Caloglyphus* sp. MJ (Mori et al., 1996). The female pheromones of *A. ovatus* (Kuwahara et al., 1992) and *C. rodriguezi* (Mori et al., 1995), were found only in minor amounts in the nymphal stages, however. Before concluding that the female sex pheromone of astigmatid mites is present in both sexes, additional examples need to be studied. Furthermore, it would be interesting to determine whether astigmatid mites are able to discriminate conspecific females from males and nymphs.

We report here the identification of a female sex pheromone that triggers mounting behavior of male *Caloglyphus* sp. (tentatively named *Caloglyphus* sp. HP). The distribution of the female sex pheromone within sexes and developmental stages are confirmed, and possible sexual recognition mechanisms between males and females are discussed.

METHODS AND MATERIALS

Mites. An unnamed acarid mite, *Caloglyphus* sp. HP (Acarina: Acaridae) was collected from the yellowish elongated chafer, *Heptophylla picea* (Coleoptera: Scarabaeidae). The deutonymphal hypopi of the mite are found in abundant numbers on the body of *H. picea* larvae in tea plantations of Saitama Prefecture, in East Japan. According to Ishikawa (personal communication), adults and nymphs of the mite attack the grubs of *H. picea* and devour them. This mite species is a new one to science and will be described later by K. Kurosa. The strain was maintained in a Petri dish (85 mm ID, 20 mm high) at 25°C with dried yeast as a nutrient. Moistened filter paper was placed in the Petri dish to maintain a humidified atmosphere.

Gas Chromatography (GC), Mass Spectroscopy (MS), and Nuclear Magnetic Resonance (NMR) Analyses. GC analyses were performed on a Hewlett-Packard 5890 plus chromatograph equipped with an HP-5 column (30 m × 0.25 mm; 0.25 μm), operated in the splitless mode at 60°C for 2 min and then programmed at 10°C/min to 290°C and held at this temperature for 5 min. Mass spectra were measured with a Hitachi M-80 gas chromatograph-high resolution mass spectrometer operated at 70 eV in a low resolution mode, with the same

capillary column and the same conditions as above. ^{1}H NMR spectra were recorded with TMS as an internal standard at 90 MHz on a Jeol JNM-FX 90 Q spectrometer.

Extraction and Isolation of Pheromone. Fifty females (undetermined whether virgin or mated) were collected with the tip of a needle and dipped into hexane (100 μl) for 3 min. The hexane extract was separated on a conventional silica gel column (500 mg, Wako-gel C-200), by sequential elution with 3 ml each of hexane, 5% ether–hexane, 20% ether–hexane, 50% ether–hexane, and finally, pure ether. For quantitative studies, one female, one male, and 10 protonymphs were separately collected on a needle and placed in a tube with a conical bottom (hand-made, 8 mm OD × 30 mm high). Each group of mites was soaked for 3 min in hexane (5 μl), containing tetradecane as an internal standard (10 ng/μl). One microliter of each hexane extract, corresponding to either 0.2 female equivalents (FE), 0.2 male equivalents (ME), or 2 nymph equivalents (NE) was examined by gas chromatography. For evaluating biological activity against males, three females were dipped into 300 μl of hexane for 3 min and 1 μl of the aliquot corresponding to 0.01 FE was bioassayed.

Biological Assays. Two bioassay methods were applied in mini-Petri dishes (10 mm ID × 5 mm high). The bottom surface of the dish was covered with a small piece of damp filter paper and small amounts of moistened dry yeast were added as a nutrient source. Method 1 was designed to quantify the activity of the sex pheromone. The chamber, after the introduction of a group of 10 males (pleomorphic male; males cited hereafter are pleomorphic males), was closed with a cover glass to prevent mites from escaping and conditioned for a minimum of 1 hr. A piece of filter paper (3 × 3 mm) impregnated with whole-mite extract or separated fraction was then placed in the center of the chamber. The number of mounting attempts by males was counted for 2 min by using a stereoscopic microscope. Tests were conducted at room temperature and ambient humidity. Hexane was used as the control, and the assay was repeated 10 times.

Method 2 was designed to examine whether male mites were able to discriminate females and was carried out as follows: to the same chamber as used for method 1, one male was introduced, and the other mite (female or male) was then introduced with minimum disturbance. Initially, the numbers of chambers where mounting behavior was observed within 14 min were compared between the male or the female introductions. In addition, in the case of responding pairs, the time required for initiating mounting attempts, after introduction, were compared. This assay was repeated 40 times for each male or female introduction.

Data Analysis. The pheromone content of both sexes was analyzed by a Mann-Whitney U test. For method 1, the results were processed to evaluate significant differences in the numbers by a Mann-Whitney U test or a Kruskal-Wallis test followed by Dann's multiple-comparison test. The number of

responding and nonresponding pairs in the two cases in method 2 were statistically examined by a χ^2 test for independence. The mounting attempts initiated after introduction were processed by a Mann-Whitney U test. All values were expressed as the mean ± SEM.

Synthesis. Rosefuran was synthesized by procedures described by Leal et al. (1989b), starting from 3-methylfuran and 4-bromo-2-methyl-2-butene. The ^{1}H NMR ($CDCl_3$) spectral data are as follows: δ 1.70 (d, 3H, J = 1.31 Hz), 1.72 (d, 3H, J = 1.31 Hz), 1.96 (s, 3H), 3.26 (d, 2H, J = 7.11 Hz), 5.26 (triple heptet, 1H, J = 7.11 Hz and 1.31 Hz), 6.15 (d, 1H, J = 1.86 Hz), 7.20 (d, 1H, J = 1.86 Hz).

RESULTS

Identification of Pheromone. When male mites were exposed to a portion of filter paper containing 0.2 FE of the hexane extract, they were sexually aroused and began mounting one another around the introduced paper. The average number of mounting attempts stimulated by 0.2 FE hexane extract was 6.3 ± 0.60 (N = 10), while in control mountings were 3.6 ± 0.37 (N = 10). This represents a significant difference (Mann-Whitney U test, $P < 0.01$).

The GC profile of the hexane extract is shown in Figure 1A. By GC-MS, peak 1 (R_t 5.80 min) gave an M^+ ion and a base ion at m/z 150 (100%) with the following diagnostic ions: 135 (99.9%), 107 (63.7%), 95 (70.2%), 91 (75.4%), 82 (47.8%), 79 (63.9%), 65 (26.0%), 41 (70.7%), and 39 (60.7%) (Figure 1B). Synthetic rosefuran gave a mass spectrum and GC R_t identical to that of peak 1. Peak 2 gave an M^+ ion at m/z 234 (2.0%) and the base ion at m/z 69 (100%) with diagnostic ions at 219 (1.1%), 216 (2.2%), 191 (7.5%), 136 (16.2%), 109 (10.6%), 81 (16.1%), and 41 (41.2%). Thus, we conclude that peak 1 is rosefuran. The structure of peak 2, however, remains unknown. The mounting activity of the extract at 0.2 FE was recovered in the hexane eluate of peak 1. All the other column eluates were found to be inactive as shown in Figure 2.

Evaluation of Synthetic Sex Pheromone. The dose-response relationship with synthetic rosefuran is summarized in Figure 3. Numbers of mounting attempts were 3.8 ± 0.49, 3.6 ± 0.43, 7.4 ± 0.75, 7.9 ± 0.86, 6.8 ± 0.61, and 4.7 ± 0.60 (in each case, N = 10) for doses of synthetic rosefuran in the range 0.01, 0.1, 1, 10, 100, and 1000 ng, respectively. The use of 10 ng ($P < 0.01$), 1 ng, and 100 ng (the latter two both $P < 0.05$) gave significant activity, while levels of 0.01, 0.1, and 1000 ng showed no significant activity compared to the control. As a result, the dose-response curve for activity showed a convex shape as reported for *C. rodriguezi* (Mori et al., 1995) and an unidentified species, *Caloglyphus* sp. MJ (Mori et al., 1996).

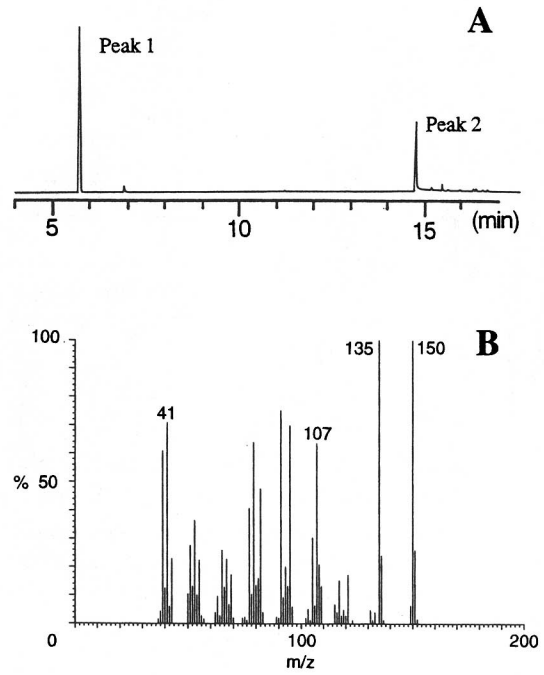

FIG. 1. (A) Typical gas chromatogram of the hexane extract (0.2 FE) from an HP-5 capillary column (30 m × 0.25 mm, programmed from 60 to 290°C at 10°C/min). (B) EI-MS of peak 1 at R_t 5.80 min.

Quantitative Determination of Pheromone. The GC profiles of the hexane extracts obtained from females, males, and protonymphs are shown in Figure 4. The pheromone, rosefuran, was identified as a major component of the hexane extract from both sexes, while only traces were detected in the extract from protonymphs. This species is similar to *A. ovatus* (Kuwahara et al., 1992) and *C. rodriguezi* (Mori et al., 1995) in that the pheromone is mainly present in adult females. The pheromone content was 87.6 ± 14.2 ng ($N = 20$) for females and 10.4 ± 2.5 ng ($N = 20$) for males. This represents a significant difference between sexes (Mann-Whitney U test, $P < 0.01$, $N = 40$).

Ability of Males to Discriminate Between Sexes. The numbers of responding and nonresponding pairs, respectively, in the male introductions in bioassay 2 were 26 and 14, and 34 and 6 for female introductions. These results indicate that the number of mounting attempts between an introduced female and a conditioned male is significantly more than between two males (ψ^2 test, $P < 0.05$). Furthermore, the distribution pattern of lag times required for the initi-

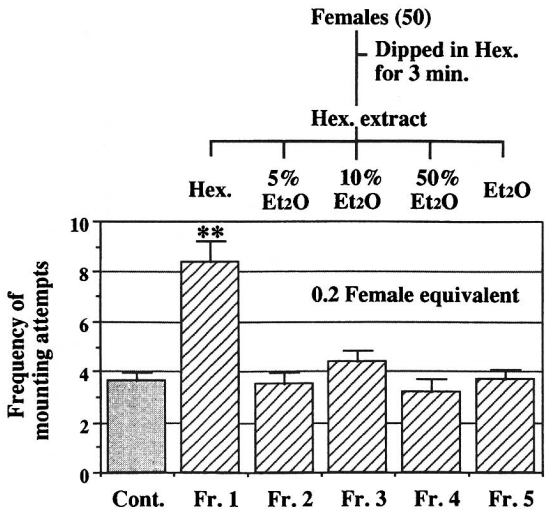

FIG. 2. Bioassay results for the female sex pheromone and the response of *Caloglyphus* sp. HP males to isolated fractions. Fraction 1 is significantly different from the hexane control (Kruskal-Wallis test followed by Dann's multiple-comparison test, $**P < 0.01$). Error bars are standard errors of mean.

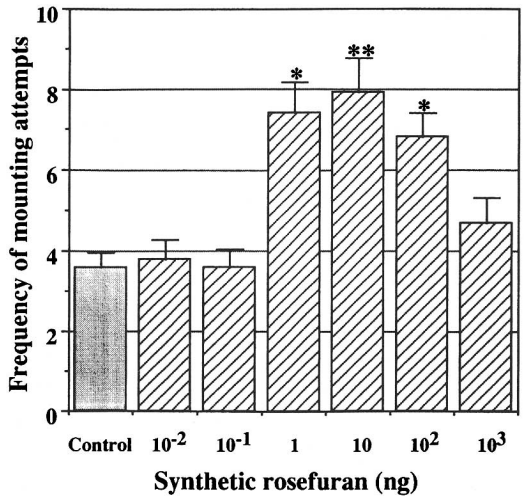

FIG. 3. Dose–responses of mounting attempts by *Caloglyphus* sp. HP males to synthetic rosefuran. Significant differences were determined by Kruskal-Wallis test followed by Dann's multiple-comparison test, $**P < 0.01$, $*P < 0.05$. Error bars are standard errors of mean.

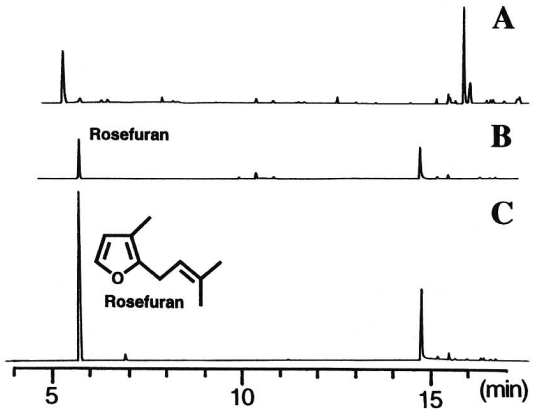

FIG. 4. Typical gas chromatograms of extracts from (A) 2 protonymphal equivalents, (B) 0.2 male equivalents, and (C) 0.2 female equivalents. Chromatograms were obtained with an HP-5 capillary column (30 m × 0.25 mm, programmed from 60 to 290°C at 10°C/min).

ation of mounting attempts showed that the mounting attempts occurred significantly earlier with introduced females than with introduced males (Figure 5). These results indicate that males are able to discriminate a conspecific female from a male.

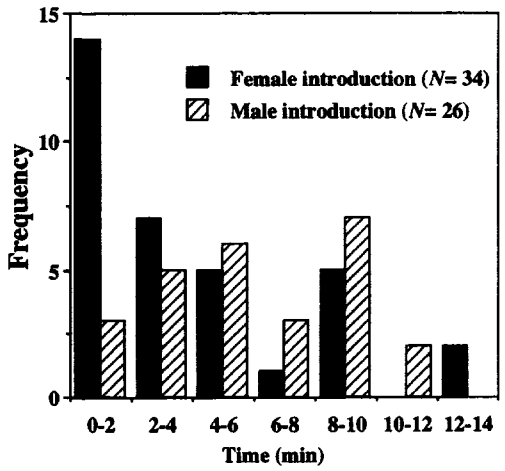

FIG. 5. Frequency distribution of time required for the initiation of mounting attempts in males or females introduce to conditioned males (Mann-Whitney U test, $P < 0.05$, $N = 60$).

DISCUSSION

Secretory components from the opisthonotal gland are species specific, and their GC profiles provide insight into the chemotaxonomy of astigmatid mites (Leal et al., 1989b; Kuwahara et al., 1990; Kuwahara, 1991). Thus far, we have examined the GC profiles for six species of the genus *Caloglyphus*, including *C. polyphyllae* (Leal et al., 1989a), *C. rodriguezi* (Mori et al., 1995), *C. moniezi* (unpublished), and three unidentified species including *Caloglyphus* sp. MJ (Mori et al., 1996, details of the other two unidentified species are unpublished). The present *Caloglyphus* species uses rosefuran as the female sex pheromone and possibly an unidentified compound (M^+: at *m/z* 234). The GC profile is completely different from that of the other six species.

Since the male mites could be sexually aroused by exposure to the 0.2 FE hexane extract and since the sex pheromone content was determined to be 87.6 $\pm$ 14.2 ng/female, this suggests that 17.5 ng of rosefuran is contained in the extract. This value is consistent with the fact that synthetic rosefuran is active at doses of 1–100 ng. Furthermore, sexual behavior stimulated by synthetic rosefuran was the same as that observed when a female was introduced to conditioned males in the assay chamber. Based on these results, we conclude that rosefuran is the sex pheromone of this *Caloglyphus* species. Although males of *C. polyphyllae* (Leal et al., 1989a) or of *Caloglyphus* sp. MJ (Mori et al., 1996) are attracted to the pheromone source in the glass chamber, males of this species were not attracted as reported for the case of *C. rodriguezi* (Mori et al., 1995). We assume that this dissimilarity may be due to differences in volatility of the sex pheromone component.

The female sex pheromone was detected, not only in females, but also in males. However, 8.4-fold (female/male) differences exist in the pheromone content between the sexes. We conclude that this difference could function to aid males in discriminating conspecific females from males, as reported for the case of *C. rodriguezi* (Mori et al., 1995), while males of *Caloglyphus* sp. MJ cannot discriminate conspecific females from males and there is only a 1.4-fold (female/male) difference (Mori et al., 1996). A comparison of the present *Caloglyphus* sp. HP and *C. rodriguezi* with *Caloglyphus* sp. MJ clearly shows that males of the former two species have developed a more efficient strategy to detect females than *Caloglyphus* sp. MJ and that this is accomplished, in part, by localizing the pheromones to females.

The distribution of the female sex pheromone among developmental stages can be categorized into two types. In type 1, the pheromone is present in both sexes and in nymphal stages as a major component, while in type 2, the pheromone is distributed in both sexes, but it is not a major component in nymphal stages. The *Caloglyphus* species studied in this report belongs to type 2. It will be interesting and provide insight into the evolution of sex pheromones in mites

to further define the distribution of female sex pheromones, the ratio of the pheromone between sexes, and male ability to discriminate females among species belonging to the genus *Caloglyphus*.

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INSECT FEEDING STIMULANTS FROM THE LEAF SURFACE OF *Populus*

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Abstract—Leaf surface chemicals from a beetle-preferred poplar clone, Eugenei (*Populus deltoides* × *Populus nigra*), induce feeding in adult cottonwood leaf beetles, *Chrysomela scripta*. The feeding stimulants were isolated and identified as *n*-beheryl alcohol (C_{22}), *n*-lignoceryl alcohol (C_{24}), *n*-hexacosanol (C_{26}), *n*-octacosanol (C_{28}), *n*-triacontanol (C_{30}), and α-tocopherylquinone [2-(3-hydroxy-3,7,11,15-tetramethyl-hexadecyl)-3,5,6-trimethyl-2,5 cyclohexadiene-1,4-dione] (α-TQ). It is the first time that α-TQ has been reported as a feeding stimulant for an insect. Fatty alcohols or α-TQ alone do not induce beetle feeding significantly, but a mixture of alcohols and α-TQ synergistically stimulates beetle feeding. The role of these feeding stimulants in insect feeding behaviors and possible use in a pest management program is discussed.

Key Words—*Populus*, cottonwood leaf beetle, *Chrysomela scripta*, insect feeding stimulant, leaf surface chemicals, *n*-primary alcohols, α-tocopherylquinone.

INTRODUCTION

It is generally accepted that host-plant selection behaviors or feeding preferences of phytophagous insects are largely mediated by the presence and distribution of secondary chemicals in plants. Plant leaf surface chemicals have an important role as mediators of insect–plant interactions because, after being attracted by visual and olfactory cues, insects first contact those compounds present on the

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plant surface. Plant epicuticular waxes have been reported to affect feeding behavior differently for various insects (Städler, 1986; Chapman and Bernays, 1989; Espelie et al., 1991; Eigenbrode and Espelie, 1995). Phytophagous insects usually make a sensory exploration of the leaf surface as a prelude to biting. Insects have a finely tuned sensory capacity to perceive nonpolar substances on the leaf surface (Chapman and Bernays, 1989). Epicuticular lipids have been reported to stimulate insect feeding (Städler, 1986). For example, the surface lipids of the host plant *Poa annua* induce nymphs of *Locusta migratoria* to bite, whereas hydrocarbons stimulate the pea aphid, *Acyrthosiphon pisum*, to feed on an artificial membrane (Eigenbrode and Espelie, 1995). *n*-Hexacosanol and octacosanol in the leaves of the mulberry, *Morus alba*, stimulate feeding in larvae of the silkworm, *Bombyx mori* (Mori, 1982). To some extent, plant surface chemicals may help insects to determine if the plant is suitable as a host.

Several species of chrysomelid beetles also are stimulated to feed by *n*-alkanes and fatty alcohols isolated from the leaf surface of their host plants (Adati and Matsuda, 1993). Similarly, we find that the leaf surface chemicals from *Populus* clones preferred by the cottonwood leaf beetle induce adult feeding. Adults of the cottonwood leaf beetle, *Chrysomela scripta* Fabr. (Coleoptera: Chrysomelidae), show varied feeding preference among poplar clones, *Populus sp.* (Salicaceae), and among leaves of different development stages (Bingaman and Hart, 1992, 1993; Harrell et al., 1981; Caldbeck et al., 1978). We posit that leaf surface chemicals play an important role in host plant selection by the cottonwood leaf beetle. In this report, we characterize the beetle feeding stimulants from the leaf surface of *Populus*.

METHODS AND MATERIALS

Plant Material. A beetle-preferred poplar clone, Eugenei (*P. deltoides* × *P. nigra*), was planted at the Hind's Farm, Ames, Iowa, in summer 1994. During the growing season in both 1994 and 1995, immature leaves were harvested for biological evaluations and characterization of leaf surface feeding stimulants.

Biological Evaluations. Egg masses or larvae of the cottonwood leaf beetle were collected from a plantation near Ames, Iowa, and reared in a growth chamber under a photoperiod of 16L:8D and a temperature regime of 24°C:16°C on greenhouse-grown *P. deltoides* leaves. Adults, at 36–48 hr after eclosion, with no prior feeding experience, were used to evaluate plant residues and chromatographic fractions or authentic standards as described.

Three pairs of adult males and females were exposed to artificial leaf discs in a Petri dish; each disc was made from half pieces of Whatman No. 1 filter paper, 42.5 mm in diameter. Discs were fixed to each wax-coated glass Petri dish (100 × 15 mm) with insect pins. Two layers of towel paper and one layer

of Whatman No. 1 filter paper (7 cm diameter), completely soaked with deionized water, were used to keep the humidity high in each Petri dish. For each dish, two discs were treated with plant residue (21.1 $\mu g/cm^2$) or chromatographic fraction (11.2 or 8.5 $\mu g/cm^2$) and positioned alternately with two discs treated with solvent control (chloroform) for biological activity screening tests. Assays were conducted in the dark for 24 hr. The dark condition was used to avoid any possible visual cues. Three replications, each with three pairs of beetles in a Petri dish, were run for each test. Bite marks were counted for each test. Each beetle was used for only one test and then discarded.

Artificial leaf discs containing one of the following four treatments were prepared: (1) 50 μg *n*-beheryl alcohol (C_{22}) (Sigma), (2) 5 μg synthetic α-TQ, (3) a mixture of 50 μg *n*-beheryl alcohol and 5 μg α-TQ, and (4) the solvent control (chloroform). Four leaf discs, one with each treatment, were placed in a Petri dish. Three pairs of beetles were added to the dish, which was then placed in the incubator. The assay was replicated 70 times under the same conditions as those for the screening tests. The bite marks were counted and evaluated by analysis of variance (ANOVA) and *t* tests. Responders were tested with χ^2 (SAS, 1985).

Reference Compounds. Synthetic α-TQ and *n*-beheryl alcohol (C_{22}), *n*-lignoceryl alcohol (C_{24}), *n*-hexacosanol (C_{26}), *n*-octacosanol (C_{28}), and *n*-triacontanol (C_{30}) (Sigma) were used as chromatographic standards and in biological evaluations. α-TQ was prepared from *d*-α-tocopherol (Issidorides, 1951; Weng and Gordon, 1993), and the acetates of authentic alcohols and alcohols from the plant were prepared by using acetate anhydride.

Instrumentation and General Methods. Intact fresh leaves were dipped into hexane for 30 sec at room temperature and the hexane extract was filtered. The filtrate was concentrated under reduced pressure at 35°C, and 5.1 g of the concentrated extract was subjected to open-column chromatography on 200 g Florisil (60–100 mesh, Fisher) that was deactivated with 7% water by weight. Solvents (1 liter for each solvent) used to elute materials from the column were hexane, benzene, chloroform, and methanol (Figure 1 below). The fraction that eluted with benzene retained the biological activity. Then, 1.98 g of the benzene fraction concentrated material was applied on 200 g Florisil and eluted with an ascending series of 20% (800 ml), 25% (800 ml), 33% (600 ml), and 50% (800 ml) chloroform in hexane. The fraction that eluted with 50% chloroform in hexane held the activity. This fraction was purified further by applying 40 mg on 10 g Florisil and eluting with chloroform (total of 80 ml; 10 ml for one fraction, fractions 3–7 were combined). The active fraction in chloroform (14.5 mg) from open column chromatography was applied on TLC silica gel plates (Merck 60 F254, 250 μm thick, 20 × 20 cm), that were developed by solvent system I (hexane–diethylether–formic acid, 50:50:0.5) followed with solvent system II (chloroform). Fractions were scraped from the plate and desorbed with chloroform. The procedure was repeated until 1.2 mg of α-TQ was obtained.

Gas Chromatography–Mass Spectrometry (GC-MS). A sample of TLC fractions possessing biological activity was injected on a 30-m × 0.25-mm-ID, 0.25-μm film thickness, DB-5, 5% phenyl methyl silicone column (J&W Associates) in an HP 5890 series II gas chromatograph. Gas flow rate for He was 1.3 ml/min; the temperature program was 100°C for 1 min, programmed to 295°C at 10°C/min, and maintained at 295°C for 10 min. The flame ionization detector temperature was 300°C, the injector temperature 250°C, and the split ratio 100:1.

GC-MS data were recorded from an HP 5890 series II gas chromatograph coupled with an HP 5972 mass spectrometer operated at 70 eV. Chromatography was performed on a DB-1 column, temperature: 100°C (0 min), 10°C/min, 325°C. Helium was the carrier gas at 1 ml/min. MS data also were recorded on a Magnum GC-MS with a 30-m, 0.25-mm ID, 0.25-μm film thickness DB-5 column with He as the carrier gas. The temperature program was 100°C for 1 min, 15°C/min to 298°C, and held 15 min.

Fourier transformed-infrared spectrometry (FT-IR). An FT-IR spectrum of the active fractions was obtained on a Buker IR 98 spectrophotometer with samples on a NaCl crystal, and the evaluation was between 4000 cm^{-1} and 500 cm^{-1}. UV spectra were determined in absolute ethanol with a UV 160u Shimadza UV/Vis spectrophotometer, scan range 200–400 nm. A 300-MHz ^1H NMR was recorded in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal standard with a Varian Vax 300 MHz NMR.

RESULTS

The yield of leaf surface wax varied through the growing season and ranged from 10 to 20 $\mu g/cm^2$ leaf area. The extract applied at 21.2 $\mu g/cm^2$ on the filter paper induced adult beetle feeding behavior, whereas the solvent chloroform did not. The protocol outlined in Figure 1 was used to isolate and identify the components with feeding stimulant activity. After open-column chromatography and TLC, an active fraction was obtained that contained a UV (254 nm) quenching band and a band with no UV absorption, suggesting the presence of at least two classes of compounds.

Characterization of Active Components. The compound with no UV absorption had a TLC $R_f = 0.58$ in solvent system I and $R_f = 0.32$ in system II. The purified compounds were colorless crystals in chloroform. IR absorption at 3300, 2920, 2850, 1465, and 720 (cm^{-1}) indicated that the compounds were alcohols. Analysis by GC showed that five compounds were present and they coeluted with C_{22}, C_{24}, C_{26}, C_{28} and C_{30} primary alcohols (Sigma). The acetates of the alcohols from plants also separated into five peaks that coeluted with the acetates of C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} primary alcohols. Final confirmation of

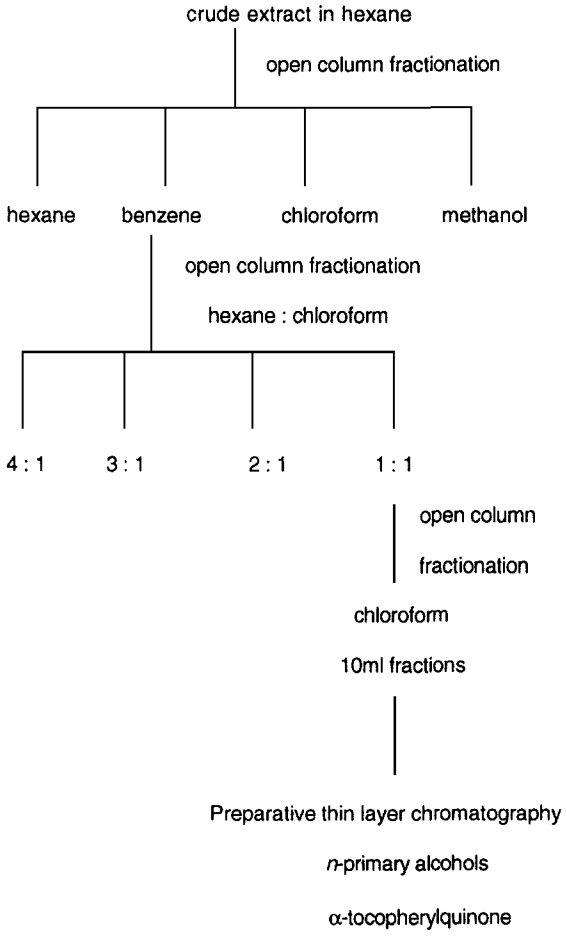

FIG. 1. The separation and purification scheme for the beetle feeding stimulants.

the five primary long-chain alcohols was by GC-MS. The mass fragmentation patterns of the plant alcohols and their corresponding acetates were identical with those of authentic long-chain alcohols and their acetates.

The band that quenched UV (254 nm) on the TLC had $R_f = 0.58$ in solvent system I and $R_f = 0.18$ in system II. The pure compound was a lemon-yellow oil and was identified as α-TQ by its UV, IR, GC-MS, and ^{1}H NMR spectra and confirmed by comparison with the synthetic α-TQ.

Ultraviolet (nm): 203, 262, 268. Infrared (cm^{-1}): 3522.7, 2952.8, 2925.2, 2851.5, 1682.7, 1643.8, 1463.4, 1375.8, 1307.2, 714.8. GC-MS (*m/z*): 447

(M+1, 1.3), 446 (M, 4.3), 431 (2.1), 430 (3.2), 428 (5.1), 221 (100), 203 (11.7), 180 (25.3), 179 (21.1), 178 (73.6), 165 (22.4), 150 (27.2), 135 (9.6). ^{1}H NMR (δ ppm): all proton signals were between 0.80 and 2.60.

Biological Activities. α-TQ (56.3 ng/cm^2) from the plant together with (8.45 μg/cm^2) alcohols from the plant at a ratio of 1:150 showed high feeding-stimulant activity; the mean $\pm$SE (N = 3) number of bite marks was 74 $\pm$ 17. Activity decreased with higher or lower amounts of α-TQ with a fixed amount of alcohols. At ratios of 1:60, 1:100, and 1:200, the mean numbers of bite marks were 10 $\pm$ 5, 11 $\pm$ 6, and 1 $\pm$ 1, respectively.

Any one of the commercially available C_{22}, C_{24}, C_{26}, C_{28}, or C_{30} primary alcohols, mixed with synthetic α-TQ, stimulated beetle biting activity. Bioassays with C_{22} and synthetic α-TQ showed that the beetles had varied responses to the chemicals, but a significantly higher percentage of beetles responded to a mixture of C_{22} alcohol and α-TQ than to either compound alone (Figure 2). Neither C_{22} alcohol nor α-TQ alone was significantly different from the control in inducing biting behavior, but a mixture of both compounds elicited a significantly higher number of bite marks than the control or either of the compounds alone (Figure 3). These observations confirm the synergistic feeding-stimulant activity of the plant natural products.

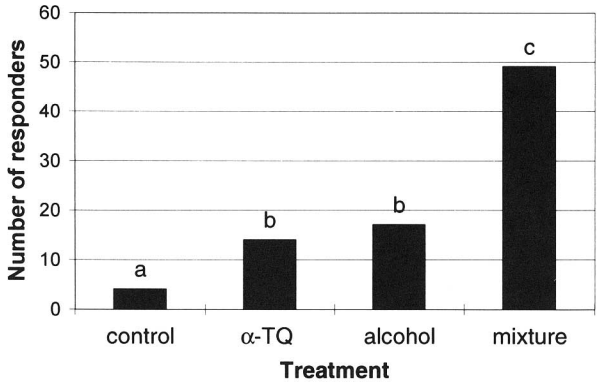

FIG. 2. Number of beetles responding to four treatments (control, α-TQ, C_{22} alcohol, and α-TQ plus C_{22} alcohol) in 24 hr. Beetles were categorized as responders when bite marks were $\geq$ 10 and as nonresponders when the bite marks were $<$ 10 in one replication. Values with different letters indicate percentages (N = 70) that are significantly different (α = 0.01) (χ^2 = 25.0 (control vs. α-TQ), 42.3 (control vs alcohol), 506.3 (control vs. mixture), 0.6 (α-TQ vs. alcohol), 87.5 (α-TQ vs. mixture), 60.2 (alcohol vs. mixture); df = 1).

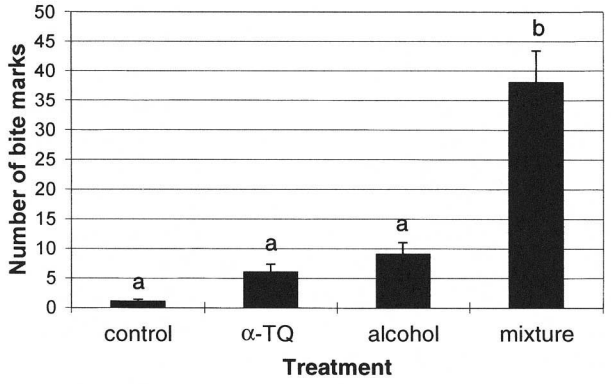

FIG. 3. Mean number of bite marks on discs (N = 70) with four treatments (control, α-TQ, C_{22} alcohol, α-TQ plus C_{22} alcohol) in 24 hr. The mean number of bite marks on disks is significantly different (F = 38.43, $P < 0.001$). Error bars are SE. Values with different letters indicate means that are significantly different (α = 0.01), as determined by t tests.

DISCUSSION

Long-chain fatty alcohols are found commonly as components in plant epicuticular lipids (Walton, 1990). The fatty alcohols stimulate feeding in larvae of the silkworm *Bombyx mori* (Mori, 1982), in the chrysomelid beetle *Chrysolina anrichalcea* (Adati and Matsuda, 1993), and the chrysomelid beetle *C. scripta* in our study. α-TQ, which has not been identified previously from the poplar leaf surface, acts as a feeding synergist for *C. scripta* when combined with the fatty alcohols from the epicuticular lipids of *Populus*. Seemingly, *C. scripta* recognizes at least two classes of poplar natural products and uses a blend of these compounds to evaluate the host plant suitability during gustation. To our knowledge, it is the first time that α-TQ has been reported as an insect feeding stimulant.

The main composition of the fatty alcohols from Eugenei are C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} primary alcohols. The beetles respond to any one of them when mixed with α-TQ. Data from the bioassays indicate the possibility that the beetles can differentiate among the aliphatic alcohols and that not all concentrations and ratios of alcohols and α-TQ induce strong feeding activity. More tests are in progress to evaluate the optimum ratio or the optimum concentration of these compounds. We speculate that poplar clones with a high concentration of fatty alcohols and α-TQ, and at an appropriate ratio for optimum gustatory stimulation, are susceptible to heavy attack by the cottonwood leaf beetle.

Thirty percent of the beetles did not respond to the mixture of C_{22} alcohol and α-TQ. This may indicate that the beetles have genetic differences in gustatory responses or that physiological regulation made the beetles unresponsive to the chemicals. It is also likely that there may be other still unidentified feeding stimulants in *Populus* leaf surface extracts. In bioassays in which synthetic α-TQ was used either with authentic primary alcohols or with plant-extracted alcohols to induce beetle feeding, a higher concentration of synthetic than natural α-TQ was always needed. The GC profile of the plant α-TQ fraction from the TLC purification step showed that, besides α-TQ, there were several compounds in much smaller concentrations in addition to α-TQ that had GC retention times close to α-TQ. Some of these minor compounds may be analogs of α-TQ and also may have some stimulating effect on beetle feeding. Lack of these compounds in the synthetic material, or differences between stereoisomerism of synthetic α-TQ and naturally occurring α-TQ, may account for the observed lower feeding responses.

The yield of wax from poplar varied during the growing season, although we do not know how the composition changes for the leaf surface feeding stimulants. Plant leaf surface chemicals can vary in quality and quantity among different plant tissues, during maturation, and because of fluctuating environmental conditions (Städler, 1986; Walton, 1990). Cottonwood leaf beetle adults feed on the adaxial surfaces of leaves and prefer immature leaves (Bingaman and Hart, 1992); therefore, these leaves probably have a high concentration of alcohols and α-TQ. Whether the chemicals have a stimulating effect on larval feeding has not yet been tested. Evaluating how leaf surface chemicals are distributed in paplar plants and their turnover dynamics will assist in understanding the relationship between poplar leaf surface chemicals and beetle feeding behavior.

α-TQ occurs widely in photosynthetic organisms, in animal tissues, and in bacteria. Metabolic functions have been ascribed to it, including participation in photosynthetic electron transport and antioxidative pathways. Many investigators have suggested that plant α-TQ is limited to the chloroplasts and that the content of α-TQ is dependent on light intensity, tissue age, and other factors (Kruk and Strzalka, 1995). The extraction method of the leaf surface chemicals in this study makes us confident that the α-TQ was from the leaf surface, showing that the distribution of α-TQ in plant tissues varies and may depend on genetics and environmental conditions.

Populus selections have been developed as preferred choices for short-rotation woody crop plantings throughout much of the world because of desirable characteristics such as rapid juvenile growth and biomass production, ease of vegetative propagation, ease of tissue and cell culture propagation, ease of breeding, large species diversity, availability of unmanaged natural populations, and

excellent growth on a wide range of sites (Dickmann and Stuart, 1983; Ceulemans, 1990). The cottonwood leaf beetle is one of the potential limiting factors for poplar plantations in North America (Wilson, 1976; Brown, 1956; Burkot and Benjamin, 1979). The phagostimulants existing on the leaf surface of the host poplar leaves and their variation may regulate beetle feeding behavior and can be used as an important tool in the management of *C. scripta*. Through breeding, selection, or genetic transformation, it may be possible to reduce or remove the feeding stimulants from the leaf surfaces and thus improve poplar resistance to the cottonwood leaf beetle.

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CHEMICAL ECOLOGY OF COTTONWOOD LEAF BEETLE ADULT FEEDING PREFERENCES ON *Populus*

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Abstract—The effect of select *Populus* clones on adult cottonwood leaf beetle feeding preferences was examined. Field-planted University of Washington poplar pedigree materials—parent clones ILL-129 (*Populus deltoides*) and 93-968 (*Populus trichocarpa*), F_1 clones 53-242 and 53-246, and 87 F_2 selections—were used. Both field cage feeding tests with parent and F_1 clones, and leaf disc feeding tests with all 91 clones were performed. Feeding stimulants on the leaf surface, long-chain fatty alcohols and α-tocopherylquinone (α-TQ), and phenolic glycosides, tremulacin and salicortin, were analyzed to correlate chemical abundance with cottonwood leaf beetle *Chrysomela scripta* adult feeding preference. The beetles showed varied feeding preferences among parent clones, F_1 clones, and F_2 clones. Contents of alcohols, tremulacin, and salicortin did not explain adult beetle feeding preference. Content of α-TQ on the leaf surface did explain the adult beetle feeding preference. The beetles preferred to feed on clones with α-TQ rather than clones without α-TQ. As the amount of α-TQ increased, the feeding preference increased, and then decreased as the amount of α-TQ increased further.

Key Words—*Populus*, cottonwood leaf beetle, *Chrysomela scripta* Fabr., insect feeding stimulants, leaf surface chemicals, long-chain fatty alcohols, α-tocopherylquinone, phenolic glycosides, tremulacin, salicortin.

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INTRODUCTION

The cottonwood leaf beetle, *Chrysomela scripta* Fabr. (Coleoptera: Chrysomelidae), is a major defoliating pest of *Populus* in North America (Burkot and Benjamin, 1979; Wilson, 1979; Harrell et al., 1981). Both adults and larvae feed on young leaves. The adult stage is highly mobile and well adapted for the wide dispersal of the species and for host-plant selection. The beetle usually rejects species or clones from the Leuce section and shows a preference for clones containing Aigeiros and Tacamahaca parentage (Caldbeck et al., 1978; Harrell et al., 1981; Bingaman and Hart, 1992). The beetle also discriminates between leaf age classes, preferring to feed on young leaves (LPI 3–5) over mature leaves (Harrell et al., 1981; Bingaman and Hart, 1992). No correlation between beetle preference and poplar leaf nitrogen levels, carbohydrate levels, leaf thickness, toughness, or surface physical characteristics is known to occur (Harrell et al., 1981, 1982).

In Salicaceae, *Salix* spp. and *Populus* spp., phenolic glycosides are family-specific secondary compounds (Palo, 1984), well known to influence the susceptibility of plants to both insect and mammalian herbivory (Rowell-Rahier and Pasteels, 1982; Smiley et al., 1985; Rowell-Rahier et al., 1987; Denno et al., 1990; Lindroth, 1991; Kelly and Curry, 1991; Rank, 1992, 1994; Matsuki and Maclean, 1994; Kolehmainen et al., 1994, 1995). Salicin and salicortin content do not negatively influence cottonwood leaf beetle host selection, but tremulacin may (Bingaman and Hart, 1993). However, no clear and direct correlation between these phenolic glycosides and feeding or oviposition preference has been established.

Numerous studies have shown that plant surface chemicals are involved in enhancing or deterring insect movement, oviposition, and feeding and also affect herbivores indirectly by influencing predatory and parasitic insects. In most cases, the behaviorally active components are common lipophilic compounds (Städler, 1986; Woodhead and Chapman, 1986; Chapman and Bernays, 1989; Espelie et al., 1991; Städler and Roessingh, 1991; Eigenbrode and Espelie, 1995). Wax components of host plant leaves stimulate some chrysomelid beetles to feed (Adati and Matsuda, 1993). We found, however, that long-chain fatty alcohols and α-tocopherylquinone (α-TQ) on the leaf surface of a preferred *Populus* clone Eugenei (*Populus deltoides* × *Populus nigra*) stimulate adult cottonwood leaf beetle adult feeding behavior (Lin et al., 1998).

In this study, we investigated whether levels of long-chain fatty alcohols and α-TQ among poplar plants explained the adult cottonwood leaf beetle feeding preference among trees. Moreover, we further elucidated the role of phenolic glycosides on host-plant selection. By using University of Washington poplar pedigree material, we evaluated the effects of phenolic glucosides, fatty alcohols, and α-TQ on the cottonwood leaf beetle adult feeding preference.

METHODS AND MATERIALS

Plants. We tested for variation in phenolics and leaf surface chemicals (α-TQ and long-chain fatty alcohols), as well as cottonwood leaf beetle feeding preferences, among clones in a poplar pedigree generated by the University of Washington (Bradshaw and Stettler, 1993). This pedigree material was chosen because information generated by our work could be submitted for inclusion by the University of Washington poplar genome study. The pedigree materials used included: two parent clones, 93-968 (*Populus trichocarpa*) and ILL-129 (*Populus deltoides*); two F_1 generation clones, 53-246 and 53-242; and 87 F_2 generation selections.

Trees were grown from softwood cuttings taken from greenhouse stock plants. The cuttings were dipped in 500 ppm indolebutyric acid for 5 sec and planted in saturated peat pellets under mist until roots formed. Plants then were transplanted into pots containing vermiculite, perlite, and milled peat (1:1:1) (Faltonson et al., 1983). In May 1994, the two parent and two F_1 clones were planted at the Iowa State University (ISU) Hind's Research Farm, in Ames, Iowa, for field cage feeding tests. Four plants were planted as a group, with one of each of the four clones planted randomly within the group; 12 such groups were planted. Spacing was 2 m between each group and 1 m between each plant within a group. In April 1995, all 91 clones were planted in the field at the ISU Institute for Physical Research and Technology in Ames, Iowa, for leaf disc feeding tests. These trees were planted in eight blocks; within each block, one of each of the 91 clones was planted randomly with interplant spaces of 1 m within the row and 3 m between the rows.

Insects. For each experiment, cottonwood leaf beetle larvae were collected from a plantation 10 mi southeast of Ames, Iowa, and reared in a growth chamber under a photoperiod of 16L:8D and a temperature regime of 24°C:16°C on greenhouse-grown *P. deltoides* leaves. Previously, we found that larval feeding experience did not affect beetle feeding preference (unpublished data). Adults were used in all bioassays, 36–48 hr after eclosion and with no prior feeding experience.

Bioassays. For field cage feeding preference tests, the parent and F_1 clones planted at the ISU Hind's Research Farm were used. In September 1994, three groups of four trees were selected. The four trees in each group were covered with a cage that was 2 × 2 m. Five pairs of male and female beetles were released into the center of each cage. After four days, all leaves with feeding damage were collected and the leaf area consumed from each plant was measured with an area meter in the laboratory.

A leaf disc feeding assay was used to test beetle feeding preferences among the 91 clones. Plants from three field blocks were used; each field block was considered a replicate. For each block, branches with active terminal growth

were collected from each tree, placed on ice, and transferred to the laboratory. Leaf discs, 224 mm^2 (22.7 mm diameter), were punched from leaves LPI 3–5 with a No. 12 cork borer. An incomplete Latin-square design was used (Cochran and Cox, 1957). Each test chamber consisted of one plastic Petri dish (100 × 15 mm) with two layers of paper towel moistened with distilled water, and 10 leaf discs, each from a different clone, were placed in a circle around the perimeter of each petri dish. A total of 91 dishes were used per block and leaf discs from each clone appeared in 10 of these 91 Petri dishes. Five pairs of beetles were released in the center of each Petri dish. Dishes were placed in a growth chamber under the same conditions as described. After 24 hr, the leaf area consumed from each disc was measured with an area meter. For all three field blocks, the bioassays were finished within eight days, allowing two days for each field block.

Chemical Analysis. Leaves for bioassay and chemical analyses were collected at the same time. Leaves, LPI 3–5, were chemically analyzed immediately following collection. For the parent and F_1 clones used in the field cage feeding tests, leaves were collected from four trees of the same clone and mixed together, then two leaf samples were used for chemical analysis. For the clones used in leaf disc feeding tests, leaves LPI 3–5 from each individual tree were used for chemical analysis.

Fatty alcohols and α-TQ levels were analyzed by GC after solid-phase extraction. Erucyl alcohol (*cis*-13-docosen-1-ol) (Sigma), ca. 99% by capillary GC, was used as an internal standard (IS). Briefly, 300–600 cm^2 intact fresh leaf material was dipped in 250 ml hexane at room temperature for 30 sec. Each sample was filtered with Whatman No. 1 filter paper and dried under a stream of nitrogen gas. The plant extract (ca. 10 mg), along with 5 μg of erucyl alcohol, was dissolved in 220 μl 5% ethyl ether in hexane. The sample and IS were washed onto ca. 335 mg Supelclean LC-Florisil (Supelco) solid phase in a 15-cm-long disposable flint Pasteur glass pipet (Fisher). The feeding stimulants and minor impurities were eluted from the solid phase with 4 ml of 30% ethyl ether in hexane (Figure 1). The eluant was dried under a stream of nitrogen gas. The residue was dissolved in 10 μl chloroform. A 1-μl aliquot was injected on a 30-m × 0.25 mm-ID, 0.25-μm film thickness, DB-5, 5% phenyl methyl silicone column (J&W Associates) on a HP 5890 series II gas chromatograph. Gas flow rate for He: 1.3 ml/min; temperature program: 100°C for 1 min, then to 295°C at 10°C for 15 min; flame ionization detector: detector temperature, 300°C, injector temperature 250°C; split ratio 100:1. Standard curves were determined for erucyl alcohol, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} alcohols (Sigma), and synthetic α-TQ (Lin et al., 1998). The Gc retention times relative to erucyl alcohol were used to identify the feeding stimulants. The integrated area of each feeding stimulant was compared to its standard curve to determine the amount in the

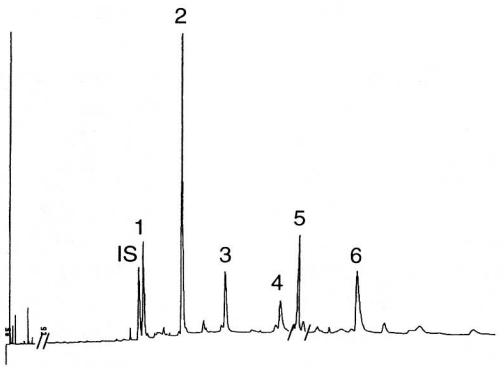

FIG. 1. GC profile for leaf surface feeding stimulants. IS, internal standard. 1, C_{22}-OH. 2, C_{24}-OH. 3, C_{26}-OH, 4, C_{28}-OH. 5, α-TQ. 6, C_{30}-OH. The attenuation was changed from $2^{\wedge} = 1$ to $2^{\wedge} = -3$ between the second slashes.

sample injection. The amount of the stimulant was adjusted to match the recovery of erucyl alcohol from the solid phase for each injection. The amount of each stimulant was then calculated for a 10-mg leaf-wax sample. Finally, the concentration (nanograms per leaf area in square centimeters) was obtained according to the leaf area used to get the 10-mg leaf-wax sample.

The phenolic glycosides were analyzed by following their UV absorption at 220 nm. Fresh leaves, ready for extraction after they were cut at the petiole, were placed on ice, transported to the laboratory, and vacuum-dried (Orians, 1995). The dry leaves were pulverized in a plastic bag and the leaf powder (25 mg) was sonicated (Sonicor ultrosonic tank, Whatman) in 1 ml ice cold MeOH for 15 min. The sample was centrifuged (5000*g*, 6 min) and filtered through cotton to remove particles, and 15 μl of the extract was applied to analytical grade silica gel plates (Merck 60 F254, 250-μm thick, 10 $\times$ 10 cm). Two-dimensional TLC (solvent I: CH_2Cl_2–MEOH, 80:20; and solvent II: CH_2Cl_2–MeOH–THF, 60:10:10) was used to separate the phenolic glycosides. The chemicals were then desorbed from the silica in 1 ml ethanol, filtered, and measured at 220 nm. Standard curves were determined for tremulacin and salicortin. To determine the recoveries for tremulacin and salicortin from TLC plates, 15 μl of 0.5 mg/ml tremulacin and salicortin standards in MeOH were applied to TLC plates. After two-dimensional development, the chemicals were desorbed in 1 ml ethanol, filtered, and measured at 220 nm ($N = 3$). The concentrations were determined by using the standard curve, and recoveries were calculated for tremulacin (0.73) and salicortin (0.61). The amounts of tremulacin and salicortin in the plant samples were calculated according to the standard

curves, modified by the overall recovery, and expressed as micrograms per milligram of leaf dry weight.

Isolation of Phenolic Glycoside Standards. To obtain tremulacin and salicortin, tips of twigs with immature leaves attached were collected from a wild *Populus alba* hybrid, put on ice, transported to the laboratory, and vacuum-dried. Dried leaves and twigs (490 g) were powdered and extracted in cold MeOH with sonication for 20 min. After filtration, the extract was concentrated at 35°C. A hexane wash was used to eliminate lipid materials from the residue. The method of Picard et al. (1994) was used to recover the crude phenolic glycoside extract. The residue (45.5 g) was dissolved in 900 ml H_2O and 800 ml 40% $(NH_4)_2SO_4$. Undissolved material was filtered away with Whatman No. 4 filter paper. The solution was partitioned with three volumes of hexane, and the water phase was extracted with four volumes of ethyl acetate. The ethyl acetate extract was dried with $MgSO_4$ and concentrated. Nine grams of phenolic glycoside crude extract were recovered. Vacuum liquid column chromatography on silica gel (10–40 μm, Sigma) was used to separate the phenolic glycosides. An ascending series of MeOH in $CHCl_3$ (0, 10%, 20%, and 30%) was used to elute the material. Preparative TLC (Merck 60 F254, 250 μm thick) was used to purify tremulacin and salicortin. The solvent system $CHCl_3$–MeOH (80:20), was used for tremulacin purification, and $CHCl_3$–Me_2CO–MeOH (70:15:15), was used for salicortin. Chemicals from TLC were desorbed in acetone. ^1H NMR spectra ($CDCl_3$, 400 MHz) of both tremulacin and salicortin were identical to those reported by Lindroth et al. (1987).

Statistical Analysis. The percentage of leaf area consumed among parent and F_1 clones in field cage feeding tests was evaluated by ANOVA and *t* tests. Leaf area consumed in leaf disc feeding tests for 91 clones was evaluated by GLM. Beetle feeding activity and chemical variations among individuals of the same clones planted in the three field blocks were evaluated by correlation analysis (SAS, 1985). The correlations between the feeding preference and chemical contents were analyzed by using data from each of the three blocks instead of using the average data of the three blocks.

RESULTS

Parent and F_1 Clones. The field cage feeding test results showed that the beetles easily distinguished among the four clones. The order of preference for consumption was 53-242, 53-246, ILL-129, and 93-968. The percentage of leaf area consumed was 59.0 ± 1.9, 31.2 ± 1.4, 8.2 ± 1.1, and 1.6 ± 1.6, respectively ($F = 286.2$; $df = 3, 8$; $P < 0.001$).

The leaf content of alcohol and α-TQ varied among the four clones (Table 1). Parent clone ILL-129 had the highest content of alcohol on the leaf surface.

TABLE 1. CONTENT OF SECONDARY PLANT COMPOUNDS IN PARENT AND F_1 CLONES[a]

Clone	Feeding stimulants (ng/cm^2)		Phenolic glycosides (μg/mg)	
	Alcohol	α-TQ	Tremulacin	Salicortin
53-242 (F_1)	1370.7	5.4	17.07	42.31
53-246 (F_1)	1429.8	2.5	12.87	34.90
ILL-129 (P)	1772.7	17.2	12.79	21.23
93-968 (P)	1187.9	not detected	14.19	40.53

[a] Detection limit = 0.2 ng.

The two F_1 clones, 53-242 and 53-246, had alcohol contents between those of the parents. Clone ILL-129 had a high content of α-TQ on the leaf surface, while α-TQ could not be detected in the leaf surface of clone 93-968. The two F_1 clones had α-TQ content between those of the two parents. The content of tremulacin and salicortin in F_1 clones and parents was variable (Table 1). The chemical variation pattern, however, did not correspond to the feeding preference pattern.

Parents, F_1, and F_2 Clones. The beetle showed significantly different feeding responses to the 91 clones for all three blocks ($F = 5.21$ for block 1, 10.41 for block 2, 4.42 for block 3; $df = 90, 819$ for all blocks; $P < 0.001$ for all blocks). Average leaf area consumed ranged from 18.4 ± 8.8 to 210.8 ± 16.3 mm^2 in block one, 0.3 ± 0.9 to 208.6 ± 10.1 mm^2 in block two, and 6.6 ± 7.5 to 193.3 ± 29.4 mm^2 in block three.

Only 56 of the 87 F_2 clones planted produced sufficient foliage for chemical analyses. The correlation coefficients were low for beetle feeding activity measured by the average ($N = 10$) leaf area consumed, for the content of α-TQ on the leaf surface, and for the tremulacin content per leaf dry weight, although the coefficients were relatively higher for the amount of alcohol on the leaf surface and salicortin per leaf dry weight (Table 2). Both beetle feeding responses and the chemical content, especially α-TQ and tremulacin, were highly variable among individuals within each clone.

Leaf surface alcohol content was not correlated with beetle feeding. The beetles preferred clones with α-TQ over clones without α-TQ. Moreover, as the amount of α-TQ increased to approximately 2.5 ng/cm^2, the beetles consumed more leaf area. When α-TQ levels increased further, however, consumption decreased (Figure 2).

Leaf content of tremulacin and salicortin was not correlated with leaf area consumed by the beetles. Although contents of tremulacin and salicortin varied

TABLE 2. PEARSON CORRELATION COEFFICIENT BETWEEN BLOCKS

	Block 1 vs. block 2	Block 1 vs. block 3	Block 2 vs. block 3
Feeding activity	0.10	0.19	0.43
Alcohol	0.66	0.54	0.66
α-TQ	0.35	0.25	0.45
Tremulacin	0.40	0.42	0.53
Salicortin	0.53	0.62	0.65

both between and within clones, especially for tremulacin, the variation did not explain beetle feeding preference differences.

DISCUSSION

A combination of leaf surface long-chain fatty alcohols and α-TQ stimulate adult beetle feeding behavior (Lin et al., 1998). Our field cage and leaf disc feeding tests also showed that the beetles preferred to feed on clones with moderate levels of α-TQ (1.00–5.00 ng/cm^2). In both the field cage and leaf disc feeding tests, beetles preferred to feed on poplar plants with α-TQ and long-chain fatty alcohols both present on the leaf surface. Beetles preferred trees with moderate α-TQ content. The variation of α-TQ content on the leaf surface

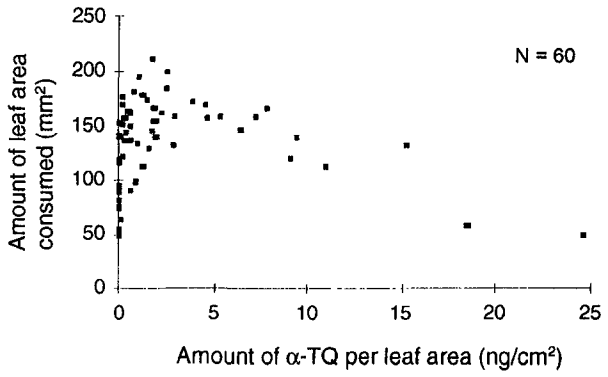

FIG. 2. Relationship of amount of leaf area consumed (N = 10) and α-TQ on leaf surface (N = 60) for data from block 1. The same relationship was found for data from blocks 2 and 3.

was correlated with cottonwood leaf beetle adult feeding preference among poplar plants.

In the cottonwood leaf beetle, oviposition preference patterns are known to correspond with feeding preference patterns, both among clones and leaf ages (Bingaman and Hart, 1992). In our leaf disc bioassays, gravid female feeding preference was based on stimulant content, but oviposition was random on all the discs and on the Petri dish. Such oviposition behavior is typical for this species when confined in a restricted environment and does not reflect oviposition preference in the field (unpublished data). As with the willow-feeding leaf beetle, *Chrysomela aeneicollis* (Rank, 1992), the cottonwood leaf beetle probably locates favorable hosts through acceptance or rejection by feeding adults. Adult feeding activity, therefore, may be important in determining the beetle abundance in the field.

Current practices for population suppression of cottonwood leaf beetle rely heavily on the use of insecticides. With the knowledge that the beetles prefer to feed on poplar plants with α-TQ and long-chain fatty alcohols together on the leaf surface, we may be able to find alternative ways to manage the beetle. In both the field and the laboratory, we found that adults exhibited aggregation behavior (unpublished data). Beetle feeding stimulants, α-TQ and long-chain alcohols, together with knowledge of the beetle aggregation behavior, may allow us to develop an effective bait for the beetle, as has been done for diabroticite beetles (Metcalf and Metcalf, 1992). The poplar trees with optimum α-TQ and long-chain alcohol content could also be used as a trap crop for the beetle. Selecting and breeding poplar clones without α-TQ on the leaf surface, or using molecular biology tools to block secretion of α-TQ onto the leaf surface, could increase poplar natural resistance to the cottonwood leaf beetle.

Larvae of the cottonwood leaf beetle use salicin and its derivatives from their host plants to make their own defensive chemicals against generalist predators (Wallace and Blum, 1969; Pasteels et al., 1989). In some willow-feeding, salicin-using leaf beetles, the phenolic glucosides influence beetle feeding behavior (Kolehmainen et al., 1995; Rank, 1992). In our observation and in the study of Bingaman and Hart (1993), it seems that for the cottonwood leaf beetle, phenolic glycosides do not influence adult feeding behavior to any extent. They may have some subtle effects on oviposition behavior, and most probably much more significant effects on tritrophic relationships, such as offering chemical protection from predators and pathogens (Wallace and Blum, 1969; Pasteels et al., 1989).

Fatty alcohols are very common leaf surface wax components (Walton, 1990), and they are abundant on the poplar leaf surface. α-TQ is a commonly occurring phytochemical (Kruk and Strzalka, 1995), and in poplar clones it occurs in the leaf surface wax matrix. The alcohol content, however, is less variable than α-TQ among individuals of the same clones. In the summer of

1996, when our poplar plantation was affected by flooding, different clones and individuals of the same clones were subjected to varied microenvironmental conditions. These may have significantly affected α-TQ content of the leaf surface of some clones and changed susceptibility to the beetles. Understanding how α-TQ and long-chain fatty alcohols content is affected by leaf maturation, environmental factors, and phenology will increase our knowledge of beetle feeding behavior and may serve to strengthen our pest resistance breeding program.

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ISOLATION AND STRUCTURAL IDENTIFICATION OF SUCROSE ESTERS FROM CORN SPURREY (*Spergula arvensis*); INHIBITION OF SEED GERMINATION

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Abstract—Polar as well as nonpolar extracts of *Spergula arvensis* (corn spurrey) were shown to cause inhibition of seed germination. The polar methylene chloride extracts were subjected to solvent partitioning and separations by a combination of silicic acid and Sephadex LH-20 column chromatography. All chromatographic fractions were monitored by a proso-millet seed germination assay, which resulted in the isolation of a group of sucrose esters (SE). The identity of the ester acids was determined by gas chromatography (GC) of their butyl esters. Further characterization of the SE by GC-mass spectrometry of their trimethylsilyl derivatives showed the presence of four different SE groups with two to seven acyl groups attached to sucrose. The SE isomers in greatest abundance contained either octanoic or dodecanoic acid along with two smaller branched-chain acids (butanoic and pentanoic acids). All four SE groups were inhibitory to seed germination. This is the first report of SE outside the family Solanaceae.

Key Words—*Spergula arvensis*, corn spurrey, sucrose esters, seed germination inhibition, *Panicum milliaceum*.

INTRODUCTION

Corn spurrey (*Spergula arvensis* L.: Caryophyllaceae) is an agronomically important weed with worldwide distribution (Holm et al., 1977; New, 1961). The plants severely retard growth of various crops in the field, such as cole

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crops, peas, and forage legumes (Lemieux and Deschenes, 1984). Corn spurrey tissue homogenates, incorporated into a potting medium, reduced the growth of broccoli and pea seedlings and tissue extracts strongly inhibited proso-millet seed germination and broccoli seedling growth (Harrison and Peterson, 1997). Tsuzuki and Araki (1984) reported that an acidic fraction contained inhibitory substances that had similar properties to caffeic and ferulic acids. Preliminary HPLC analysis in our laboratory of a methanol extract of freeze-dried corn spurrey, utilizing diode array spectral scanning of eluted peaks, failed to detect caffeic or ferulic type compounds other than in trace quantities. However, extraction of fresh corn spurrey plants with methylene chloride (to obtain the cuticular waxes) resulted in a neutral fraction that strongly inhibited the germination of proso-millet seeds. Extracts obtained with hexane proved inhibitory to seed germination as well. The identity of the active compounds in these hexane extracts is not known, although it was shown that they are not sucrose esters. These compounds will be included in future research. This paper reports the isolation and identification of a series of sucrose esters from whole plant corn spurrey that were shown to suppress seed germination strongly and that undoubtedly are a major factor in the plant's inhibitory properties.

METHODS AND MATERIALS

Chemicals. All solvents were analyzed reagent grade. Chloroform (with 0.75% ethanol by volume, Resi-Analyzed grade) was from J. T. Baker (Phillipsburg, New Jersey). Dimethylformamide (DMF) and *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were silylation grade (Pierce Chemical Co., Rockford, Illinois).

Plant Materials. Corn spurrey was harvested from natural, vigorously growing populations and either placed in methylene chloride or freeze-dried. In both cases all aboveground parts were sampled. The freeze-dried material was ground in a Wiley mill to pass a 40-mesh screen.

Fractionation of Freeze-Dried Corn Spurrey. Freeze-dried corn spurrey (100 g) was sequentially extracted with hexane (0.7 liters; fraction 1), CH_2Cl_2 (1.1 liters; fraction 2), MeOH (1.1 liters; fraction 3) and water (0.16 liters; fraction 4). Fractions 2 and 3 were combined, and 50 g of silicic acid (SA, Mallinckrodt, 100 mesh, washed with methanol and activated at 155°C for 1 hr) was added and evaporated to dryness to produce a SA–sample deposited mixture that was submitted to SA column chromatography. The glass column (6 × 80 cm) was packed with 250 g SA in hexane and the sample was applied to the top of the column as a SA–sample deposited mixture. The column was eluted with the following solvents: fraction 5 hexane–CH_2Cl_2 (3:1; 1 liter),

fraction 6 hexane–CH_2Cl_2 (1:1; 2 liter), fraction 7 CH_2Cl_2 (1 liter), fraction 8 acetone–CH_2Cl_2 (1:9; 1.5 liter), fraction 9 acetone–CH_2Cl_2 (3:7; 0.5 liter), fraction 10 acetone (1 liter), fraction 11 acetone–MeOH (2:8; 0.5 liter), and fraction 12 MeOH (0.5 liter). Fractions 8–11 were combined and rechromatographed on silicic acid as above. The column was eluted with the following solvents (all solvent volumes were 0.5 liter: fraction 13 hexane, fraction 14 hexane–CH_2Cl_2 (9:1), fraction 15 hexane–CH_2Cl_2 (3:1), fraction 16 hexane–CH_2Cl_2 (1:1), fraction 17 hexane–CH_2Cl_2 (3:7), fraction 18 CH_2Cl_2 fraction 19 acetone–CH_2Cl_2 (1:3), fraction 20 acetone–CH_2Cl_2 (1:1), fraction 21 acetone, fraction 22 acetone–MeOH (8:2), fraction 23 MeOH.

Fractionation of Corn Spurrey Methylene Chloride Extract. Whole freshly harvested *Spergula arvensis* plants (1084 g fresh weight = 197 g dry weight; water content was determined separately) were soaked in 4 liter of methylene chloride for several weeks and then filtered. The filtrate was evaporated to dryness to give 9.5 g of residue. The residue was partitioned between hexane (450 ml) and acetonitrile (300 ml). The acetonitrile solution was deposited onto 10 g of SA as above and chromatographed on a 100-g SA column (3 × 40 cm) with the following solvents: fraction 24 hexane (0.8 liter), fraction 25 hexane–CH_2Cl_2 (1:1; 2 liter), fraction 26 hexane–CH_2Cl_2 (1:3; 1 liter), fraction 27 CH_2Cl_2 (1.5 liter), fraction 28 acetone–CH_2Cl_2 (1:9, 3 liter; 0.9 g dry weight), fraction 29 acetone–CH_2Cl_2 (2:8; 4 liter; 1.03 g), fraction 30 acetone–CH_2Cl_2 (2:8; 3 liter; 0.31 g). Fractions 28 and 30 were evaporated to dryness and each submitted to Sephadex LH-20 column chromatography. The chromatographic system consisted of one glass Cheminert LC column (2.54 × 54 cm; Valco Instruments Co., Inc., Houston, Texas) packed with Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, New Jersey) in $CHCl_3$. The samples were dissolved in $CHCl_3$ and applied to the column with a loop injection valve. Flow rate was 2 ml/min, and 4-ml fractions were collected. Eluting fractions were monitored by GC and GC-MS and combined as follows: From fraction 28, subfractions 8–21 gave fraction 31 (designated group Ia SE, 0.337 g), subfractions 22–38 gave fraction 32 (designated group Ib SE, 0.192 g), subfractions 39–62 gave fraction 33 (designated group Ic SE, 0.231 g); and from fraction 30, subfractions 50–87 gave fraction 34 (designaed group II SE, 0.129 g).

Analysis of Sucrose Ester Acids. Acids esterified to sucrose were analyzed as their butyl esters after base hydrolysis according to the method of Kays et al. (1994). Relative levels of the acids were determined from chromatographic response data obtained from standard butyl esters.

Sucrose Ester GC Analyses. Gas chromatographic analyses of the sucrose esters were performed on a Hewlett-Packard 5890 GC fitted with a DB-5 (25 m × 0.32 mm ID) capillary column (0.52 μm film thickness) (J&W Scientific, Folsom, California); injector 250 C, detector 350 C. Sucrose esters were

analyzed as their trimethylsilylated derivatives (TMS) prepared from BSTFA/DMF (1:1) and heated at 75°C for 30 min; GC oven temperature program 100–300 at 8°C/min.

Seed Germination Bioassay Procedure. Seed of proso-millet (*Panicum milliaceum* L.) were obtained from Valley Seed Co., Fresno, California. The seed was cleaned of damaged, discolored, or foreign material. Fractions to be tested were dissolved in MeOH and pipetted into 9-cm glass Petri dishes containing two Whatman No. 1 filter papers. The filter papers were prewashed with methanol twice, followed by rinsing with water and drying. This procedure removes germination-inhibiting substances (Peterson and Harrison, 1991). Each fraction was tested at a concentration of 20 mg (dry wt tissue) equivalent extract per milliliter of germination water. The MeOH was completely evaporated and 5 ml of water added. One hundred prosomillet seeds were added and the seeds were incubated at 25°C for 39 hr. Each fraction was tested five times (five replications) and controls had 15 replications. All tests were subsequently repeated. At the end of the incubation period the dishes were quickly frozen to stop any further germination. Germination was considered to have occurred when the radicle length was equal to or exceeded the diameter of the seed. Results are expressed as percent inhibition compared to controls.

RESULTS AND DISCUSSION

Since we observed strong growth inhibition of cole crops and English pea in field and greenhouse experiments caused by corn spurry (Harrison and Peterson, 1997), we isolated and characterized a major part of compounds inhibitory to seed germination. All crude extracts and fractions of freeze-dried sample were assayed, thus accounting for as much inhibitory potential as possible.

Sequential extraction of this material with solvents of increasing polarity (hexane followed by methylene chloride, methanol, and water) resulted in fractions that were bioactive (Table 1). The hexane extract (fraction 1) and the combined methylene chloride and methanol extracts (fractions 2 and 3, respectively), strongly inhibited proso-millet seed germination. The hexane extract was not further characterized; however, it tested negative for the presence of sucrose esters (SE). The methylene chloride and methanol combined extract was separated by silicic acid (SA) column chromatography twice to give a refined subfraction (fraction 19) that strongly inhibited seed germination. Preliminary GC-MS analysis of this subfraction (as a TMS-derivative) indicated the presence of SE as the major constituents. These results prompted us to reisolate these compounds by a more efficient procedure, which separates SE better. The method was developed for the isolation of SE from *Nicotiana* species by Severson et al. (1994) as modified by Chortyk et al. (1996). This entailed partitioning of a

TABLE 1. PROSO-MILLET SEED GERMINATION INHIBITION OF CORN SPURRY FRACTIONS

Fraction[a]	Inhibition (%)[b]	Fraction[a]	Inhibition (%)[b]	ppm[c]
1	92	17	−1	
2+3	90	18	15	
4	−3	19	94	
5	0	20	31	
6	19	21	29	
7	19	22	45	
8–11	88	23	42	
12	0	31 (Ia SE)[d]	90	34.2
13	3	32 (Ib SE)	90	19.4
14	11	33 (Ic SE)	94	23.5
15	8	34 (II SE)	88	13.1
16	16			

[a] See Methods and Materials section. Fractions 24–30 were obtained via the more efficient method. All of these fractions were monitored (GC-MS) for the presence of SE. Since SE were absent, these fractions were not bioassayed.

[b] Relative to control. Fractions tested at 20 mg eq/ml. [1 mg eq/ml represents extract obtained from 1 mg (dry weight) of tissue per milliliter of germination water].

[c] The actual weights of the purified SE groups (fractions 31–34) were determined gravimetrically.

[d] Groups of sucrose esters are listed in the order in which they eluted from a Sephadex LH-20 column (see Methods and Materials and Table 3).

methylene chloride extract of fresh corn spurry plants between hexane and acetonitrile. The sucrose esters are preferentially partitioned into acetonitrile and subsequently separated by SA and Sephadex LH-20 column chromatography. A GC trace of the original methylene chloride (TMS derivatives) solubles is shown in Figure 1. Hydrocarbons were removed by the hexane–acetonitrile partitioning and the sucrose esters separated from other materials such as the fatty acids by SA chromatography. SA chromatography produced pure sucrose ester (SE) isolates, designated group I and group II. The total SE isolates represented 1.1% of the dry weight of corn spurry. Group I (as described below) was characterized by multiple (four to seven) acyl groups on the sucrose moiety whereas, group II SE contained mainly only two or three acyl groups per sucrose (see Table 3 below).

In order to separate the many different SE isomers into related groups, lipophilic gel chromatography utilizing Sephadex LH-20 chromatography was employed. Sephadex LH-20 with chloroform as solvent will efficiently separate compounds based on the degree of hydrogen bonding between the LH-20 gel and the solute (Snook and Fortson, 1979; Snook et al. 1979). This type of separation has been demonstrated for a number of classes of organic compounds,

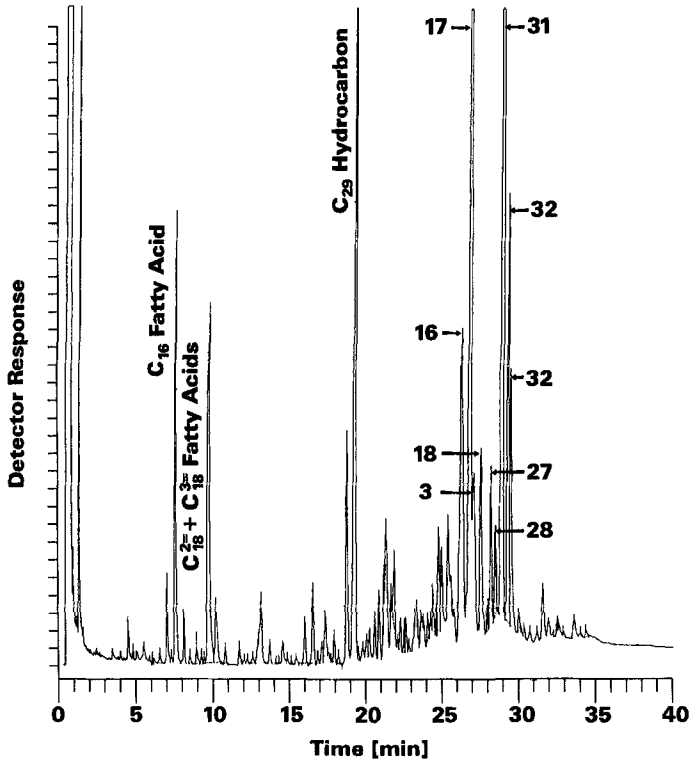


FIG. 1. GC trace of the methylene chloride solubles from *Spergula arvensis* (TMS derivatives). Fractions 31–34 refer to groups of sucrose esters in the order in which they eluted from a Sephadex LH-20 column (see Methods and Materials).

including SE from tobacco (Severson et al., 1985). Gas chromatograms of selected gel fractions (TMS derivatives) are shown in Figure 2 and indicate the unusual complexity of the Se of corn spurry. A chromatogram of group II SE is given in Figure 3. Many of the different SE isomers have similar GC retention times and the analysis of individual LH-20 fractions allowed for a more complete identification of the SE than would otherwise be possible.

The SE types were identified by GC-MS of their trimethylsilyl (TMS) derivatives (Table 3). Additional data for the identification of SE types was obtained by determination of the different kinds of acids present in the total ester fraction. Relative abundances of acids obtained by hydrolysis were determined by GC as their butyl esters by the method of Kays et al. (1994) and are given in Table 2. The most abundant acid was dodecanoic (C_{12}, 35.3%) fol-

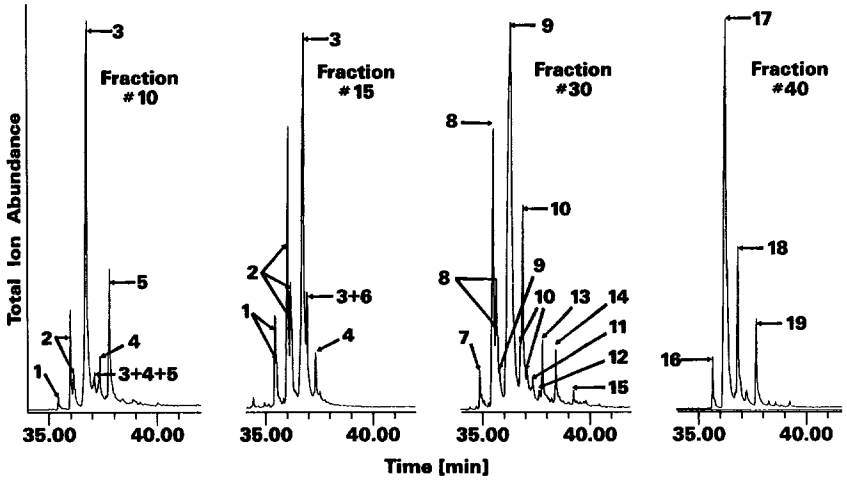


FIG. 2. GC-MS total ion chromatograms of selected lipophilic gel (Sephadex LH-20-chloroform solvent) chromatographic fractions of group I SE (see Methods and Materials section; TMS derivatives). See Table 3 for peak numbers.

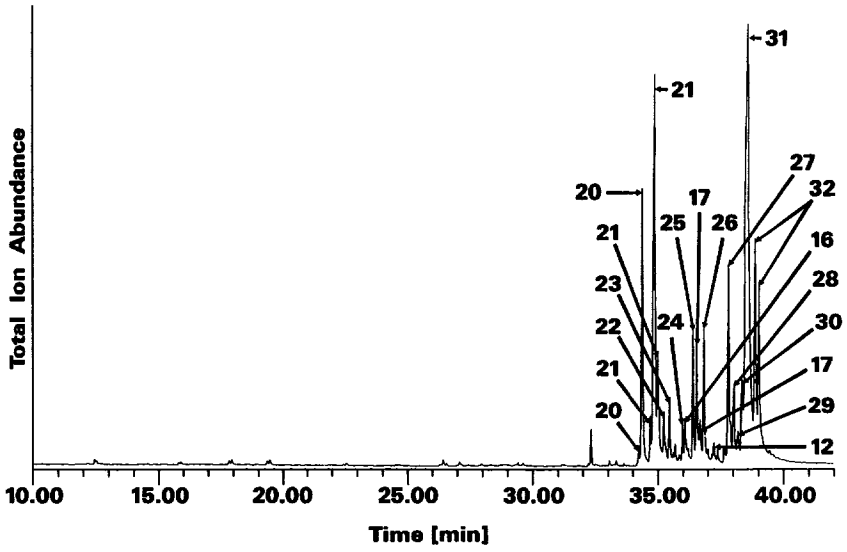


FIG. 3. GC-MS total ion chromatogram of group II SE (TMS derivatives). See Table 3 for peak numbers.

TABLE 2. ACYL COMPOSITION OF CORN SPURRY TOTAL SUCROSE ESTERS

Acyl group	Relative abundance	Distribution (%)
C_2	0.04	1.3
2-Me-C_3	0.50	17.8
2-Me-C_4	0.06	2.0
3-Me-C_4	0.84	29.4
3-Me-C_6	0.04	1.2
C_6	0.01	0.4
C_8	0.31	10.8
C_{10}	0.06	2.0
C_{12}	1.00	35.3

lowed by 3-methylbutanoic (3-Me-C_4, 29.5%) and 2-methylpropionic (2-Me-C_3, 17.9%). These three acids accounted for almost 83% of the acid groups on the SE.

An extensive discussion of the structural identification of TMS-SE by GC-MS has been published (Arrendale et al., 1990). Examples of the GC-MS of the two most abundant SE in corn spurry are given in Figure 4. The spectrum of peak 17 exhibited characteristic ions representing simple cleavage of the glucose and fructose parts to give *m/z* 505—fructose containing one C_8–acyl group and three TMS groups designated C_8-3XTMS-Fru; and *m/z* 473—glucose containing one C_4- and two C_5-acyl groups and one TMS group ($C_4C_5C_5$-TMS-Glc). In addition to these simple cleavage ions, esters with a TMS group at position 6 of the fructose exhibit a strong rearrangement ion by the loss of neutral formaldehyde (Arrendale, et al., 1990; see Figure 5 below) that aids in the determination of which acids are present on the fructose and which are on the glucose. Thus, the MS of peak 17 also contains a strong rearrangement ion at *m/z* 491 and is designated C_8-3XTMS-Fru(Rearr.). Corresponding ions for peak 31 (the most abundant SE, Figure 4) were found at *m/z* 573 (C_5C_{12}-2XTMS-Fru), 559 [C_5C_{12}-2XTMS-Fru(Rearr.)], and 449 (C_4-3XTMS-Glc). An example of a SE whose MS does not exhibit a fructose rearrangement ion is also shown in Figure 4 (peak 3). The absence of a rearrangement ion indicates that one of the acyl groups occupies position 6 of the fructose. Acyl groups on position 6 do not rearrange possibly due to steric requirements inherent in the bulky TMS group. Thus, MS data will only indicate the number and types of acyl substitution on glucose or fructose (Table 3) and in special cases if the 6 position of fructose is occupied. Other specific SE-acyl positions will require detailed NMR studies (Arrendale et al., 1990).

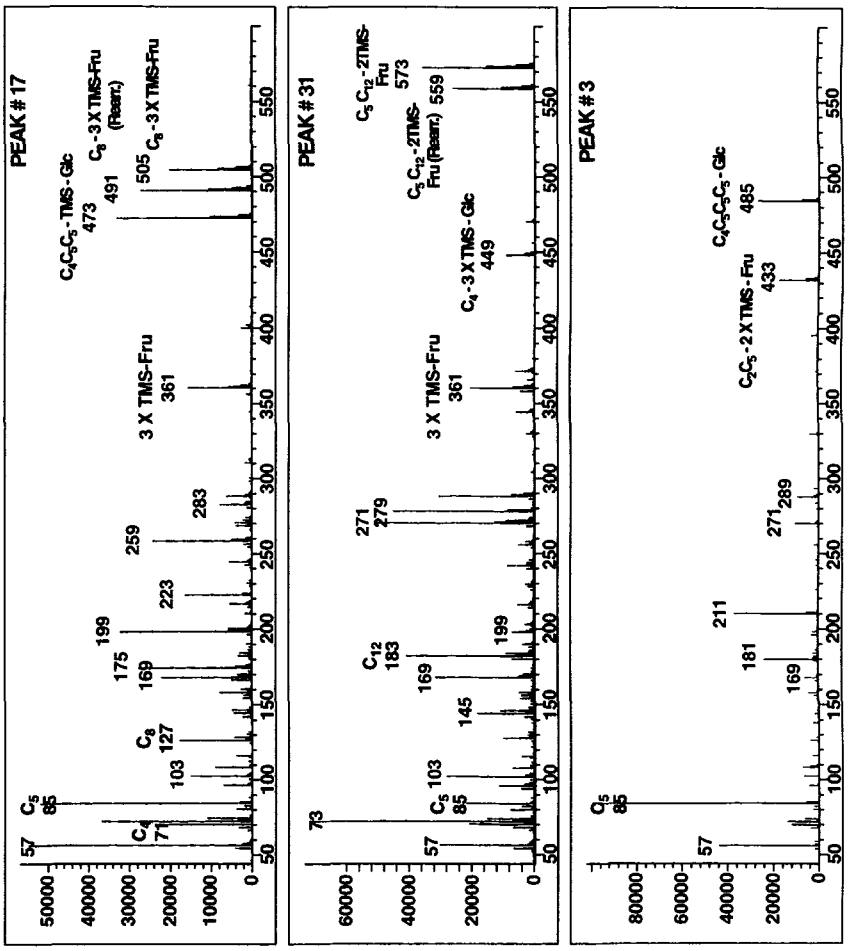
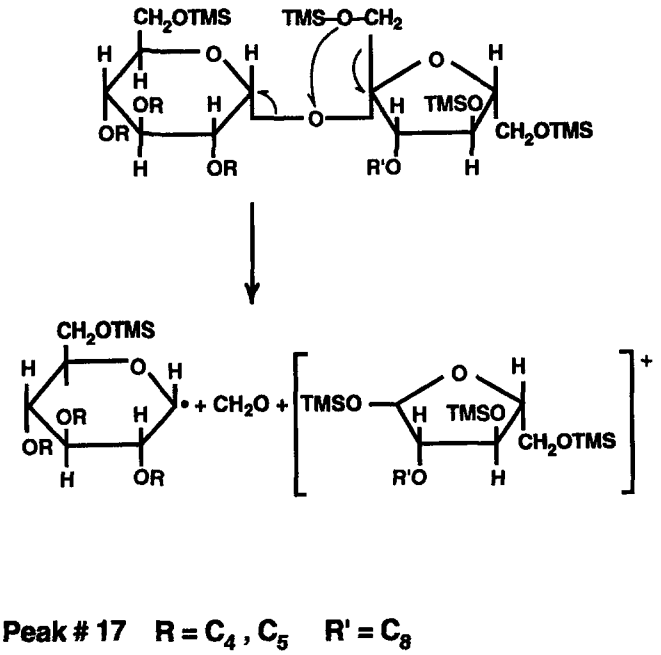


FIG. 4. GC-MS of GC peaks 3, 17, and 31 (TMS derivatives).



Peak # 17 R = C_4, C_5 R' = C_8

FIG. 5. TMS-ether derivatives: Formation of the rearrangement ion (Arrendale et al., 1990). Peak 17 has one C_4 and two C_5 acyl groups on glucose and only one C_8 acyl group on fructose.

Knowing the structures of the SE allowed one to rationalize their elution behavior in the various chromatographic systems. On silicic acid, the most highly substituted esters (least polar) eluted first. Those with four to seven acyl groups (groups Ia, Ib, Ic) eluted from SA with acetone-CH_2Cl_2 (1:9) while SE with two and 3 acyl groups (group II) required a 2:8 ratio of acetone-CH_2Cl_2 solvent. The presence of long-chain acyl groups (C_{10} and C_{12}) on the SE of group II was undoubtedly the reason group II eluted from SA with only a small increase in solvent polarity (1:9 to 2:8). Sephadex LH-20 chromatography with chloroform as eluent also separated the Se based on the number of acyl groups attached (Figure 2). The highly substituted 6- and 7-acyl SE eluted in gel fraction 10 (i.e., $C_4C_5C_5C_5$-Glc-*O*-C_2C_5-Fru; 26 acyl carbons; Gc peak 3) while 4-acyl SE were found in gel fraction 40 (i.e., $C_4C_5C_5$-Glc-*O*-C_8-Fru; 22 acyl carbons; GC peak 17). Sephadex LH-20 gave a more efficient separation of the esters than SA but is most useful after SA chromatography has removed other materials.

TABLE 3. SUCROSE ESTER COMPOSITION OF ISOLATED GROUPS (GC-MS TRIMETHYLSILYL ETHER DERIVATIVES)

SE group	GC peak[a]	SE molecular weight[b]	Glucose moiety		Fructose moiety		Acyl groups (*N*)	Acetyl carbons (*N*)
			Associated ions	Acyl substitution	Associated ions	Acyl substitution		
Ia SE		908	459	$C_4C_4C_5$	433, 419,[c] 85	C_2C_5	5	20
		922	473	$C_4C_5C_5$	433, 419,[c] 85	C_2C_5	5	21
	6	964	473	$C_4C_5C_5$	475, 461,[c] 127	C_2C_8	5	24
	1	906[d]	457	$C_4C_4C_4C_5$	433, 419,[c] 85	C_2C_5	6	24
	2	920[d]	471	$C_4C_4C_5C_5$	433, 419,[c] 85	C_2C_5	6	25
	3	934[d]	485	$C_4C_5C_5C_5$	433, 419,[c] 85	C_2C_5	6*[f]	26
	4	948	499	$C_5C_5C_5C_5$	433, 419,[c] 85	C_2C_5	6	27
	5	904	485	$C_4C_5C_5C_5$	403, 389,[c] 85	$C_2C_2C_5$	7	28
Ib SE	7	936	457	$C_4C_4C_4C_5$	463, 449,[c] 85	C_5	5	22
	8	950	471	$C_4C_4C_5C_5$	463, 449,[c] 85	C_5	5	23
	9	964	485	$C_4C_5C_5C_5$	463, 449,[c] 85	C_5	5*	24
	10	978	499	$C_5C_5C_5C_5$	463, 449,[c] 85	C_5	5	25
	13	992	471	$C_4C_4C_5C_5$	505, 491,[c] 127	C_8	5	26
	14	1006	485	$C_4C_5C_5C_5$	505, 491,[c] 127	C_8	5	27
	11	1020	499	$C_5C_5C_5C_5$	505, 491,[c] 127	C_8	5	28
Ic SE		952[d]	473	$C_4C_5C_5$	463, 449,[c] 85	C_5	4	19
		966	473	$C_4C_5C_5$	477, 463,[c] 99	C_6	4	20
	16	980	459	$C_4C_4C_5$	505, 491,[c] 127	C_8	4	21
	17	994	473	$C_4C_5C_5$	505, 491,[c] 127	C_8	4**	22
	18	1008	487	$C_5C_5C_5$	505, 491,[c] 127	C_8	4	23
		1008	459	$C_4C_4C_5$	533, 519,[c] 155	C_{10}	4	23
	19	1012	473	$C_4C_5C_5$	533, 519,[c] 155	C_{10}	4	24
		1040	473	$C_4C_5C_5$	561, 547,[c] 183	C_{12}	4	26
II SE		928[d]	449	C_4	463, 85	C_5	2	9
		942[d]	463	C_5	463, 85	C_5	2	10
		970	449	C_4	505, 491,[c] 127	C_8	2	12
		984	463	C_5	505, 491,[c] 127	C_8	2	13

TABLE 3. CONTINUED

SE group	GC peak[a]	SE molecular weight[b]	Glucose moiety: Associated ions	Glucose moiety: Acyl substitution	Fructose moiety: Associated ions	Fructose moiety: Acyl substitution	Acyl groups (*N*)	Actyl carbons (*N*)
	20	968	447	C_4C_4	505, 491,[c] 127	C_8	3	16
	21	982	461	C_4C_5	505, 491,[c] 127	C_8	3	17
	23	996	475	C_5C_5	505, 491,[c] 127	C_8	3	18
	22	982	449	C_4	517, 503,[c] 127	C_5C_8	3	17
		998	449	C_4	533, 519,[c] 155	C_{10}	2	14
		1012	463	C_5	533, 519,[c] 155	C_{10}	2	15
	24	996	447	C_4C_4	533, 519,[c] 155	C_{10}	3	18
	25	1010[d]	461	C_4C_5	533, 519,[c] 155	C_{10}	3	19
		1024	475	C_5C_5	533, 519,[c] 155	C_{10}	3	20
	26	1010	449	C_4	545, 531,[c] 155	C_5C_{10}	3	19
	12	1024[d]	463	C_5	545, 531,[c] 155	C_5C_{10}	3	20
		1024	447	C_4C_4	561, 547,[c] 183	$C_{12}CC_5$	3	20
	28	1038[d]	461	C_4C_5	561, 547,[c] 183	C_{12}	3	21
		1052	475	C_5C_5	561, 547,[c] 183	C_{12}	3	22
	27	1024	449	C_4	559, 545,[c] 183	C_4C_{12}	3	20
	29	1038	463	C_5	559, 545,[c] 183	C_4C_{12}	3	21
	31	1038	449	C_4	573, 559,[c] 183	C_5C_{12}	3***	21
	15	1008	419	C_2C_4	573, 559,[c] 183	C_5C_{12}	4	23
	30	1022	433	C_2C_5	573, 559,[c] 183	C_5C_{12}	4	24
	32	1052[d]	463	C_5	573, 559,[c] 183	C_5C_{12}	3	22

[a] Peak numbers designated on Figures 1–3.
[b] Trimethylsilyl ether molecular weight.
[c] Contains fructose rearrangement ion in major abundance.
[d] Two isomers present.
[e] Fructose rearrangement ion is present in minor to trace abundance.
[f] *, **, and ***: relative abundance.

All groups of SE (Ia, Ib, Ic, and II) inhibited the germination of proso-millet about equally. This was somewhat unexpected since we previously found that seed germination inhibition is dependent on the number and chain length of acyl groups present (Peterson et al., 1997). However, in those studies, the SE were mixtures of primarily mono-, di-, and tri-C_7 to -C_{10} esters. Thus, for example, the total acyl carbon number for the C_7 SE was a composite of 7 to 21 while that for C_{10} ranged from 10 to 30. The total acyl carbon numbers for the SE from corn spurry are in agreement with the most active ester mixtures tested in the previous studies. Matsuzaki et al. (1988) isolated two types of SE from *Nicotiana glutinosa* and tested these on tobacco (*Nicotiana tabacum*) seed germination. Both groups were strongly inhibitory to germination of tobacco seed as well as seedling growth. These results cannot easily be compared with those reported here since the compounds are complex mixtures of SE with varying degrees of acylation and mixed chain lengths of the acyl groups. It may be of significance, however, to note that in the two SE reported by Matsuzaki the most abundant acyl chain length was 7 (34.0 and 45.5%). The most active synthetic SE tested on germination of proso-millet (*Panicum milliaceum*) and velvetleaf (*Abutilon theophrasti*) seed, were heptanoyl and octanoyl esters (Peterson et al., 1997). Of related interest is the finding by Perez and De Vivar (1989) that there are three triacyl glucose compounds with acyl chain lengths ranging from two to seven in the nonsolanaceous plant *Bahia schaffneri*. No biological activity of these compounds has been reported to date.

CONCLUSION

A wide variety of sucrose esters has been isolated and identified from *Spergula arvensis* and shown to strongly inhibit seed germination. The results, together with reported crop growth attenuation in the field and greenhouse studies using soil incorporated corn spurry tissue, strongly implicate sucrose esters in this plant's inhibitory effects on cole and other crops. This is the first report of naturally occurring sucrose esters outside the family Solanaceae.

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MALE-RELEASED SEX PHEROMONE OF THE STINK BUG *Piezodorus hybneri*

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Abstract—Male-released semiochemicals of the stink bug *Piezodorus hybneri* (Heteroptera: Pentatomidae) elicit attraction of male and female bugs and homosexual behavior in males. Three active components were isolated from the airborne volatiles of males by flash chromatography, with the activity monitored by GC-EAD and behavioral bioassay. The pheromone system was characterized as a mixture of β-sesquiphellandrene, (*R*)-15-hexadecanolide, and methyl 8-(*Z*)-hexadecenoate (ratio: 10:4:1), and the activity of the semiochemicals was assessed with authentic samples. Enantiomerically pure samples of the *R* and *S* macrolactones were obtained by Yamaguchi's and Mitsunobu's macrolactonization of a key intermediate, (*R*)-15-hydroxyhexadecanoic acid. The nonnatural *S* stereoisomer was neither a beneficial nor a behavioral antagonist. Individual constituents or binary mixtures were active, but the optimal male response was elicited only by the full mixture. Behavioral observation and the fact that the onset of pheromone production is coincident with ovarian development strongly suggest that these semiochemicals are, in fact, sex pheromones.

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Key Words—Homosexual behavior, aggregation pheromone, β-sesquiphellandrene, (*R*)-15-hexadecanolide, methyl 8-(*Z*)-hexadecenoate.

INTRODUCTION

Chemical communication requires the release of specific chemicals from a producer (emitter), the transmission of these chemicals to a receiver, and the processing of these signals leading to appropriate behavioral responses in the receiver (Roelofs, 1995). Among the insect groups in which chemical attraction is a major means of sexual recruitment, namely Lepidoptera, Coleoptera, Hymenoptera, Orthoptera, Diptera, and Homoptera, females predominantly are the emitters and males the receivers (Cardé and Baker, 1984) of the signal referred to as a sex pheromone. When a member of one sex produces a signal that attracts others of the same sex, the cue is termed an aggregation signal, such as the aggregation pheromone of bark beetles (Borden, 1985). However, the assumption that male signals have evolved to attract other males deserves close scrutiny (Thornhill and Alcock, 1983).

Hitherto, male-released pheromones of true bugs (Heteroptera) have been identified for a few species (Aldrich et al., 1984, 1991, 1994; Baker et al., 1987; Oliver et al., 1972; Leal et al., 1995, 1996c; Sugie et al., 1996) and are referred to as aggregation pheromones, implying a male-to-male attraction. We describe here the characterization of a male-released sex pheromone of a stink bug, *Piezodorus hybneri* (Heteroptera: Pentatomidae). Interestingly, the semiochemicals elicit a clear precopulatory behavior in males, which in the absence of females is directed toward males.

METHODS AND MATERIALS

Extracts and Isolation of Pheromone. Airborne volatiles from laboratory-raised adult *P. hybneri* (25°C, 16 hr light, 8 hr dark photoperiod) were trapped on a Super Q column (Alltech, Deerfield, Illinois) set in an all-glass aeration apparatus (Leal et al., 1996a). The column was washed with hexane and the extracts concentrated to 0.1 insect equivalent per microliter. Crude male extracts were subjected to flash column chromatography on silica gel (Wakogel C-200, Tokyo, Japan) by successive elution with hexane–ether mixtures in the following order: 100:0, 95:5, 90:10, 80:20, 50:50, 0:100.

Bioassays. The activity of crude extracts and of fractions separated by column chromatography was monitored in a wind tunnel (2 m long × 30 cm ID). Five females per experiment were placed in the downwind end of the tunnel and observed for 15 min at an airflow of 30 cm/sec at 25°C. Behavioral responses of male and female bugs were also observed in a Y-olfactometer (long arm, 24

cm; side arms, 16 cm; 2.6 cm ID; airflow, 300 ml/min). Behavioral data were statistically analyzed with JMP Software, Version 2 (SAS Institute, Cary, North Carolina).

Analytical Procedures. Gas chromatography (GC) was carried out on Hewlett-Packard 5890 II Plus instruments equipped with split/splitless injector, electronic pressure control, flame ionization detector, and HP 3365 Series II Chemstation. High-resolution GC analyses were performed with polar and nonpolar capillary columns (0.25 mm × 0.25 μm). The polar columns, HP-Innowax (30 m) and BP-20 (25 m), were operated at 50°C for 1 min, increased to 150°C at a rate of 4°C/min, held at this temperature for 1 min, increased to 230°C at a rate of 10°C/min, and finally held at this temperature for 10 min. The nonpolar columns HP-5MS and DB-5 (30 m × 0.22 mm × 0.25 μm) were operated at 50°C for 1 min, increased to 180°C at 5°C/min, held for 1 min, increased to 270°C at 10°C/min, and finally held at this temperature for 10 min. Chiral resolution of the enantiomers of 15-hexadecanolide was achieved on a CP-Chirasil-DEX CB capillary column (25 m × 0.25 mm: 0.25 μm, Chrompack), operated at 150°C and with a (helium) head pressure of 1.17 kg/cm^2 (2.39 ml/min; 61.4 cm/sec). Low-resolution mass spectrometry (MS) was carried out with HP 5890 II Plus GCs linked either to a mass selective detector MSD 5972, a Mass Engine 5989B, or a GC electron ionization detector GCD (Hewlett-Packard) and with a HP 5890 GC linked to a Finnigan-MAT (San Jose, California) ion-trap detector 800. Vapor-phase Fourier transform infrared (FTIR) spectra were recorded with a GC HP6890 (Hewlett-Packard) coupled to a light pipe interface, FTS-40A, GC/C32 (Bio-Rad). GC coupled with an electroantennographic detector (GC-EAD) was carried out on a previously described system (Leal et al., 1996b). ^{1}H NMR spectra were recorded on Varian XL-400 and Jeol JNM-A500 spectrometers.

Chemical Derivatizations. Dimethyl disulfide (DMDS) adducts of the monoene methyl ester were obtained by reaction of dimethyl disulfide DMDS (70 μl) with the isolated natural product (20 μl; ca. 10 ng of the natural ester per microliter of sample), catalyzed by iodine. The mixture was stirred for 1 hr at 85°C in a 1-ml conical vial containing a magnetic spin vane and closed with a Teflon-lined cap. After the mixture was cooled to room temperature, hexane (100 μl) was added, and iodine was removed by washing with sodium thiosulfate. The organic phase was dried over anhydrous sodium sulfate, concentrated to 10 μl, and analyzed by GC-MS. Methyl palmitoleate was derivatized by the same procedure as a standard. The macrolactones (ca. 5 μg) were converted to the corresponding hydroxy methyl esters by overnight reaction with boron trifluoride diethyl etherate (8 μl) in methanol (100 μl) at 70°C. After cooling to room temperature and addition of hexane (100 ml), the reaction mixture was washed (water, 2 × 50 μl), dried over anhydrous sodium sulfate, and concentrated. After GC-MS analyses, the mixture was dried under argon and redis-

solved with CH_2Cl_2 (200 μl). Pyridinium dichromate (PDC; 1.5 mg) was added and the mixture was stirred for 50 min. This was diluted with hexane (500 μl) and filtered through a Pasteur pipet loaded with silica gel.

Synthesis. Methyl 8-(Z)-hexadecenoate. 1-Nonyne was coupled with 1-bromo-5-chloropentane to give 1-chloro-6-tetradecyne. The isolated product reacted with the anion obtained by the reaction of diethyl malonate with sodium hydride in dimethyl sulfoxide (DMSO) to yield diethyl 6-tetradecynylmalonate. Hydrolysis of the malonate followed by heating (135°C) gave 8-hexadecynoic acid, which was esterified with boron trifluoride diethyl etherate in methanol to generate methyl 8-tetradecynoate. Its catalytic hydrogenation (5% $Pd/BaSO_4$; quinoline) in methanol gave the desired methyl 8-(Z)-hexadecenoate (43% yield from 1-nonyne).

(R)-15-Hexadecanolide (Asymmetric Methylation). A mixture of cyclopentadecanone (0.69 g, 3 mmol) and (*S*)-(−)-1-amino-2-methoxymethylpyrrolidine (SAMP) (0.4 g, 3 mmol) was heated at 70°C for 20 hr under an argon atmosphere. After cooling to room temperature, the reaction mixture was dissolved in methylene chloride (20 ml) and dried over $MgSO_4$. The solution, after removal of $MgSO_4$ by filtration, was concentrated by vacuum to provide the hydrazone product (1 g, 99%).

To a solution of lithium diisopropylamide (LDA), prepared by addition of *n*-BuLi (1.6 M in hexane, 0.6 ml 1 mmol) to a solution diisopropylamine (200 mg, 2 mmol) in ether (5 ml), was slowly injected the above hydrazone (327 mg, 1 mmol) in ether (1 ml) at 0°C under an argon atmosphere. After stirring for 10 hr at 0°C, the mixture was cooled to ca. −110°C by an ethanol–liquid N_2 bath. Methyl iodide (150 mg, 1.2 mmol) was injected slowly, and the reaction mixture was allowed to stand at this temperature for 2 hr. After the reaction mixture was warmed to 25°C, methyl iodide (1 ml) was added, and the reaction mixture was heated to about refluxing temperature for 20 hr. Removal of the excess methyl iodide gave a brown residue that was further treated with HCl (3 N, 4 ml)–pentane (20 ml) for 3 hr at 25°C. The organic layer was separated and concentrated to give a crude product that was further purified by flash chromatography over silica gel (2% ethyl acetate in hexane) to provide (*R*)-2-methylcyclopentadecanone (64 mg, 27%) and recovered cyclopentadecanone (105 mg).

To a solution of (*R*)-2-methylcyclopentadecanone (10 mg, 0.04 mmol) in methylene chloride (1 ml) was added *m*-chloroperbenzoic acid (20 mg). After heating to ca. 60°C for 48 hr, the reaction mixture was subjected to flash chromatography separation over silica gel (2% ethyl acetate in hexane) to provide the desired product (4.4 mg, 42%).

(R)- and (S)-15-Hexadecanolide (Linear Approach). (*R*)-3-(*t*-Butyldimethylsilyloxy)butanal, obtained in two steps from ethyl (*R*)-3-hydroxybutyrate in 63% yield (*tert*-buthyldimethylsilyl chloride, imidazole, dimethylformamide;

diisobutylaluminium hydride, toluene), was subjected to the Wittig reaction with the ylid prepared by treating a solution of (11-carboxyundecyl)triphenylphosphonium bromide in tetrahydrofuran–hexamethylphosphoramide (4:1) with potassium bis(trimethylsilyl)amide (0.5 M in ether, 2 eq) to give an olefinic product with *Z*-geometry (Dawson and Vasser, 1977). Deprotection (HF, acetonitrile) and catalytic hydrogenation (H_2, Pd-C, EtOH) of the product gave (*R*)-15-hydroxyhexadecanoic acid (62% in three steps). Macrolactonization of the hydroxy acid by the Yamaguchi method (Inanaga et al., 1979) (2,4,6-trichlorobenzoyl chloride, Et_3N, 4-dimethylaminopyridine, toluene) afforded (*R*)-(−)-15-hexadecanolide ($[\alpha]_D^{25}$ − 18.2 (c = 1.2, hexane) (literature $[\alpha]_D^{20}$ = −16.5 (c = 1.029) (Kaiser and Lamparsky, 1978) in 84% yield, while Mitsunobu lactonization conditions (Kurihara et al., 1976) gave rise to the concomitant inversion of configuration to give (*S*)-(+)-15-hexadecanolide in 31% yield ($[\alpha]_D^{25}$ = +18.7 (c = 1, hexane).

Further detail of organic synthesis will be published elsewhere (Kuwahara et al., 1998).

RESULTS AND DISCUSSION

In order to study chemical communication in *P. hybneri*, we collected airborne volatiles from groups of males and females and tested the activity of these extracts in the wind tunnel. Females clearly responded to whole extracts of airborne volatiles from male bugs (36 ± 4% attraction; control 0%; N = 5), but not a single male was attracted to female extracts in comparable tests.

In order to isolate the semiochemicals involved in this chemical communication system, we separated the whole extract of airborne volatiles from males on a silica gel column and monitored the activity of each fraction in a wind tunnel. Females were attracted only to the hexane–ether (90:10 and 95:5) fractions. The response to the former fraction was clear-cut (42 ± 9%; N = 3). Although the attraction of the latter fraction was weaker (7 ± 5%), this fraction elicited wing fanning in females (45 ± 10%), a precopulatory behavior.

GC-EAD experiments showed electrophysiological activity of two male-specific compounds that were eluted in the hexane–ether (90:10) fraction. Interestingly, both male and female antennae responded to these semiochemicals. In the course of this study, one of us (H.H.) observed in field experiments with caged *P. hybneri* that males induced aggregation of both males and females (Higuchi, 1996). In our indoor bioassays, males also responded to the same semiochemicals involved in male-female attraction.

The two male-specific EAD-active compounds [retention times (R_t) 18.46 and 18.74 min] appeared only in the hexane–ether (90:10) fraction, whereas the other biologically active fraction [hexane–ether (95:5)] also had a male-specific compound (R_t 14.75 min), which did not give any EAD response.

Considering that these three compounds were male-specific, we decided to identify all of them and to assess biological activity of synthetic samples.

The compound with R_t 14.75 min gave a MS with the base peak at *m/z* 69 and the molecular ion at 204 (12%), and matched closely with the spectrum of (1′*R*, 6*S*)-2-methyl-6-(4′-methylenecyclohex-2′-enyl)-hept-2-ene (β-sesquiphellandrene) in the Wiley library. Other fragments were: 41 (59%), 55 (25), 77 (34), 91 (49), 93 (60), 109 (23), 120 (25), 133 (26), and 161 (32). A pure sample of this sesquiterpene from the oil of gingiber, *Zingiber officinale* (Connell and Sutherland, 1966), was indistinguishable from the natural product in terms of retention time as well as the MS profile. Although we did not determine analytically the absolute configuration of the bug-derived sesquiterpene, it is likely that it has the same stereochemistry as the plant-derived β-sesquiphellandrene (see bioassay below).

The MS [base peak, 55; 41 (75%), 43 (56), 69 (61), 74 (75), 87 (48), 97 (41), 110 (23), 123 (17), 137 (11), 141 (8), 152 (14), 194 (17), 236 (14), 237 (12), and M^+ 268 (3)] and IR [$\nu_{C=O}$, 1760 cm^{-1}, medium; ν_{CH} 2937 strong; $\nu_{=CH}$ (*cis*), 3013, weak] data for the EAD-active peak at 18.74 min suggested that the natural product was a methyl hexadecenoate, with the double bond in *cis* configuration. Although these data were similar to those of methyl palmitoleate, the two compounds showed slightly different retention times. DMDS derivatization of the pheromone, followed by MS analyses showed that these two compounds were in fact isomers. The DMDS adduct of the semiochemical gave a MS with the base peak at *m/z* 203, an outstanding peak at 159, and the molecular ion peak at 362. Interpretation of these data suggests that the original double bond was located in position 8. A sample of synthetic methyl 8-(*Z*)-hexadecenoate was identical to the natural product in the MS, IR, and GC data; the synthetic ester was also biologically active (see below).

Finally, the MS and IR data for the second EAD-active compound (R_t 18.46 min) suggested that it was either 15- or 16-hexadecanolide. MS data: base peak, 105; 41 (83%), 43 (54), 69 (53), 71 (32), 83 (42), 97 (41), 111 (22), 125 (13), 152 (11), 192 (9), 194 (7), 210 (10), 236 (17), and M^+ 254 (2). IR data: $\nu_{C=O}$ 1747 cm^{-1}, medium; ν_{CH} 2937, strong. Derivatization of this macrolactone with boron trifluoride diethyl etherate in methanol gave a product with a peak at *m/z* 45, suggesting the occurrence of a secondary alcohol in the product. This was confirmed by further oxidation of this derivative product with pyridinium dichromate, which gave a peak at *m/z* 58 (McLafferty rearrangement). This evidence for the presence of a methyl ketone moiety suggested that the original macrolactone would be 15-hexadecanolide. A sample of this macrolactone was prepared by asymmetric methylation of a hydrazone obtained by condensation of cyclopentadecanone with (*S*)-(−)-1-amino-2-(methoxymethyl)pyrrolidine (SAMP), followed by hydrazone cleavage and Baeyer-Villiger

oxidation of the resulting 2-methylcyclopentadecanone. The synthetic and natural macrolactones were indistinguishable by GC, MS, and IR.

The method of chiral amplification (Shi et al., 1996) was used to determine the absolute configuration of the natural product. The original macrolactone was derivatized with trifluorobromide in methanol to generate a related secondary alcohol. Diastereoisomers of the methyl 15-hydroxyhexadecanoate were obtained by reaction with (*R*)- and (*S*)-α-methoxy(α-trifluoromethyl)phenylacetyl chloride (Mosher's reagent). Unfortunately, diastereoisomeric resolution was not achieved on GC, probably due to the high molecular weight, and, consequently, long retention time. This problem could be overcome by the use of high-performance liquid chromatography (HPLC) or by the resolution of the macrolactones on a chiral column.

Enantiomeric resolution was achieved on a CP-Chirasil-DEX CB capillary column. The synthetic lactone obtained by asymmetric methylation as the key step showed a low enantiomeric purity (Figure 1A and B). The *R* enantiomer

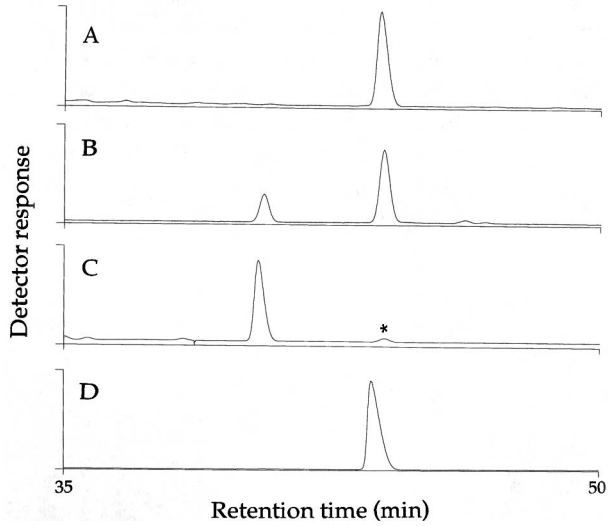

FIG. 1. Enantiomeric resolution of 15-hexadecanolide on a CP-Chirasil-DEX CB capillary column at 150°C. The natural product (A) gave a single peak that corresponded to the major peak of the synthetic sample (B) that gave predominantly (*R*)-15-hexadecanolide. (*S*)-15-Hexadecanolide obtained by an unambiguous synthesis (C) confirmed the identity of the earlier eluting peak. The chemical impurity (*) was removed by further purification on a silica gel column. (D) Enantiomerically pure (*R*)-15-hexadecanolide obtained by a linear approach.

gave the same retention time (R_t 43.93 min) as the natural product whereas the *S* stereoisomer appeared at 40.56 min.

Enantiomerically pure stereoisomers were prepared by Yamaguchi or Mitsunobu macrolactonization of (*R*)-15-hydroxyhexadecanoic acid, which was obtained from ethyl (*R*)-3-hydroxybutyrate via the Wittig chain-elongation reaction. As demonstrated by chiral GC (Figure 1 C and D), both enantiomers were obtained in high optical purity.

In the olfactometer bioassay, both males (Figure 2A) and females (Figure 2B) were significantly attracted to a mixture of β-sesquiphellandrene, (*R*)-15-hexadecanolide, and methyl 8-(*Z*)-hexadecenoate (500 ng of a 10:4:1 mixture). Interestingly, males were not only attracted to the pheromone source, but they also displayed a clear precopulatory behavior. While walking towards the pheromone source, males mounted other males they encountered and tried to effect copulation. When males were released individually, they displayed a sequence of behavior in response to the pheromone: walking towards the pheromone source, searching around the filter paper impregnated with these compounds, and exposing the pygophor (Figure 3).

In order to investigate whether all these semiochemicals are involved in chemical communication of *P. hybneri*, bioassays were conducted in the olfactometer with all combinations of the three compounds. Males were attracted more strongly to the full mixture than to any binary combination or individual component (Figure 4A). Although (*R*)-15-hexadecanolide was not attractive per se, it is required for the optimal male response. The precopulatory behavior (exposure of male's pygophor) on the other hand, is elicited even by individual components with responses equivalent to the full mixture (Figure 3B). The

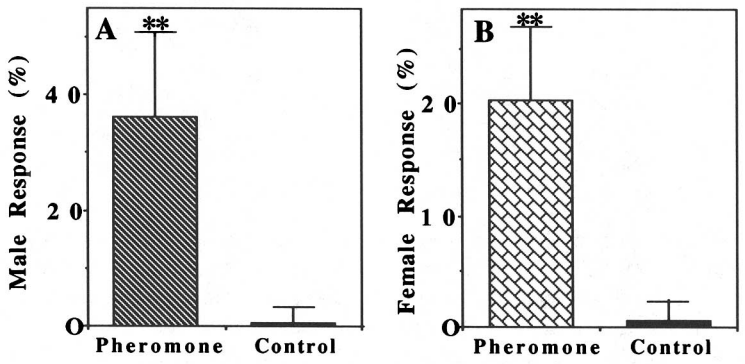

FIG. 2. Responses of *P. hybneri* males (A, $N = 13$) and females (B, $N = 8$) to the synthetic pheromone mixture (ca. 2 male equivalents) in the olfactometer. $^{**}P < 0.01$ (*t* test).

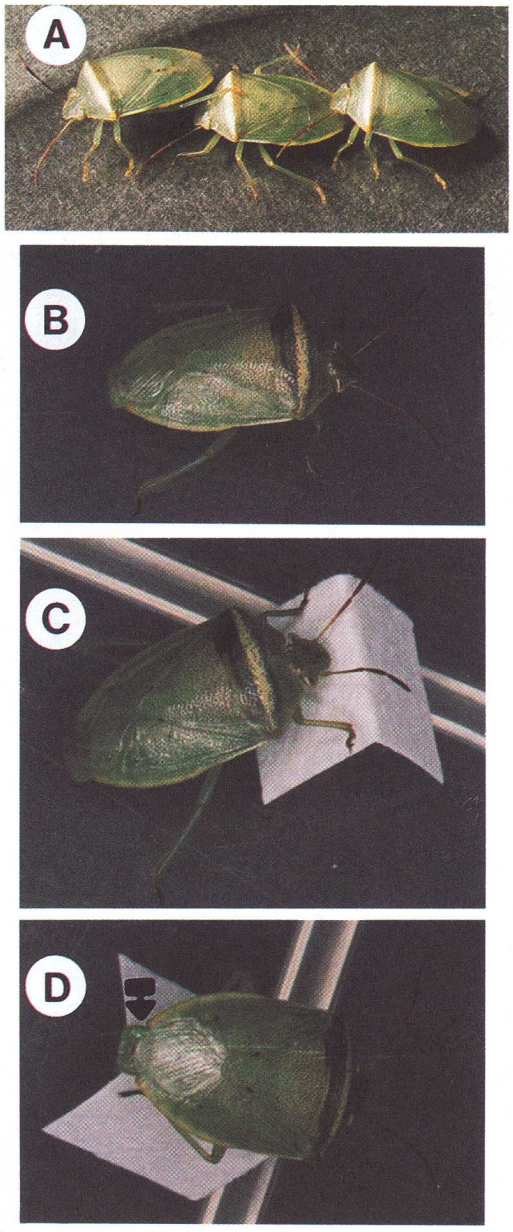

FIG. 3. Responses of *P. hybneri* males elicited by the male-released sex pheromone. (A) Males trying to mount other males they encountered en route to the pheromone source. (B) Male walking towards the pheromone source, (C) touching the pheromone-laden filter paper and starting searching behavior, and (D) showing a precopulatory behavior by exposure of the pygophor (arrow).

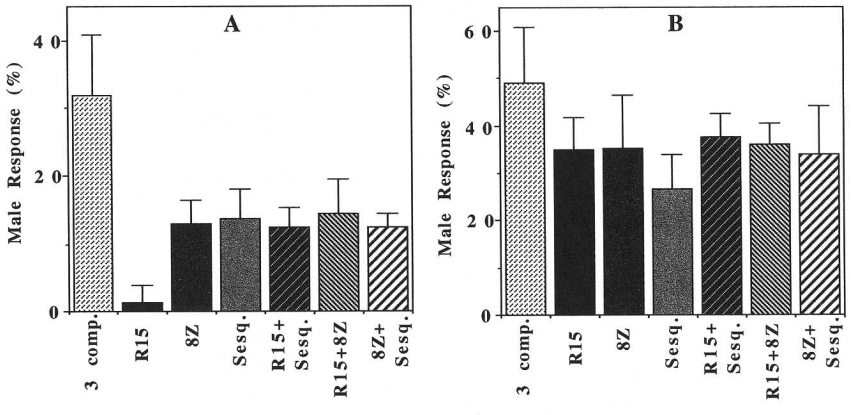

FIG. 4. Behavioral responses of *P. hybneri* males to various combinations of the semiochemicals isolated from bugs of the same sex ($N = 4$ for each candidate lure). (A) Attraction in the olfactometer and (B) precopulatory behavior characterized by the exposure of pygophor.

nonnatural (*S*)-15-hexadecanolide did not elicit any response in males, but it was not a behavioral antagonist. Male responses to the full mixture containing the (*R*)-15-hexadecanolide were not significantly different from those displayed when the sample was spiked with an equimolar amount of the *S* enantiomer, i.e., a racemic mixture of the lactone (Figure 5A and B).

In conclusion, the sex pheromone system for the stink bug *P. hybneri* is composed of at least three constituents, β-sesquiphellandrene, methyl 8-(*Z*)-hexadecenoate, and (*R*)-15-hexadecanolide, that are released by males in a 10:4:1 ratio. The observation that *P. hybneri* males responded to the male-released sex pheromone system with a clear precopulatory behavior sheds light on the evolution of chemical communication in this group of insects and strongly suggests that referring to semiochemicals that attract both sexes as aggregation pheromones may be misleading.

Because laboratory bioassays with true bugs often do not consistently yield any quantifiable orientation behavior, one is normally tempted to carry out field tests with candidate chemicals and look at the overall response in terms of catches in pheromone-baited traps (in comparison to control traps) (Aldrich et al., 1984, 1991; Leal et al., 1995, 1996c; Sugie et al., 1996). It seems that the male–male sexual behavior elicited by male-released pheromones has not been observed before in other hemipterans because no one looked for it. This behavior, which was first observed in aposematic beetles, *Lycus loripes* (Eisner and Kafatos, 1962), strongly suggests that the semiochemicals are involved in sexual recruitment.

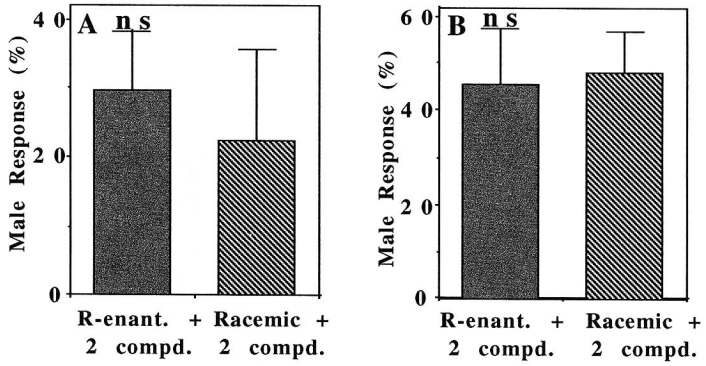

FIG. 5. Effect of 15-hexadecanolide stereochemistry on male responses: (A) attraction, (B) precopulatory behavior. (ns, not significant by Wilcoxon/Kruskal-Wallis test; $N = 5$).

In various species for which females are the pheromone-emitters, sex pheromone may elicit homosexual behavior. Males responding to synthetic chemicals try to copulate with each other in the absence of females. The fact that the male-released pheromone of *P. hybneri* elicited male–male sexual behavior suggests that the semiochemicals are sex pheromone constituents. In addition, the onset of pheromone production in *P. hybneri* males is coincident with the ovarian development in females. Pheromone production starts with 6- to 8-day-old bugs (40 ng/male/day), increases to 250 ng/male/day (at 13 days old) and remains at this level throughout the observation time (up to 25 days old). At 26°C and under the same light conditions at which we collected the airborne volatiles from males, oviposition of *P. hybneri* starts six days after emergence (or at seven days at 24°C) (A. Kikuchi, personal communication).

It is not known how males evolved the ability to respond to their own semiochemicals, but it is likely that responding males are exploiting the signal emitted by other males for the recruitment of females. This scenario may be an example of an alternative mating strategy for males to pool their pheromonal resource in order to increase their individual chances of getting a mate. It would be interesting to pursue further studies on the chemical communication of *P. hybneri* to address the questions of whether any one male could be either a "caller" or a "joiner" at different times in the field, the factors that determine which strategy will be pursued, and the comparative mating success of a joiner versus a caller.

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EVIDENCE FOR THE SITE OF FEMALE SEX PHEROMONE PRODUCTION IN *Periplaneta americana*

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Abstract—We used bioassays to determine the sex pheromone production site in the female American cockroach (*Periplaneta americana*). Bioassayed extracts from various body parts aroused sexual responses from unmated males. However, the extract of hindgut (including both colon and rectum) induced strong responses. Colon extract stimulated the strongest sexual response. To compare the sex pheromone contents of atrial glands, pygidium, rectum, and colon, we recorded the EAG responses of male antennae to these extracts. Among the four tissues, colon extract caused the strongest EAG response. From gas chromatographic (GC) analysis, the quantities of periplanone-A (PA) and periplanone-B (PB) (the main sex pheromone components in female American cockroach) were 0.34 and 8.31 ng, respectively, in the colon of a virgin female. The ratio of PA to PB was ca. 1:24. The present results are different from those reported in a previous study, in which it was concluded that atrial glands are the sites of sex pheromone production in *P. americana*, and from GC analysis the quantity of PB was estimated to be 60 ng/female. There was no difference in sexual behaviors induced by colon and atrial glands of calling virgin females. It is unclear whether the colon is the sole sex pheromone source in the female American cockroach.

Key Words—American cockroach, colon, atrial glands, alimentary canal, EAG; bioassay, *Periplaneta americana*.

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INTRODUCTION

Virgin female adults of *Periplaneta americana* emit a specific sex pheromone (Roth and Willis, 1952). This substance adheres to paper and other surfaces that females contact. Males manifest sexual behavior to paper contaminated with sex attractant, as well as to virgin females. Previously, the female head was postulated to produce this pheromone, as the head was found to elicit sexual responses from males (Sturckow and Bodenstein, 1966). A later report (Bodenstein, 1970) suggested that the alimentary canal was the organ responsible for secretion of the pheromone. The presence of sex pheromone in extracts of the alimentary tract (Persoons et al., 1976; Chow et al., 1978; Talman et al., 1978) and midgut (Takahashi et al., 1976) also has been reported.

In their pioneering studies, Persoons et al. (1974) isolated and elucidated two major chemical structures of the sex pheromone from extracts of feces and alimentary tracts of virgin female American cockroaches. The compounds were named periplanone-A (PA) (Persoons et al., 1982; Hauptmann et al., 1986) and periplanone-B (PB) (Persoons et al., 1979). Other biologically active components also have been reported, including periplanone-C, and periplanone-D (Biendl et al., 1989; Persoons et al., 1990).

Synthetic periplanone-A and periplanone-B both induce great excitement in males. Male displays are characterized by increased antennal waving, active searching for the pheromone source, wing raising, wing fluttering, and copulatory attempts (Persoons et al., 1979; Adams et al., 1979; Okada et al., 1990b). In behavioral bioassays, the active responses to PB increased at concentrations of 10^{-5} to 10^{-2} ng and to PA at concentrations of 10^{-3} to 10^{-1} ng (Okada et al., 1990a,b).

The "search for the sex pheromone production site of the American cockroach has been very controversial because there is neither direct evidence of secretory gland cells nor observation of calling pose" (Takahashi et al., 1976). Abed et al. (1993) reported that the female virgin adopts a characteristic calling posture to emit the pheromone. During this time, two white turgescent masses, the atrial glands, usually folded into the genital atrium, are everted. They further demonstrated that extracts from atrial glands of calling females are highly attractive and induce more sexual wing-raising behavior than extracts from other body parts. From GC-MS analyses, they estimated that amount of PB was about 60 ng/virgin female (30 ng/gland). These workers concluded that the atrial glands are the sex pheromone production site. In addition, they suggested that fecal material contained no sex pheromone, but can act like filter paper to adsorb the pheromone. We repeated their experiments, traced the feces in the rectum, and reproduced their results showing that the last two abdominal segments (containing atrial glands) induced strong sexual response from unmated males. In contrast, we found that extracts of the feces in the rectum, as well as extracts made of other sections of the alimentary tract, induced strong sexual behavior in males.

A sensitive pipet bioassay and electroantennogram (EAG) technique were employed to study the diffusion pathway of the pheromone and the connection between atrial glands and alimentary tract. Our results showed that the colon seems to be the only organ that produces sex pheromone in virgin female *P. americana*.

METHODS AND MATERIALS

Experimental Insects. Periplaneta americana were reared in 20-liter plastic buckets and maintained at a temperature of 26 ± 2°C with 75 ± 10% relative humidity, under a 12L:12D photoperiod. They were provided with dry dog food and water ad libitum. Last instars were taken from the colonies and kept under the same conditions. Newly emerged adults were separated once every three days. Females were put into 2-liter glass bottles in groups of three to five individuals. Males were reared in glass aquaria (30 × 30 × 30 cm) in groups of 25–30 individuals and kept in a room that was isolated from females and female odor (Yang et al., 1992). Virgin females of 10–38 days of age were used for studies to determine the pheromone production site.

Preparation of Pheromone Extracts for Bioassay. To determine the pheromone production site, the atrial glands and other body parts of females were collected in the first half of scotophase for the preparation of extracts (Schal and Bell, 1985; Abed et al., 1993). Sampling included: (1) the last two abdominal segments (containing atrial glands), the entire alimentary canal, and the remainder of the abdomen; (2) fecal pellets of virgin females, collected and extracted at 0, 6, 12, 18, 24, and 24–48 hr after being excreted; (3) feces from the rectum, and genitalia, atrial glands, rectum, colon, midgut, foregut, the remainder of the abdomen, and head and thorax; (4) foregut, midgut, hindgut, and atrial glands from 20 virgin females that were maintained for six days with fresh food and water daily, and with feces removed daily to prevent pheromone contamination; (5) colon and rectum, dissected from 10 virgin females after treatment as in (4), cut longitudinally, and washed four times with 1 ml saline (Ryan and Karp, 1993) before extracting any pheromone; and (6) colons and atrial glands of 48 virgin calling females (Abed et al., 1993) of different ages extracted with *n*-hexane and bioassayed individually.

All body parts were extracted in 0.5–1.0 ml *n*-hexane (according to the size of the body part) by shaking at 120 rpm for 3 hr. Scissors and forceps were used once before being washed with detergent and heated in an oven (80 ± 1°C) for more than 24 hr to avoid contamination. The dissecting instruments could induce only a little antennal waving in test males after such heat-treatment and extraction with *n*-hexane.

Bioassay. The pipet method (Chow et al., 1976; Chow and Wang, 1981) was used for studying unmated male sexual response. Test extracts were injected

into the tip of the pipet with a microsyringe. After evaporation of *n*-hexane, the pipet was puffed 10 times with a 1-ml rubber bulb toward the 25–30 test males within the first half of scotophase at intervals of 5 min (Chow et al., 1976; Hawkins and Rust, 1977; Rivault, 1981; Seelinger, 1984; Zhukovskaya, 1995).

Sexual Response Index. The sexual responses of male roaches were categorized as: (1) no sexual response (−); (2) less than half of the males showing waving of antennae (±); (3) all males showed waving of antennae and less than half of the males moving slightly by walking (+); (4) more than half of the males walking and searching for the pheromone source (++); (5) all males running and searching, and some showing wing-raising and wing-fluttering response (+++); and (6) all males running, searching, and some showing copulatory attempts (homosexual behavior or backward movement with protruding genitalia) (++++).

EAG Technique. For the EAG tests, 50 virgin females in the first half of scotophase were dissected. The colon, rectum, pygidium, and atrial glands were removed and extracted according to the above procedure. The antenna for recording the EAG remained attached to a male roach, reared as described above, that was held on a glue plate in a Petri dish with Nikki nontoxic modeling clay. The distal tip of the flagellum was cut off and the antenna was fixed on a rubber base with insect pins. The distal portion was inserted into a saline-filled (Ryan and Karp, 1993) glass capillary (A-M Systems Inc.; 1.1 mm ID, 1.8 mm OD), which served as a recording electrode, and connected to the headstage of a DC pre-amplifier (NL 102G, NeuroLog system, Digitimer Ltd.), with a chloridized silver wire. Another chloridized silver wire served as an indifferent electrode and was inserted into the hemocele between the roach's mid- and posterior thorax. The amplified signals were digitized and measured on a personal computer (IBM compatible PC 586) with a data acquisition system (Digidata 1200A, Axon Inc.).

To clean the antenna, a continuous airstream (40 ml/sec) was delivered by a Pasteur capillary pipet (opening 1 mm diameter), with airflow supplied by an ambient air pump. Air flowed perpendicular to the antenna. The distance between the pipet tip and the middle part of the immovable antenna (Nishino and Takayanagi, 1979) was about 5 cm. The tip of another pipet, connected to a different ambient air-pump, was treated with different dosages of test extract. The test airstream (also 20 ml/sec, 1-mm-diameter tip) from this pipet was supplied as above but puffed from the distal portion of the antenna so that the puffed pheromone covered the whole antenna. The distance between the pipet tip and the distal portion of the antenna was 0.5 cm. The two air puffs were regulated by two electromagnetic valves (MAC 35TYP, New Zealand), which were controlled manually with a pulse generator (Module NL300, NeuroLog system, Digitimer Ltd.). The pheromone puff duration was set at 30 msec. The pheromone plume covered the entire antenna, and the continuous airstream was off

when the test puff was on. For the control, the same procedure was followed except that no pheromone or body extract was used.

Tests were repeated at 3- to 7-min intervals with increasing doses. An antenna was used once for all tests from low to high dosages. Ten repeats were performed for each test.

Purification and GC Analysis of Extracted Pheromone. The colons were dissected from 100 virgin females, combined, and extracted with *n*-hexane. The extract was concentrated to 4 ml with a flow of nitrogen, and 0.4 ml was subjected to liquid chromatography on a Sep-pak® silica cartridge (Waters, part No. 51900) for purification. Fractions were eluted with 10 ml each of 100% hexane, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% dichloromethane in hexane, and 100% dichloromethane. The active fractions were combined, concentrated, and analyzed by HPLC in a Shimadzu 6A HPLC fitted with a Phenomenex Ultremex 5 Silica column (250 × 4.6 mm, 5 μm). Fractions were eluted with 5% tetrahydrofuran in hexane for 20 min, at 2 ml/min, and separated into 40 tubes.

The active eluates were combined, concentrated, and analyzed by GC with a Varian GC 3400 containing a DB-23 fused silica capillary column (30 m × 0.25 mm ID). Column temperature was increased at 2°C/min from 130 to 220°C, and then held at 220°C for 5 min. The temperatures of injector and detector were 220 and 240°C, respectively. Flow rate of nitrogen carrier gas was 1.1 ml/min. For quantitative determination of the pheromone, synthetic PA (2.31 ng) and PB (2.74 ng), as well as 100 ng *n*-octadecane as standards, were added to the purified colon fraction and the mixtures were subjected to GC.

SEM Preparation of Atrial Glands and Anal Opening. To observe the possible connection between atrial glands and the alimentary canal, the last two abdominal segments of virgin females were dissected and treated according to the scanning electron microscope (SEM) procedures described by Tsai et al. (1997).

RESULTS AND DISCUSSION

The extract of the last two abdominal segments induced the strongest sexual activity in unmated males at 0.001–1.000 dilution of the tissue extract (Table 1). The extract of the alimentary canal caused a sexual response as well, although not as strong as that of the last two abdominal segments. A 0.100 dilution induced 30 males to run and search for the pheromone source, and an extract without dilution induced wing-raising and wing-fluttering behaviors in the test males. The remainder of the abdomen induced little sexual response from the males.

Although the atrial glands have been reported to be the sex pheromone production site (Abed et al., 1993), our evidence shows that the alimentary tract

TABLE 1. MALE SEXUAL RESPONSES TO EXTRACTS OF VIRGIN FEMALE BODY PARTS

Dilution	Tissue		
	Last two abdominal segments[a]	Alimentary canal	Remainder of abdomen
0.001	++	+	−
0.010	+++	+	±
0.100	++++	++	+
1.000	++++	+++	+

[a] *Contained atrial glands.*

extract induced strong male sexual activity (Table 1). Feces can also induce strong sexual behavior from males, as reported by many investigators (Persoons et al., 1974, 1976; Waldow and Sass, 1984; Seelinger, 1985; Seelinger and Gagel, 1985). Abed et al. (1993) suggested that they were porous and moist and acted as an adsorbent for the pheromone. To check the adsorbing effect of fecal material, we tested the effectiveness of newly discharged feces and feces at 6, 12, 18, 24 hr, and between 24 and 48 hr after defecation. Between nine and 11 replicates of fecal pellets were tested. The test dose was 0.1 extract volume of a fecal pellet (1.8 ± 0.6 mg). All of the males manifested strong sexual behavior after the fecal extract was puffed towards them. Feces collected at different intervals gave the same activity.

Since the feces contain sex pheromone (Persoons et al., 1982), we traced and extracted feces from the rectum and other body parts of 16 virgin females. Feces in the rectum and in all regions of the alimentary canal induced strong sexual responses, but extracts of the genitalia, atrial glands, remainder of the abdomen, and head and thorax contained almost no sex pheromone. Many researchers previously have reported that the American cockroach sex pheromone comes from the alimentary tract (Bodenstein, 1970; Persoons et al., 1976, 1979; Chow et al., 1978; Talman et al., 1978; Sass, 1983) or midgut (Takahashi et al., 1976; Persoons et al., 1979). Bodenstein (1970) showed in starved females that the midgut and ceca contain about equal amounts of pheromone, while the crop is about 100 times less attractive.

We considered the possibility that pheromone is smeared on food and feces after being released. The pheromone activity in the entire alimentary canal might thus come from pheromone-contaminated food and feces through coprophagy. If pheromone comes from the atrial glands, the pathway may follow the sequence: atrial gland epidermal cells → feces and food → mouth → alimentary tract → feces. If it comes from the alimentary canal, the pathway may follow a different

sequence, such as alimentary tract cells → feces and food → mouth → alimentary canal → feces.

To determine the origin of the pheromone, we fed virgin females with fresh food and water and removed the feces daily to avoid contamination with pheromone through coprophagy. After such treatment, females were dissected, and the foregut, midgut, hindgut, and atrial glands were extracted and tested. The hindgut manifested the strongest sexual response from the males. The foregut, midgut, and the atrial glands elicited little or no pheromone response. The results indicate that the hindgut is the production site of the pheromone.

To substantiate this, we gave virgin females fresh food and water daily, and removed the feces daily for six days. Then the colon and rectum were removed, washed with saline, and extracted before testing. Colon extract showed a stronger male-stimulating activity than rectum extract and appeared to be the organ with the greatest pheromone activity.

Abed et al. (1993) indicated that atrial glands of a calling female produce 60 ng PB. We compared the responses to extracts of colon, rectum, pygidium, and atrial glands of 50 virgin calling females by the traditional EAG method. The EAG amplitude (millivolts) increased with increasing extract dosages. The colon caused the strongest response with the antenna of an unmated male (Figure 1).

In addition, we dissected 80 virgin females from mass rearing and small groups of three to five individuals. The extracts from atrial glands, pygidium,

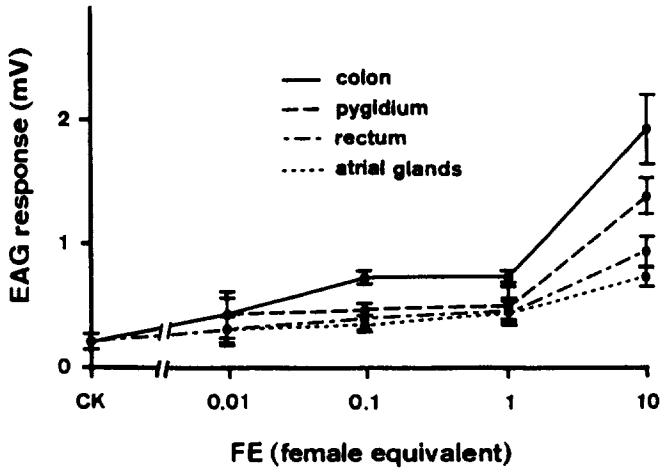

FIG. 1. The EAG response of male American cockroach induced by extracts of four organs of virgin female *P. americana* (ck: control, air). The averaged responses of each point are based on 10 measurements. Error bars are mean ± SE.

rectum, and colon were bioassayed individually. Colon extracts triggered the strongest sexual response from unmated males. In contrast, the atrial gland extracts induced only a weak sexual response, e.g., antennal waving or slight individual movement.

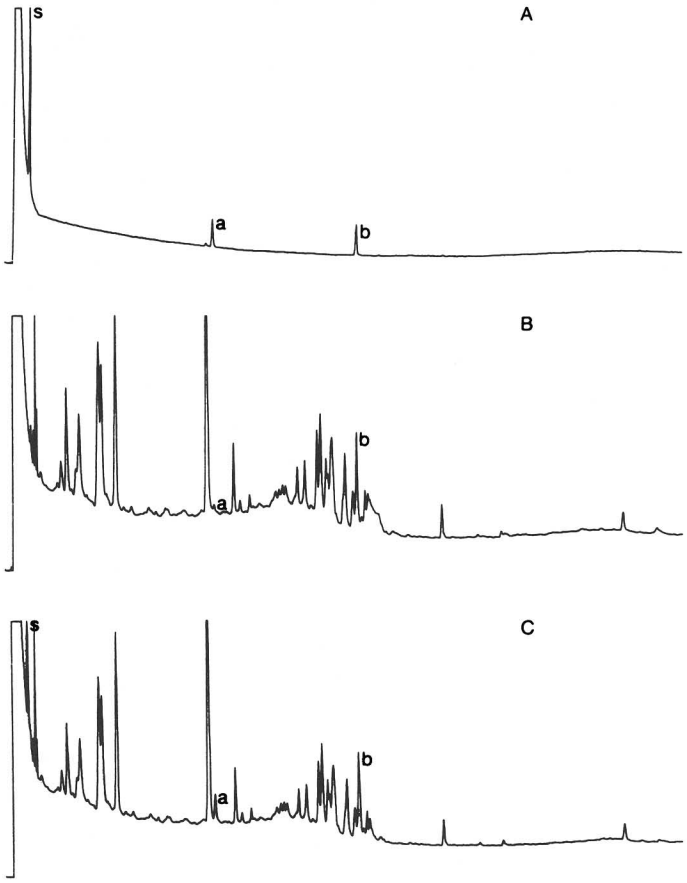

FIG. 2. Gas chromatograms of synthetic periplanone-A (PA), periplanone-B (PB), *n*-octadecane, extracts of colon, and colon extract with synthetic PA, PB, and *n*-octadecane. Operating conditions: DB-23 silica capillary column (30 m × 0.25 mm ID); column temperature, 130–220°C at 2°C/min, maintained at 220°C for 5 min. Temperatures of injector and detector, 220°C and 240°C, respectively; nitrogen carrier gas flow rate 1.1 ml/min. (A) synthetic PA, PB, and *n*-octadecane; (B) extract of colon alone; (C) extract of colon with synthetic PA, PB, and *n*-octadecane. The peaks of PA, PB, and *n*-octadecane are labelled a, b, and s, respectively.

After separation by liquid chromatography on Sep-pak silica cartridges, the colon fractions eluted with 0, 20, 30, and 40% dichloromethane in hexane elicited sexual responses from male roaches. Further purification was carried out by HPLC. Fractions from tubes 18–20 containing sex pheromone were combined and subjected to GC analysis. The natural PA and PB quantities in colon were ca. 0.34 and 8.31 ng/female, respectively, and the ratio of PA to PB was about 1:24 (Figure 2).

Since Abed et al. (1993) reported that virgin female American roaches adopt a characteristic calling posture, we extracted the pheromone from colon and atrial glands of virgin calling females of different ages. Male sexual responses induced by these two body parts were similar at all ages (Table 2). Student's *t* test indicated no significant differences in male sexual response between the colon and atrial glands.

Talman et al. (1978) and Persoons et al. (1979) reported that fecal extracts contain PA and PB in a ratio of ca. 1:10, and extracts of alimentary tracts mainly contain PB with little PA. A total of 200 μg PB was isolated from fecal material of 15,000–20,000 females and 32,000 alimentary tracts (Persoons et al., 1979). Sass (1983) measured an average of ca. 10 ng PB and 10 PB-equivalent amount ng PA in each female gut based on an electrophysiological method. Nishino et al. (1983) estimated by GC analysis the quantities of PA and PB from feces and shelters of 2130 females to be 6.8 and 1.5 μg, respectively. The ratio of PA to PB in that study was 9:2, and the quantities of PA and PB per female were 3.2 and 0.7 ng, respectively. Nishino et al. (1988) also isolated PA (24 μg) and PB (15 μg) from the dirty filter papers and feces of

TABLE 2. MALE SEXUAL RESPONSES TO EXTRACTS OF VIRGIN CALLING FEMALE BODY PARTS[a]

Female age (days)	*N*	Body part	Male sex responses (*N*)			
			+	++	+++	++++
5–10	7	Colon	4	3	0	0
		Atrial glands	2	4	1	0
11–20	25	Colon	16	5	2	2
		Atrial glands	14	8	2	1
21–30	8	Colon	3	5	0	0
		Atrial glands	6	1	1	0
31–60	8	Colon	6	2	0	0
		Atrial glands	8	0	0	0

[a] Student's *t*-test indicates no differences in the sexual behaviors induced by colon and atrial glands from females of the same age.

TABLE 3. PA AND PB QUANTITIES AND PA/PB RATIOS OF VIRGIN FEMALE AMERICAN ROACHES FROM DIFFERENT REFERENCES

Source	PA (ng/female)	PB (ng/female)	PA/PB	Quantifying method	Reference
Excreta			1:10	GLC/MS	Talman et al. (1978)
Alimentary tracts	Very little	Main		GLC/MS	Talman et al. (1978)
Feces			1:10	GC	Persoons et al. (1979)
Alimentary tracts	Very little	Main		GC	Persoons et al. (1979)
Fecal material (and alimentary tracts)	0.2-0.3	2-2.7	1:10	GC/MS	Persoons et al. (1982)
Gut	10*	10	1:1[a]	EAG	Saas (1983)
Feces and shelters	3.2	0.7	9:2	GC	Nishino et al. (1983)
Feces and shelters	1.4				Hauptmann et al. (1986)
Feces and shelters	2.1	1.3	8:5	GC	Nishino et al. (1988)
Atrial glands		60		GC/MS	Abed et al. (1993)
Colon	0.34	8.31	1:24	GC	This paper

[a]*The EAG effectiveness of the PA is given as "PB-equivalent amount, in ng."*

11,500 females over the course of five years. The ratio of PA to PB was 8:5. The quantities of PA and PB per female were 2.1 and 1.3 ng, respectively (Table 3).

Our results agree with those of Talman et al. (1978) and Persoons et al. (1979), that PB is the main component of the sex pheromone. PA is found in feces only (Talman et al., 1978; Persoons et al., 1979; Nishino et al., 1983, 1988). Moreover, PA has been proven to elicit less pheromonal activity than PB, both by bioassay (Seelinger, 1985; Seelinger and Gagel, 1985; Okada et al., 1990a,b, 1991) and by EAG (Nishino and Kuwabara, 1983; Okada et al., 1990a, 1991; Yang et al., 1992). PA might be a stable but less active metabolic product of PB (Talman et al., 1978).

Our results, however, are quite different from those of Abed et al. (1993). Their report of 60 ng PB from atrial glands of a calling virgin female seems high, in light of our results.

Our data show that both atrial glands and the colon are sources of sex pheromone in the female American cockroach. Pheromone observed in other parts is likely the result of contamination. Is there any possible connection between atrial glands and feces, or between atrial glands and gut? From SEM (Figure 3), we can see that the atrial glands are close to the opening of the anus. If the colon is the pheromone-producing site, the pheromone could be transmitted easily to atrial glands. If atrial glands are the pheromone source, it is difficult to imagine the substance diffusing to the colon in a reverse direction.

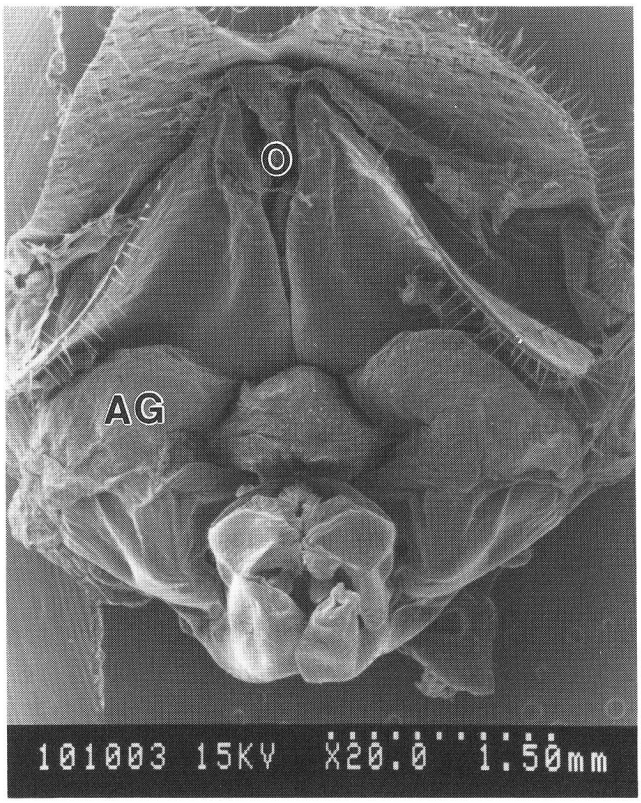

FIG. 3. SEM picture of the abdominal tip of virgin female *P. americana*, showing atrial glands (AG) and opening of anus (O).

If the atrial glands are the source, pheromone presence in the colon would more likely result from coprophagy. Further study of the sex pheromone source is certainly warranted.

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ELUTION PATTERNS FROM CAPILLARY GC FOR METHYL-BRANCHED ALKANES

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Abstract—A common and confusing problem in analyses of insect hydrocarbons is in making sense of complicated gas chromatograms and interpreting mass spectra since branched chain compounds differing by one or two carbons in backbone or chain length may elute from the column at nearly the same time. To address this confusing situation, relative gas chromatography (GC) retention times are presented for typical mono-, di-, tri-, and tetramethylalkanes comprising most of the commonly appearing series of homologous methyl-branched alkanes up to 53 carbons that are found in insect cuticular hydrocarbons. Typical insect-derived methylalkanes with backbones of 33 carbons were characterized by Kovats indices (KI); monomethyl alkanes elute between KI 3328 and 3374, dimethylalkanes elute between KI 3340 and 3410, trimethylalkanes elute between KI 3378 and 3437, and tetramethylalkanes elute between KI 3409 and 3459, depending upon the positions of substituents. A protocol is described for identification of methyl-branched hydrocarbons eluted from nonpolar polysiloxane DB-1 capillary GC columns. In this protocol, retention indices (KI values) are assigned to peaks, then the patterns in GC peaks that probably contain homologs are marked to assist subsequent GC-mass spectrometric (GC-MS) interpretation. Use of the KI allows assignment of likely structures and the elimination of others, with demonstrative consistency, as there are no known exceptions. Interpretation of electron ionization mass spectra can then proceed within narrowed structural possibilities without the necessity of chemical ionization GC-MS analysis. Also included are specific examples of insect hydrocarbons that were assembled from 30 years of the literature, and these are intended to help with confirmation of confusing or contradictory structures.

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Key Words—Hydrocarbons, alkanes, methyl-branched hydrocarbons, cuticular hydrocarbons, insects, GC-MS data, retention indices.

INTRODUCTION

Gas chromatography (GC) elution times may overlap for methyl-branched alkanes that differ by one or even two carbons in chain length, causing difficulty in interpretation of their mass spectra. Interpretation of the mass spectra of methylalkanes in many insect systems is time consuming because few synthetic standards are available for comparison of retention times, particularly for insect-produced cuticular hydrocarbons having chain lengths (carbon backbones) of 21–53 carbons. Existing mass spectral databases are very difficult to use for this purpose, because they contain few methyl-branched alkanes and computerized matching is poor, even for normal alkanes, particularly when mixtures of isomers are present in the same peak. Fortunately, the various classes of methyl-branched hydrocarbon compounds may be separated on nonpolar capillary columns by GC. Each class shows consistent elution patterns with increasing chain length and positions of methyl branching. Normal alkanes can be plotted on a nomogram as boiling point versus log of retention time (Hupe, 1965). Kovats retention indices (KI) (Kovats, 1965) are convenient for assigning relative retention times and for determining the numbers of carbons in the backbones, although this is usually done isothermally. Linear temperature programming is typically used for practical reasons, and true KIs are not obtained. However, KI values may be estimated or interpolated from consecutive *n*-alkane standards injected together on the same column with the same temperature program (Takeda, 1991).

Kissin and Feulmer (1986) described KI for the difference between the elution time of a standard *n*-alkane and a methyl-branched isomer with the same molecular weight, and systematized some examples [$KI = KI_{n\text{-alkane Cn}} - KI_{\text{isoalkane Cn}}$ (for OV-1)]. In this system the molecular weight must be known, which can be difficult to determine in hydrocarbons in general, particularly when multiple compounds coelute. Relative retention times are well documented for monomethyl-branched hydrocarbons (Kissin and Feulmer, 1986) and for some polymethylalkanes (Kissin et al., 1986) that included calculated retention times for "terminal" polyisoprenoid alkanes of less than 20 carbons. However, confusion of di-, tri-, and tetramethyl-branched hydrocarbons having different backbones and overlapping elution times remains common. Examples of specific isomers are often difficult to find in the literature for comparison of retention times, and often chromatograms are published without a useful scale or without retention indices.

We previously described insect hydrocarbons that display consistent

schemes of methyl branches with increasing chain length, often with visible differences of methyl-branching patterns between closely related species (Carlson et al., 1993). Plentiful examples can be found of mass spectra, particularly of biologically active compounds (Nelson and Blomquist, 1994). Early papers on insect methylalkanes of 20–40 carbons showed consistent increases of 0.25–0.3 carbon equivalents for each additional methyl branch when the carbon backbone was held constant (Nelson and Sukkestad, 1970; Nelson et al., 1972), with internal methylene bridges of three carbons being most common. Useful examples are found in the alkanes of the tsetse fly *Glossina morsitans morsitans* that have series of internally substituted di-, tri-, and tetramethylalkanes of 25–39 carbons in which the major components had three methylene interruptions only (Nelson and Carlson, 1986), but were later shown to include dimethylalkanes having longer interruptions (Sutton and Carlson, 1997a). Tsetse flies of other species, *G. pallidipes* and *G. tachinoides*, showed dimethylalkanes with larger interruptions of 7, 9, and 11 methylenes, and a later study of *G. palpalis* describes "terminal" trimethylalkanes (Nelson et al., 1988). Lange et al. (1989) primarily described methylalkanes and only a few dimethylalkanes.

We describe herein representative retention times on nonpolar columns for several rather complete series of di-, tri-, and tetramethylalkanes found in tsetse flies (Carlson et al., 1993; Sutton and Carlson, 1997a) and extended data for internal monomethyl alkanes to 60 carbons found in grasshoppers (Sutton et al., 1996). We include data from the literature covering most of the homologous methylalkanes from insects, where detailed retention data were included. Several novel structures from *Blattella* cockroaches (Brenner et al., 1993) are included, as are selected structures from beetles that were verified with synthetic compounds, parasitoid wasps (Bernier et al., 1998; Geden et al., 1998), mosquitoes (Carlson et al., 1997), and horseflies (Sutton and Carlson, 1997b). Work by others includes that on termites (Haverty et al., 1988), pine engraver beetles (Page et al., 1997), Colorado potato beetles (Szafranek et al., 1994), and screwworm flies (Pomonis, 1989; Pomonis et al., 1989). We describe retention times from capillary GC that were confirmed by electron ionization (EI) gas chromatography–mass spectrometry (GC-MS) with nonpolar capillary columns and chemical ionization (CI) GC-MS.

We also describe a protocol for identification of methyl-branched hydrocarbons by assigning KI (retention indices), then identifying patterns in clusters of GC peaks with the charted retention indices of hydrocarbons on a representative scale. This is the first extensive presentation of several series of di-, tri-, and tetramethyl hydrocarbons in which relative retention times were determined by capillary column GC-MS and were shown to correlate with positions of methyl substituents and with changes in lengths of interruptions between methyl branches.

METHODS AND MATERIALS

Insects. Laboratory-reared and wild insects were used as fresh or dried intact specimens for extraction of hydrocarbons. Crude hexane or ether extracts of males and females of different ages were used. Museum-deposited voucher specimens were also used as a source of hydrocarbons. Hydrocarbons were obtained from many species of tsetse flies (Nelson and Carlson, 1986; Nelson et al., 1988; Carlson et al., 1993), *Blattella* cockroaches (Carlson and Brenner, 1988), horseflies (Sutton and Carlson, 1997b), grasshoppers (Sutton et al., 1996), and parasitoid wasps (Bernier et al., 1998; Geden et al., 1998).

Chemical Samples. Cuticular hydrocarbons were usually extracted from individual specimens by rinsing with hexane (ca. 1 ml) or by soaking the insect in at least three changes of hexane over 1 hr. Pinned specimens were submerged from 1 hr to overnight in a vial containing 3 ml of hexane, removed by grasping the pin with clean forceps, and rinsed once. Pooled extracts were eluted with hexane from a 4-mm × 20-mm or 20-mm × 450-mm column of silica gel. The hydrocarbon-containing fractions that eluted with hexane were concentrated under a stream of nitrogen and subjected to argentation thin-layer chromatography or column chromatography on 5% silver nitrate impregnated silica gel to separate the alkane fraction from the smaller amounts of alkenes (Carlson and Langley, 1986). Alternatively, larger samples of total hydrocarbons were reconstituted in hexane, aliquots were eluted with hexane from small columns (4 mm ID × 40 mm) containing silver nitrate-impregnated silica gel to obtain alkanes, and alkenes and alkadienes were eluted with 1% and 5% ether in hexane, respectively.

Several synthetic samples were available from previous efforts, including tsetse fly sex pheromone compounds mentioned above, 33-, 35-, and 37-carbon backbone di- and trimethylalkanes (Carlson et al., 1978), and a series of synthetic 2,*X*-dimethylalkanes (Pomonis et al., 1989).

GC and GC-MS. Gas chromatography (GC) of the hydrocarbons was performed on a Tracor model 540 instrument fitted with a nonpolar capillary column (30 m × 0.32 mm ID, 0.25 μm film of DB-1) in the on-column mode (OCI-3, SGE, Dallas, Texas) with hydrogen carrier gas. The oven temperature was programmed from 60° to 325°C at 6°/min. The instrument was equipped with a flame ionization detector and a Nelson Analytical model 9000 PC data system. The detector was operated at 360°C with hydrogen carrier gas at 1.2 ml/min. Later analyses were performed on a Hewlett-Packard model 6890 instrument fitted with a nonpolar capillary column (25 m × 0.20 mm ID, 0.25 μm film of DB-1) in the on-column mode with hydrogen carrier gas. The oven temperature was programmed from 60° (hold 2 min) to 220°C at 20°/min, then 220°C to 310°C at 3°/min (hold 30 min). The flame ionization detector was held at 340°C, and a Perkin-Elmer Turbochrom model 4.1 PC-based system was used

to record data. All samples were diluted as necessary, and each was coinjected with consecutive *n*-alkane standards of 22–40 carbons for determination of relative retention indices. Based on KI, an *n*-alkane of 33 carbons was assigned KI 3300 and an internally monomethyl-branched alkane with 34 total carbons was assigned KI 3328 (33.28 carbons equivalent chain length or 0.28 carbons added to a 33-carbon alkane). If the column was overloaded, the KI value was 3335. Mass spectra were recorded on a Hewlett-Packard model 5988 quadrupole instrument interfaced to a HP 5890 Series II GC. The 30-m DB-1 column described above was used with an on-column injector and helium carrier gas at a linear flow rate of 35 cm/min, with a hold at 60°C for 2 min, then temperature programmed from 60° to 220°C at 20°/min, and finally programmed from 220 to 310°C at 3°/min (hold for 20 min).

We have not attempted correlate isothermal GC-calculated KIs and temperature-programmed KIs. About 80% of the data presented are from recent analyses in our laboratory, with the remaining data obtained from the literature or from unpublished data. Minor variation is possible with changes in temperature programs, column length, film thickness, possibly with injection technique, and certainly with overloading.

RESULTS

The interpretation of mass spectra of methylalkanes is difficult, particularly when a large number of isomers are present, as in many insects. Interpretation is facilitated by the recognition of patterns and can be improved by using a protocol that involves recognition of methyl branching patterns from known structures. For the discussion presented in this paper, "isomers" all have the same molecular weight but different branching patterns, whereas "homologs" have the same methyl branching pattern, but the backbones have different lengths.

The peak sizes of standards and unknowns should be as close as possible to get good KI values. Overloading the column's stationary phase should be avoided to minimize the shifting of peak centers. It may be more useful to use the front of the peak than the center, especially if methylalkanes are smeared together. The KIs provide useful information, allowing elimination of some structures, while strongly suggesting others. Polymethylalkanes with even-numbered methylene bridges are biosynthetically unlikely (de Renobles et al., 1991).

Protocol for Identification of Insect Methylalkanes

1. Assume that the most prominent peaks represent unbranched or normal *n*-alkanes with odd numbers of carbons in their chains (21–31 carbons, but rarely above 33), with smaller amounts of even-numbered homologs. These *n*-alkanes are often found in a bell-shaped distribution around a central peak,

sometimes with a separate minor distribution. Locate and mark these by comparing their retention times to standard *n*-alkanes run under the same conditions, or coinject an *n*-alkane standard (C_{20}–C_{40}) with both even- and odd-numbered backbones. Make a scale to mark the KI values for all peaks within the sample. This establishes the likely backbone length of each hydrocarbon, after which assigned KI values from GC analysis contribute significantly to interpretation of branching patterns. The protocol given in the following illustrative examples is based on straight-chain and methyl-branched hydrocarbons having 33 or 34 carbons in the backbone; 3300 refers to *n*-tritriacontane (KI 3300), and 3400 refers to *n*-tetratriacontane (KI 3400). Figure 1 shows that compounds eluting up to 160 KI units later than an *n*-alkane can have the same carbon chain length. However, there may be a bimodal distribution of peaks (C_{23-29}, C_{33-41}) if the largest peaks are mono- or dimethylbranched of more than 33 carbons.

2. Identify the odd-numbered backbone compounds first because of the relative simplicity of their mass spectra. These are generally the larger peaks, except in the 2-methylalkane series in which large peaks containing even-numbered backbone homologs may be present.

3. Locate and mark as methylalkanes the peaks ranging between KI 3328 and 3333. These are probably internally branched monomethyls (IMMs) with methyl branches near the center of the chains (typically at carbons 15, 13, and 11). The majority of methyl branches occur on odd-numbered carbons in odd-numbered backbone monomethylalkanes (15-methyl to 3-methyl, etc.), as they do in di-, tri-, and tetramethylalkanes. On the back shoulder of these peaks are 9-methyl isomers at KI 3336, then 7-methyl isomers at KI 3340, and 5-methyl isomers at KI 3350. The common 2-methyl isomers elute at KI 3362-65 just after 4-methylalkanes at KI 3358, although the latter are rare in insects. The last eluting monomethyl isomer at KI 3372-74 is the 3-methyl isomer.

4. Alkanes with even-numbered backbones will have very nearly the same last two digit KI values as do odd-numbered backbone homologs with methyl-branches in about the same location. Since one carbon may be added to either end of the carbon chain, the resulting isomers will have methyl branches residing on both even-numbered and odd-numbered carbons, while retaining the same methylene interruption. The mass spectra therefore become more difficult to interpret, particularly for those with multiple branches, so these should be addressed after finishing all odd-numbered backbone compounds. For simplicity, Figure 2 exhibits KIs for di-, tri-, and tetramethylalkanes having even-numbered backbones only.

5. Locate and interpret structures of peaks near KI 3355–3360. These are usually "terminal" dimethylalkanes (IDMs) with a short bridge having intervals (I) between methyl branch points of 3 methylenes (I = 3). Thus addition of one isoprene unit [containing three methylenes (I = 3) and a methyl-branched carbon] typically adds 0.25 carbons to monomethylalkane KI values, while longer

methylene bridges (I = 7, 9, 11, 13) add 0.33 carbons, and the compounds elute later, near 3363. Dimethylalkanes with even-numbered methylene bridges are rare, if not biosynthetically impossible, in internally branched alkanes, and intervals of five methylenes (I = 5) are rare. Common near KI 3410 are "terminal" 3,*X*-dimethyl branched alkanes (I = 3) with the first branch on carbon 3, in which addition of one isoprene unit to a starting 3-methyl alkane has typically added 0.35 carbons. For larger intervals between methylene branches (I = 9, 11, 13), the compounds elute earlier, near KI 3403, an addition of 0.28 carbons. Less common are 2,*X*-dimethyls that elute on either side of an *n*-alkane of the same chain length depending on the interruption. Thus, 2,*X*-dimethyl isomers elute near KI 3395, when the methylene bridge is longest (I = 9, 11, 13), but together near the *n*-alkane when I = 5 or 7, and elute later near KI 3405 when I = 3.

6. Locate and interpret structures of peaks near KI 3380. These may be trimethylalkanes having short methylene bridges (I = 3/3) between internal methyl branches. Mixed length bridges eluting slightly later (KI 3383) are encountered less often (I = 3/5, 3/7 and sometimes 5/3). It is necessary to first locate and eliminate fragments from 2- and 3-methyl alkanes from these spectra before attempting interpretation of the rest of the fragments. Thus, addition of a third methyl branch with one methylene bridge (I = 3) typically adds 0.25 carbons to the KI value of a dimethylalkane. Peaks eluting near KI 3416–3420 may be "terminal" trimethyl-branched even-numbered backbone alkanes (I = 3/3) with the first branch on the 4-carbon, typically a 4,8,12-trimethyl. Peaks eluting near 3437 may be I = 3/3 homologs that have one more or one less carbon in the chain, with the first branch on the 3-carbon, and typically have 3,7,11-trimethyl branching: These homologs may coelute with IMMs of the next chain length, making interpretation tricky.

7. Locate peaks that elute at KI 3405–3410, or about 0.25–0.30 carbons after internal trimethyl isomers and just after the next (34-carbon) *n*-alkane. Peaks at 3405 may be internal tetramethylalkanes with the first methyl at 13- or 11- and I = 3/3/3 or some small interval. A tetramethyl hydrocarbon with the first branch at 9- and I = 3/3/3 elutes near KI 3418, and with a first branch at 7- it elutes near KI 3430. With a first (terminal) methyl branch at 3-, it elutes near KI 3459 (I = 3/3/3). We have examples of tetramethylalkanes with I = 3/3/3, and none with longer methylene interruptions.

8. The principles and procedures outlined above can be applied to all other sets of alkanes with a single backbone chain length.

Specific Examples in Support of the Protocol and the Figures

Figure 1 illustrates elution patterns measured or estimated for typical methylalkanes of 33-carbon backbones and is meant to be general for compounds with odd-numbered backbones. It shows typical methyl-substituted tritriacon-

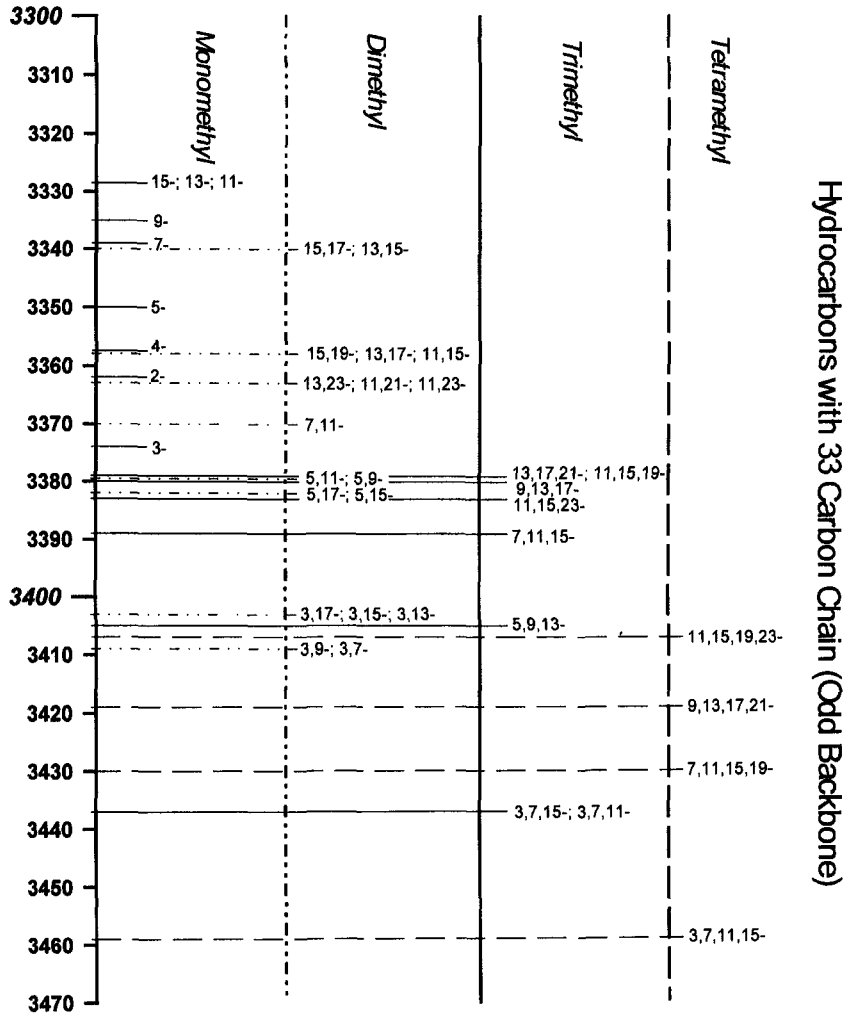

FIG. 1. Measured or estimate elution patterns of mono-, di-, tri-, and tetramethylalkanes with an odd-numbered backbone length of 33 carbons in insect hydrocarbons. KI values are shown in the legend and may be applied generally from C_{23} to C_{40} insect-derived alkanes with caveats as noted in the text.

tanes (alkanes with backbones of 33 carbons) with observed or estimated KI values, in which all monomethyl tritriacontanes elute between KI 3328 and 3374 (total 34 carbons), dimethyl tritriacontanes elute between KI 3340 and 3410 (total 35 carbons), trimethyl tritriacontanes elute between KI 3377 and 3437 (total 36 carbons), and tetramethyl tritriacontanes elute between KI 3407 and

3459 (total 37 carbons). This illustration is valid for methyl-branched alkanes of 25–40 carbons, with the caveats noted below for shorter or longer chain lengths, as illustrated specifically in Figures 3–5 below.

Figure 2 illustrates elution patterns measured or estimated for typical methylalkanes with 34 carbons in the backbone and is meant to be general for com-

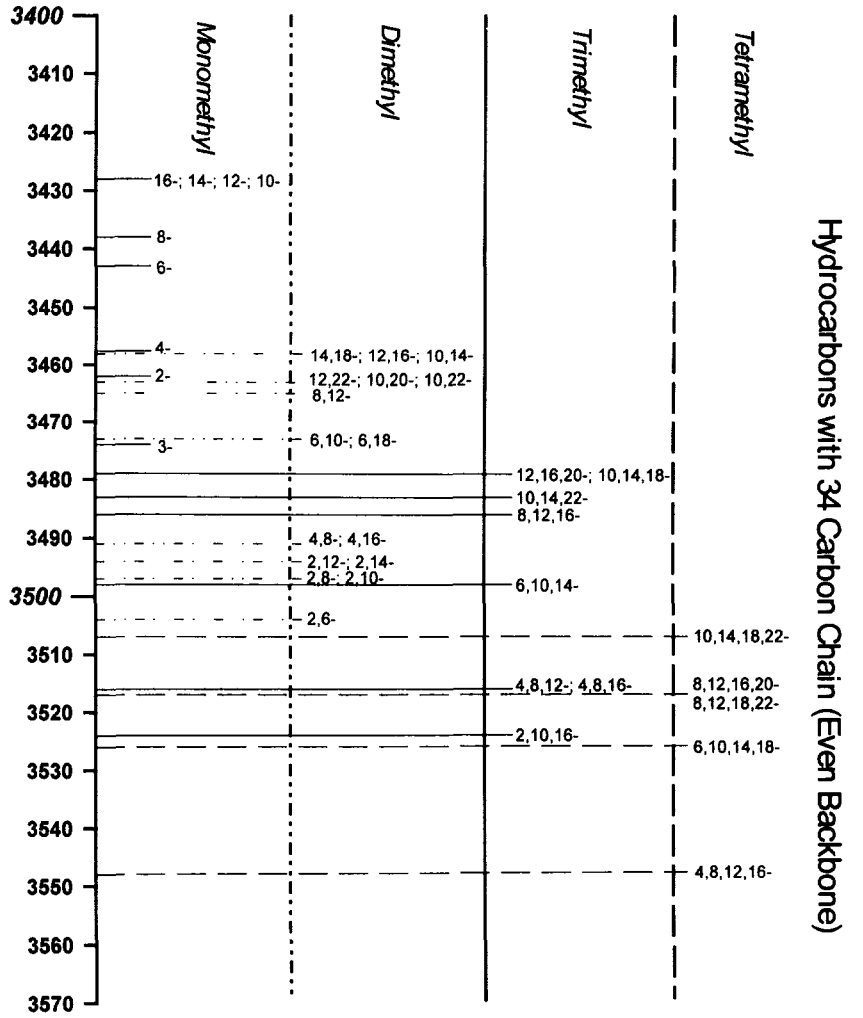

FIG. 2. Measured or estimated elution patterns of mono-, di-, tri-, and tetramethylalkanes with an even-numbered backbone length of 34 carbons in insect hydrocarbons. KI values are shown in the legend and may be applied generally from C_{23} to C_{40} insect-derived alkanes with caveats as noted in the text.

pounds with even-numbered backbones. Methyl branches at odd-numbered positions may be seen on alkanes with even-numbered backbones, but a number of compounds do not appear in Figure 2, as we did not observe them in odd-numbered backboned methylalkanes (i.e., 5- and 7-IMM, and 5,*X*-, and 7,*X*-IDM).

Methylalkanes. We concur with the assignments of Kissin and Feulmer (1986), who showed that relative retention times of methylalkanes increase smoothly when the chain length is held constant and the methyl branch is moved from the center carbon toward the 2-position at the end of the chain. 3-Methylalkanes elute last. We found close agreement with Kissin and Feulmer (1986) for plotted relative retention times of IMMs that successively eluted earlier after 30 carbons with increasing chain length, as in KI 3725 (Figure 3). The present work extends the data for IMMs to 53 carbons, with the heaviest homologs eluting at KI 5319.

Dimethylalkanes. We observed that the plotted relative retention times of IDMs decline smoothly with increasing chain length (KI 2765 to KI 3950) (Figure 4). KI values for Figure 1 were estimated from Figure 4 if an actual value was not available. Relative retention times of internal dimethylalkanes increase smoothly by about 0.14 carbons as the methylene bridge length increases (I = 7, 9, or 11) because the second methyl branch in dimethylalkanes can be considered to be moved past the center of the chain toward the other end.

The slight declines or increased shifts in retention indices observed with increasing chain length for the dimethylalkanes are real and are discussed here as they appear at the lower part of Figure 4, with the IDMs eluting earliest. The IDMs with lower interruptions (I = 3) included 9,*X*-, 11,*X*-, 13,*X*-, and 15,*X*-isomers and these showed the largest range of shifts with increasing chain length. However, peaks also containing the internally branched series with higher interruptions are split (I = 9, 11), because the latter isomers eluted considerably later than those with I = 3, typically by 8 KI units at 35, 36, and 37 carbons. This was seen in hydrocarbons from *Melanoplus* grasshoppers (Sutton et al., 1996) and tsetse flies (Sutton and Carlson, 1997a).

The 8,*X*-, 7,*X*-, and 6,*X*-series ranges were relatively narrow, with downward trends of 4 or 5 KI units observed with increasing chain length. There is a potential anomaly for 7,15-C_{27}, that appears to elute 3 KI units too early.

The values for 5,*X*-dimethyls were split, and the lower methylene-interrupted 5,*X*-series (I = 3) was shifted by 8 KI units to lower values over the range of 14 carbons, with a potential anomaly at 5,9-dimethyl-C_{35} that eluted 4 KI units too late. The 5,*X*-series with larger interruptions (I = 11) was nearly flat at KI 2192–2791, adding about 91 KI units to each backbone value. An anomaly was 5,13-Me_2C_{23} (I = 7), found at KI 2377, rather lower than expected by 7 KI units.

Relative retention times of "terminal" dimethylalkane isomers decrease

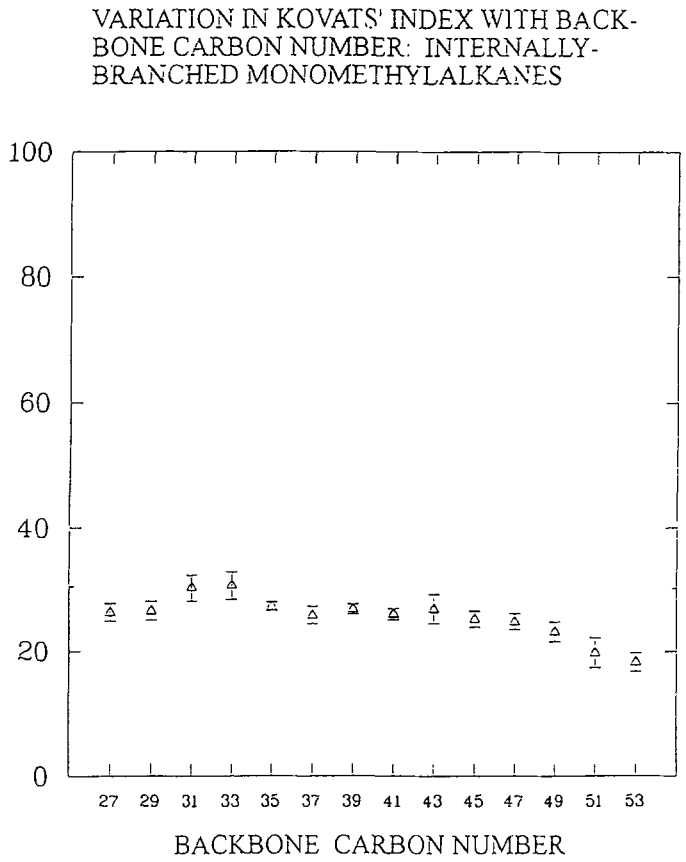

FIG. 3. Variation in KI with backbone carbon number: internal methylalkanes from *Melanoplus sanguinipes* coinjected with alkane standard up to 60 carbons (25 m × 0.20 mm ID, SBP-1, Supelco) (means of 10 analyses).

smoothly with increasing methylene bridge length because the second methyl branch can be considered to be moving toward the center of the chain. The overriding principle is that with a methyl branch moving toward the end of a chain, the isomer elutes later, and the effect of the terminal methyl is most important. With a methyl branch moving toward the center of a chain, the isomer elutes earlier. The 4,*X*-series appears to decline slightly over the range of compounds observed, from KI 2694 to 3489, changing by about 5 KI units over the range of C_{25}–C_{35}.

The 2,*X*-series was obtained from literature values, except for a synthetic 2,*X*-C_{27} series including 2,6- (KI 3005), 2,8-, 2,10- (KI 3002), 2,12-, and 2,14-

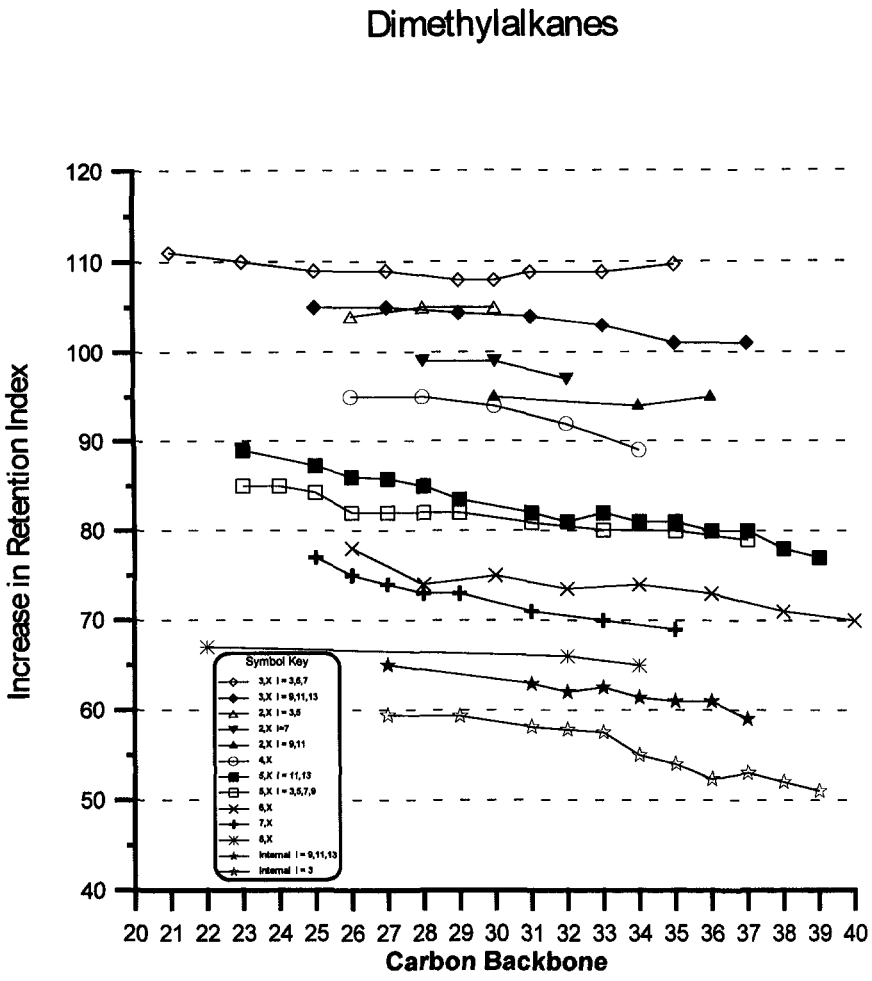

FIG. 4. Variation in KI with backbone carbon number compared to *n*-alkane standards: dimethylalkanes. Example-experimental values for internal dimethyls (I = 3/3) declined incrementally from KI 2759 (13,17-Me_2C_{27}) to KI 3952 (15,19-Me_2C_{39}).

dimethylnonacosanes (KI 2994). These results show that the lower methylene-interrupted 2,*X*-series (I = 3) did not shift to lower values over the range examined, but the 2,*X*-series with larger interruptions (I = 7, 9, 11) showed a shift downward.

The lower methylene-interrupted 3,*X*-series (I = 3, 5, 7) eluted after all other dimethyls and tended not to shift to lower values over the range examined,

declining from KI 2609 to KI 3007, then increasing slightly to KI 3609. The overall shifts in KI values were 107 to 110 KI units from C_{25} to C_{35}; this KI value is added to the carbon backbone for each homolog. Thus the measured retention value for 3,7-dimethyl C_{33} is 3409 (see Figure 1). In contrast, the 3, *X*-series with larger interruptions (I = 11) declined over the series from C_{25} to C_{37} by 8 KI units, starting at 2609 and ending at 3801. Thus, the measured retention value for 3,15-dimethyl-C_{33} is KI 3403.

Trimethylalkanes. The slight declines or declining shifts in retention indices observed with increasing chain length for the trimethylalkanes are discussed as they appear from the top of Figure 5, with the 3,*X*,*Y*-trimethylalkanes eluting

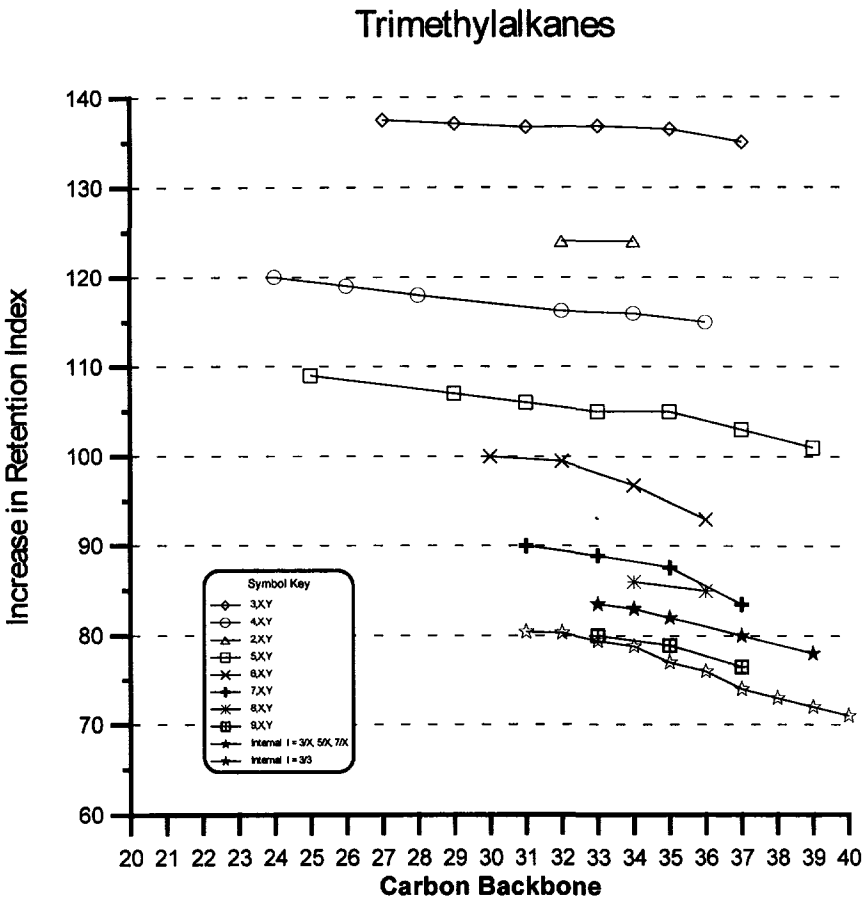

FIG. 5. Variation in KI with backbone carbon number compared to *n*-alkane standards: trimethylalkanes.

latest, and the internal trimethylalkanes eluting earliest. In each series, most of the trimethylalkanes contained two 3-methylene bridges (I = 3), but this typically varied when the corresponding dimethylalkanes had larger interruptions, as the third methyl branch was often added internally into a I = 9 or 11 methylene bridge.

Tetramethylalkanes. Internal tetramethylalkanes (I = 3/3/3) eluted near KI 3395, or 0.21 carbons after internal trimethylalkanes marked as 3374. External tetramethyl compounds such as 3,7,11,15-series eluted last at 3459, about KI 160 units later than the corresponding alkane, or 0.22 carbon equivalents after the corresponding external trimethylkanes. (Figure 6).

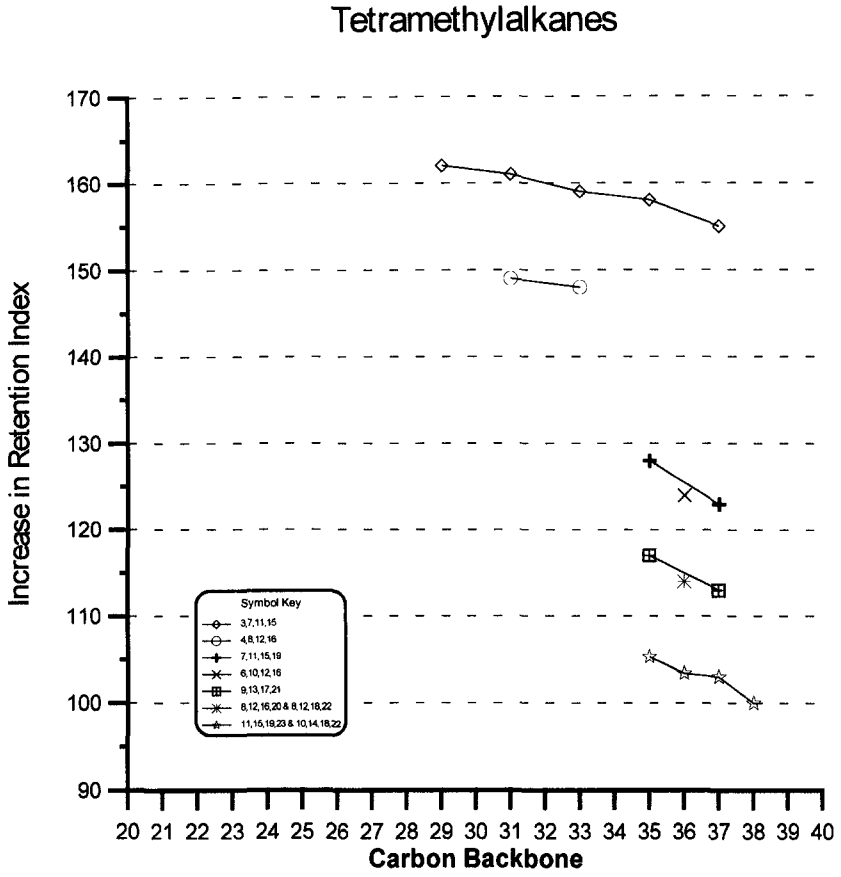

FIG. 6. Variation in KI with backbone carbon number compared to *n*-alkane standards: tetramethylalkanes.

DISCUSSION

We believe that the figures shown here will assist those attempting to decipher insect polymethylalkane structures, for which there are a restricted number of overlying patterns (Blomquist et al., 1987). These structures could be considered to rise from the apparent addition of isoprene units, although this is not meant to apply in the context of biosynthesis. The most common IDMs observed have 3-methylene interruptions, with 7-, 9-, and 11- bridges less common, while trimethylalkanes often have two 3-methylene interruptions, and all tetramethylalkanes had only 3-methylene interruptions. Most data in the literature do not include KI values or have few calculated KI values or carbon equivalents in which exact measurements were reported. In some cases, particularly GC-MS runs in which identifications were critical, sample overloading caused late elution of one or two peaks, and these late KI values were not included here. In these cases, we inspected GC-MS runs that first had been run very dilute, to get more accurate data for the larger peaks. It must be pointed out that recent KI values obtained from long narrow-bore (0.10–0.15 mm) capillary columns of 5% phenyl siloxanes such as DB-5 are substantially lower for polymethylalkanes than values described here. Observations that tri- and tetramethylalkanes elute more rapidly on these columns has not been previously reported, to our knowledge, and will be reported elsewhere.

The discussion of hydrocarbon identification by Lockey (1988) is illustrative with many examples given. However, the present effort refutes Lockey's statement that retention indices are "an unreliable indicator of carbon number owing to the effect which methyl branches have on retention times." Use of high-resolution columns and avoidance of overloading the column allow more precise, consistent, and indicative KI values to be assigned. Lockey (1988) stated that "An *n*-alkane of a hydrocarbon mixture frequently elute[s] with terminally-branched dimethylalkanes," with his examples (Figure 7, p. 625) showing 2,*X*-dimethyl nonacosanes eluting (at about KI 2895) just ahead of *n*-C_{29}, and 3,*X*-dimethyl triacontanes eluting (at about KI 3006) just after *n*-C_{30}. These statements are consistent with the present findings.

Lange et al. (1989) describe a strategy for identification of insect hydrocarbons that involves silica gel and argentation chromatography followed by GC and GC-MS with CI-GC-MS. Their mass spectral interpretation followed Nelson and Sukkestad (1970) and Nelson et al. (1972), as does ours, although we also referred to Nelson (1978) and Blomquist et al. (1987).

Methylalkanes. Lange et al. (1989) presented one table that showed carbon equivalent retention times to two decimals and contained IMMs, but only a few dimethylalkanes with mass spectra were presented. The KIs of IMM from *Reticulitermes* termites showed a decrease with increasing chain length from C_{25} (KI 2533) to C_{31} (KI 3131) with temperature-programmed values (L. Nelson,

unpublished data). Kissin and Feulmer (1986) showed similar trends for monomethylalkanes of 36 carbons or less, the upper limit of their published data.

Dimethylalkanes. The earliest-eluting dimethylalkane, 13,15-Me_2C_{29}, is a rare variant that has monomethylene spacing (I = 1). It occurs in the imported fire ant *Solenopsis invicta* and elutes near KI 2940. It occurs also in several termites including *Coptotermes formosanus* (Haverty et al., 1990), but has slightly longer retention times (KI 2947). This was ascribed to the presence of two diastereomers with very similar mass spectra. The early-eluting enantiomer appears consistently in termites, in which it elutes just after internally branched monomethylalkanes. Other GC-MS data presented for dimethylalkanes with 1-, 2-, and 4-methylene bridge carbons from *Hypoponera eduardi* ants (Lange et al., 1989) appeared dubious, but since no retention indices were presented, any suggestion of alternative structures is not possible. Although CI-GC-MS was performed in that study, we believe that even-numbered bridges in dimethylalkane structures are rare in insects, if they exist at all. However, synthetic enantiomers of 9,11-Me_2C_{29} eluted separately, just after internal methylalkanes (Pomonis et al., 1980).

The earliest eluting dimethyl isomers usually found are the symmetrical IDMs (I = 3) that elute near KI 3355, 3555, 3755, and 3955 in *G. morsitans* (Nelson and Carlson, 1986) (Figure 4). The 15,19- and 15,21-dimethyl alkanes elute at KI 3355 with I = 3 and 5 in *G. austeni*, although the latter are rare. Isomeric 9-methylene interrupted (I = 9) alkanes were present in *G. pallidipes* (Nelson and Carlson, 1986), and both natural and synthetic 13,23- and 15,25-dimethylheptatriacontane eluted at KI 3763. These compounds were incorrectly described as isomers of 13,17-dimethylheptatriacontane in another report on the same insect, although the mass spectra were consistent with the 13,23- and 15,25-isomers, as were the retention times (McDowell et al., 1981). Isomeric IDMs in *G. tachinoides* contained 11,19- and 13,21-dimethylheptatriacontane having longer methylene bridges (I = 11) that eluted at KI 3763 (Nelson and Carlson, 1986) together with synthetic 11,19-, 13,23-, and 15,25-dimethylheptatriacontane (Carlson, unpublished data). Long series of isomeric I = 9 and I = 11 IDMs were present in *Melanoplus* (Sutton et al., 1996). The 7,*X*-dimethyls also appeared to elute with this group. Several 5,*X*-(KI 3382) and 4,*X*-dimethyls (KI 3392) were found in *G. brevipalpis* (Nelson et al., 1988), and 5,*X*-dimethyls appeared in termites (Haverty et al., 1988).

Much more common are 3,7-dimethylnonacosane and its 3,*X*-isomers, such as those in *Blattella* cockroaches that coelute at KI 3010. The 3,*X*-isomers with 27 (KI 2810) and 31 (KI 3210) carbon backbones eluted at the same relative position, although we did not detect 33 carbon backbone homologs (Carlson and Brenner, 1988) (Figure 4).

Szafranek et al. (1994) described the retention times of terminal di- and trimethyl-branched alkanes from two species of Coleoptera and the mass spectral

interpretation by utilizing daughter ion mass spectra. Daughter ions derived from the molecular ion were usually observed as a large fragment from cleavage internal to the longest alkyl chain, but smaller fragments were not diagnostic. They observed several 2,*X*-dimethylalkanes that elute close to *n*-alkanes and their trimethyl homologs. Curiously, the major components had even-carbon-numbered backbones. Szafranek et al. (1994) showed KIs quite consistent on OV-I columns for 2,10-dimethylalkanes as well as those with higher interruptions that eluted slightly earlier (KI 2899, 3096, 3297, 3497) and 2,6-dimethylalkanes (KI 2704, 2905, 3105, 3305). KIs for their 2,10,18-trimethyl alkanes (I = 7/7) were internally consistent (KI 3324 and 3524) eluting about 10 KI units before internal monomethylalkanes with one more carbon backbone. Pomonis et al. (1989) described 2,6-dimethylalkane isomers that are not often seen except in screwworm flies, and obtained GC-MS data for synthetic isomers of 2,14- and 2,12-dimethylheptacosane (KI 2795), whereas 2,10- and 2,8-eluted just after the *n*-alkane (KI 2802), and the 2,6-dimethyl isomer (KI 2806) coeluted with a 3,*X*-isomer (Pomonis, 1989). We obtained essentially the same results with these synthetic isomers. Thus, it may still be useful to separate unbranched from branched alkanes with molecular sieving, especially if there is an excess of *n*-alkane. Although better separation can be obtained on a higher-resolution column, these are the only two common classes of insect-produced dimethylalkanes that could be confused with *n*-alkanes (Figure 2). Numerous other examples from different insect groups could be cited. Many are listed in Nelson (1993) and Nelson and Blomquist (1994).

Trimethylalkanes. The earliest-eluting trimethylalkane is the symmetrical internal 15,19,23-trimethylheptatriacontane at KI 3775, with homologs having 33- to 39-carbon backbones. All of these contain internally branched 3-methylene bridges (I = 3) and are present in *G. morsitans centralis* and *G.m. submorsitans* female tsetse flies (Figure 7). The series may include 13,*X*,*Y*- and 11,*X*,*Y*-isomers, with partially separated 9,*X*,*Y*-, followed by clearly separated 7,*X*,*Y*-isomers.

The next eluting isomer is the 5,9,13-trimethylnonacosane (KI 3005) in *G. tachinoides* males (Nelson and Carlson, 1986). A commonly found terminal trimethyl variation with an odd-numbered backbone is 4,8,12-trimethylheptacosane (KI 2820) and its 3,7,11-C_{29} homolog at KI 2920 in *G. tachinoides* males. A rare 2,6,10-trimethyl homolog was observed that eluted at KI 2825, just after these 4,8,12-isomers, which seems consistent with the elution patterns of the 4,8- and 4,10-isomers at KI 3392 eluting before the 2,*X*-dimethyl isomers. It seems to contradict Figure 1 of Kissin and Feulmer (1986), which has a 2-methyl alkane isomer eluting at KI 3363 but the 4-methyl isomer eluting at KI 3368, and a 3-methyl isomer at KI 3372. This was the only obvious contradiction that we found with Kissin and Feulmer (1986). A series of several other short 5,*X*,*Y*-trimethylalkanes were described from *Reticulitermes* termites (Hav-

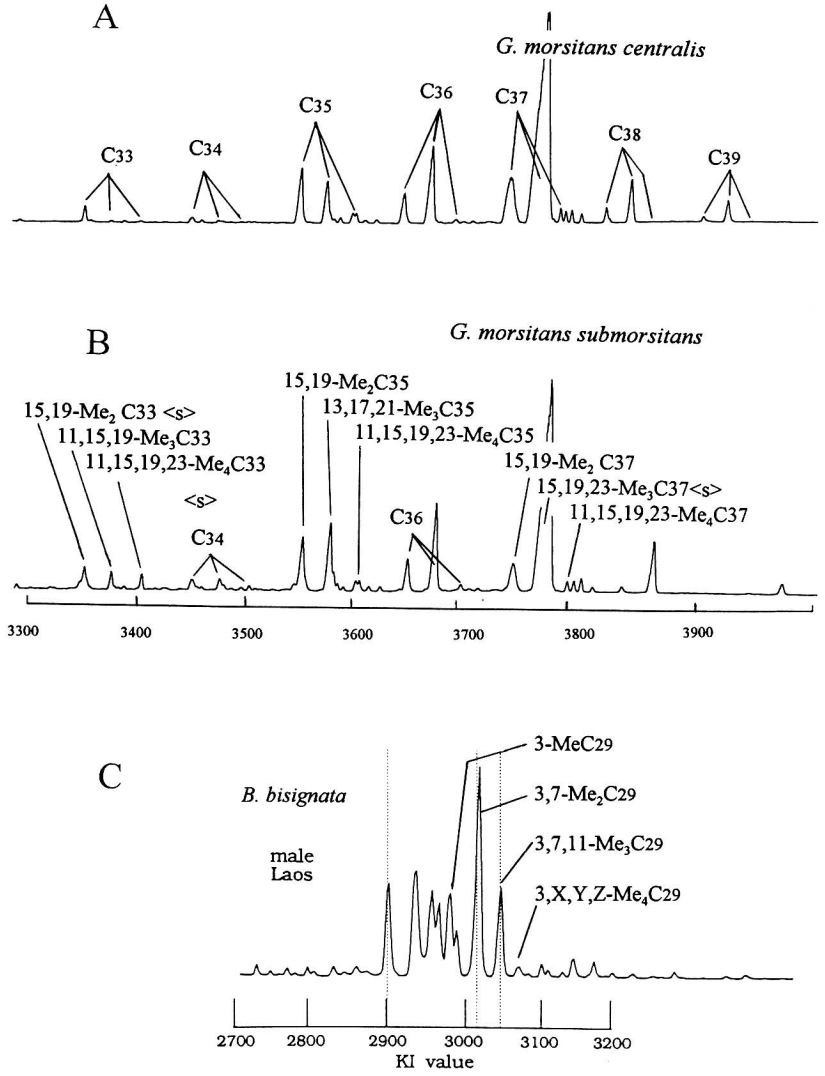

FIG. 7. Elution pattern of 15,19-dimethyl-, 15,19,23-trimethyl-, and 15,19,23,27-tetramethylalkanes of 33- to 39-carbon backbones from tsetse fly females. Add 0.20–0.25 carbons for each additional methyl branch. ⟨s⟩: symmetrically substituted. (A) *G. m. morsitans*. (B) *G. m. submorsitans*. (C) Elution pattern of 3-methyl-, 3,7-dimethyl-, 3,7,11-trimethyl- and 3,*X*,*Y*,*Z*-tetramethyl C_{29} from male *Blattella bisignata* cockroaches.

erty et al., 1996; Page et al., 1997; L. Nelson, unpublished data). The latest-eluting trimethylalkanes include "terminal" 3,7,11-trimethylhexacosane (KI 2740) and its 28-carbon backbone homolog (KI 2940) in *G. p. palpalis* males and several other species of tsetse flies in this group (Nelson et al., 1988).

Tetramethylalkanes. Internal tetramethylalkanes (I = 3/3/3) elute near KI 3390, or 0.16 carbons after internal trimethylalkanes marked as 3374. The specific example is 11,15,19,23-tetramethyl-C_{37}, found at KI 3790 in *G. m. morsitans* females (Nelson and Carlson, 1986), the first report of a homologous series of di-, tri-, and tetramethylalkanes in the same insect. An unusual terminal tetramethyl homolog eluted just after 2-methyltriacontane at KI 3065 in *Blattella* cockroaches. Reexamination of the spectra of male and female *B. asahinai* clearly shows the same homologous series that was not interpreted in the original paper (Carlson and Brenner, 1988). Inspection of the small, broad KI 3065 peak in *B. bisignata* and *B. asahinai* by EI- and CI-MS confirmed the presence of 2-methyltriacontane [*m/z* 393 (primary ion), 435 (M − 1)] that eluted just ahead of a tetramethyl homolog [*m/z* 463 (M − 1)] of the KI 3010 peak (3,7-dimethyl-C_{29}) and KI 3040 peak (3,7,11-trimethyl-C_{29}) (Figures 6 and 7C). The EI spectra contained fragment ions (*m/z* 127, 197, 224/225, 267, 295, and 365) consistent with 3,7,11,15-tetramethylnonacosane. This tetramethylalkane is the latest-eluting C_{29}-backbone compound known in insects and completes an homologous series of di-, tri-, and tetramethylalkanes that were also observed in the closely related *B. asahinai* (Brenner et al., 1993). *Glossina morsitans morsitans* females showed a nearly complete series of I = 3 internally branched, di-, tri-, and tetramethylalkanes (11,*X*-, 11,*X*,*Y*-, and 11,*X*,*Y*,*Z*-), and the monomethylalkanes were present at very low levels (Nelson and Carlson, 1986). There were externally branched series in *Muscidifurax* (Bernier et al., 1988) and *G. brevipalpis* (3-, 3,*X*-, 3,*X*,*Y*-, and 3,*X*,*Y*,*Z*-; 5,*X*-, 5,*X*,*Y*-, and 5,*X*,*Y*,*Z*-) (Nelson et al., 1988). These were also present as a complete series in *Melanoplus* (Sutton et al., 1996).

The compounds plotted here comprise most of the known insect hydrocarbon structures published or available to us that were accompanied by reference to reliable standards. The plots are mostly based on recent capillary GC-MS runs with standards. We are confident in the descriptive data and have not attempted to develop a formula to calculate retention times. If overloaded conditions are avoided, we suggest that the experimental values will fall within a few KI units of what is reported here.

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ATTRACTION OF 3-METHYL-1-BUTANOL AND AMMONIA IDENTIFIED FROM *Enterobacter agglomerans* TO *Anastrepha suspensa*

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Abstract—Tests demonstrated that volatile chemicals emitted from *Enterobacter agglomerans*, a bacterium that has been isolated from adults as well as fruit infested with larvae of the Caribbean fruit fly, *Anastrepha suspensa* (Loew) and other pest fruit flies, are attractive to female *A. suspensa* in laboratory bioassays. 3-Methyl-1-butanol and ammonia were identified as the two primary volatile chemicals released from active cultures of *E. agglomerans*. No 3-methyl-1-butanol and little ammonia (16.0 μg/hr) are released from sterile tryptic soy agar plates. *E. agglomerans*-inoculated tryptic soy agar plates, however, released an average of 1.5 ± 0.53 μg/hr 3-methyl-1-butanol and 332.9 ± 239.16 μg/hr ammonia after 24 hr of growth. 3-Methyl-1-butanol lures were formulated in a membrane-based system to provide a constant release rate of synthetic chemical. Release rates ranged from 0.046 ± 0.007 to 12.16 ± 2.76 μg/hr. In laboratory tests, equal numbers of females were captured in response to ammonium carbonate lures that released ammonia at the rate of 100 μg/hr and to 3-methyl-1-butanol lures that released 12.16 ± 2.756 μg/hr of synthetic material. The combination of the two lures was more attractive than ammonia alone. Availability of lures formulated for a range of 3-methyl-1-butanol release rates will facilitate field tests of this puta-

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tive microbial attractant and may lead to a better understanding of the role of bacteria in the ecology of pest fruit flies.

Key Words—Insecta, Tephritidae, *Anastrepha suspensa*, *Enterobacter agglomerans*, attractants, volatiles, bacteria, 3-methyl-1-butanol, ammonia, lures.

INTRODUCTION

Bacteria in the family Enterobacteriaceae have been found in association with tephritid fruit flies (e.g., Rubio and McFadden, 1966; Boush et al., 1972; Rossiter et al., 1982; MacCollom and Rutkowski, 1986; Jang and Nishijima, 1990), and bacteria in this family may be strongly attractive to fruit flies (Drew and Lloyd, 1989; Martinez et al., 1994). Bacteria on plant surfaces may serve as a protein source for adult tephritids in nature (Drew et al., 1983). Drew and Fay (1988) hypothesized that increased capture of *Bactrocera tryoni* (Froggatt) in liquid protein bait with bacteria was due to volatile metabolites produced by bacterial growth. Davis et al. (1984) demonstrated that there was greater attraction of Caribbean fruit flies, *Anastrepha suspensa* (Loew), to liquid protein bait solution in McPhail traps versus liquid protein bait placed on cotton wicks in Jackson traps and speculated that this was due in part to volatile end products from microbial breakdown that occurred in the McPhail traps.

Several studies have evaluated the attractiveness of various bacteria found in association with tephritids. Jang and Nishijima (1990) isolated 14 bacterial species from wild and laboratory-reared Oriental fruit flies, *Bactrocera dorsalis* Hendel, and most of these bacteria belonged to the family Enterobacteriaceae. They found that several bacterial species were more attractive to female than to male flies and that washed cells, (cells that were separated from the growth media) were more attractive than water or phosphate buffer. Several strains of *Staphylococcus aureus* were attractive to adults of the Mexican fruit fly, *Anastrepha ludens* (Loew) (Robacker et al., 1991). Attraction to bacterial odors was mediated by feeding history of the flies, as response to bacterial odors decreased with increased sugar hunger (Robacker and Garcia, 1993) and increased with increased protein hunger (Robacker and Moreno, 1995). Ammonia, which is known to be the primary fruit fly attractant that is emitted from liquid protein baits (Bateman and Morton, 1981; Mazor et al., 1987), is produced by microbial growth (e.g., Howell et al., 1988; Scarpati et al., 1996). Several volatile chemicals have been identified from headspace analysis of bacteria and/or culture media (Robacker et al., 1993; Lee et al., 1995; DeMilo et al., 1996), although Robacker and Flath (1995) could not determine if the chemicals that were biologically active in laboratory bioassays were produced by the bacteria or were an artifact of the analytical procedure. Thus, it is not known if there are volatile

chemicals in addition to ammonia that are attractive to fruit flies and produced by actively growing bacteria.

Enterobacter agglomerans is one of several Enterobacteriaceae isolated from adults of the apple maggot, *Rhagoletis pomonella* (Walsh), and from apple maggot-infested fruit (MacCollom et al., 1992). Washed cell preparations of an apple maggot-associated isolate of *E. agglomerans* were attractive to foraging adults in field trials (MacCollom et al., 1992). Culture plates inoculated with *E. agglomerans* are attractive to apple maggot flies in laboratory bioassays (C.R.L., unpublished data). This bacterium has been isolated from adults, larvae, and fruit infested with larvae of the Caribbean fruit fly, *Anastrepha suspensa* (Lowe), field collected in south Florida (C.R.L., unpublished data). Preliminary laboratory studies found that female *A. suspensa* are attracted to volatiles from *E. agglomerans* in laboratory bioassays. Therefore, studies were initiated to quantify ammonia release from *E. agglomerans* washed cells and culture plates and to identify other attractant chemicals released from these substrates. Based on spectroscopic analysis of volatiles, we identified 3-methyl-1-butanol (3-MeBuOH) as a major component. Release rate was quantified, and synthetic 3-MeBuOH was formulated in membrane-based controlled release lures for laboratory tests of biological activity alone and in combination with ammonia.

METHODS AND MATERIALS

E. agglomerans Culture. Stock cultures were maintained in Amherst, Massachusetts. For use in bioassays and chemical analyses, *E. agglomerans* in early log phase were plated on tryptic soy agar (TSA, Difco Laboratories, Detroit, Michigan) and sent via overnight delivery from Amherst, Massachusetts, to Gainesville, Florida. Sterile TSA plates were included as controls. Fresh cultures were shipped periodically to Gainesville and samples of cells subcultured there were shipped back to Amherst for confirmation of culture purity. Upon receipt, plates were left at room temperature overnight to ensure a minimum of 24 hr of growth. Growth of colonies on plates prior to arrival in Gainesville was affected by ambient temperatures during shipment, so there was variation in the amount of growth that occurred before the plates arrived. For comparative purposes, chemical analyses were conducted on plates allowed to grow an additional 24 hr to determine if further growth affected quantitative release of volatile chemicals. After 24 or 48 hr of growth, plates were tested immediately or placed in a refrigerator (7°C) until used. Plates removed from the refrigerator were held at room temperature for at least 1 hr, and plates were used within one week of receipt. For studies that tested washed cells, cells were scraped from the TSA and subcultured in tryptic soy broth (TSB, Difco Laboratories, Detroit, Mich-

igan) at 25°C. *E. agglomerans* was propagated by inoculating one loopful (3 mm diam) of a 24-hr subculture into test tubes containing 10 ml TSB and incubating for 24 hr at 25°C. Cells were separated from the culture medium by centrifugation for 10 min at approximately 17,000*g* (0°C), and the TSB supernatant was discarded (MacCollom et al., 1992). Cells were washed and concentrated by resuspending the pellet in sterile water (deionized), and samples were combined and centrifuged again to remove residual media from cells. This process was repeated twice. The final pellet from 40 ml subculture was resuspended in 10 ml of sterile water to obtain a fourfold increase in concentration.

A. suspensa *Culture.* Caribbean fruit flies used were obtained as pupae from the Florida Department of Agriculture and Consumer Services, Division of Plant Industry in Gainesville. Flies were maintained as reported previously (Epsky et al., 1993). Females that were 4–12 days posteclosion were used for all trials. Fully fed flies were used for all bioassays to reduce biased attraction to ammonia, which is enhanced by subjecting flies to protein starvation prior to testing (Robacker, 1991).

Volatile Collection and Chemical Analysis. Capillary gas chromatography (CGC) analyses were conducted to identify and quantify volatile chemicals from *E. agglomerans*. Headspace volatiles were collected for 1 hr at 1 liter/min airflow using purified air and were analyzed by collection systems described previously (Heath and Manukian, 1992; Heath et al., 1993). Initially, volatile collections were made from washed cells (20 ml), TSB supernatant (20 ml), and sterile TSB (40 ml). The amount of volatile chemicals released from washed cells was too low to analyze by CGC with standard on-column injection techniques. Therefore, volatiles were introduced into the CGC by thermal desorption injection. Volatiles were collected in 6-mm-OD by 120-mm-long Pyrex tubes packed with 15-mm bed length of adsorbent (Tenax TA 60/80 mesh) and were thermally desorbed in a Tekmar cryofocusing automatic desorber onto a 30-m by 0.25-μm methyl silicone capillary column interfaced to a Finnigan ion trap detection system mass spectrometer. Volatiles were analyzed by electron impact (EI) and by chemical ionization (CI) with isobutane as the reagent gas. Standard on-column injection techniques were used for analyses of sterile and *E. agglomerans*-inoculated TSA plates in a Hewlett-Packard model 5890A Series II gas chromatograph, equipped with a cool on-column capillary injector (septum injector) and flame ionization detector. Mass spectra were obtained from capillary columns, operated as described above, coupled to a Finnigan ITDS mass spectrometer in either EI or CI mode. The reagent gas used for chemical ionization was isobutane.

The release rates of ammonia from washed cell preparations, sterile, and *E. agglomerans*-inoculated TSA plates were determined with an ammonia-specific ion-selective electrochemical probe (Orion, Boston, Massachusetts). Test substrates were placed into wide-mouth Erlenmeyer flasks (500 ml), and 13 ml

of washed cells were added to 87 ml tap water for testing. TSA plates were cut into four sections to facilitate placement into the flask. Sections were removed individually from a Petri dish and placed inoculated side up around the bottom of the flask so that all sections were exposed to airflow. The flask was purged for 1 hr with an airflow of 1 liter/min, and volatiles were directed to a sparge system that consisted of a gas dispersion tube (No. 7198 Ace Glass, Vineland, New Jersey) placed in a graduated cylinder containing 100 ml of HCl solution (0.05 N). After a collection, the ionic strength of the sample solution was adjusted with 5 M NaOH/0.05 M disodium EDTA/10% methanol containing a color pH indicator. A standard ammonium calibration curve was prepared each day an analysis was done.

Formulation of Synthetic Chemicals. Ammonium carbonate was formulated to release ~100 μg/hr ammonia by packing approximately 20 mg into the bottom of a heat-sealed 200-μl glass capillary pipette (Becton, Dickinson and Co., Parsippany, New Jersey). The 3-MeBuOH was formulated by using a membrane-based formulation system described previously (Heath et al., 1996). Briefly, a lure (3 × 5 cm) with a 1.17-cm-diam. hole in the center of the front was prepared from 6-mil impermeable polyethylene. Release rate was governed by using 1-mil high-density polyethylene (membrane) film (Consep Inc., Bend, Oregon) that was placed inside the lure. The release area of the membrane was reduced to a 3-, 5-, or 10-mm-diam. circle by placing a piece of aluminum tape (United Tape Company, Cumming, Georgia) over the 1.17-cm hole in the lure. Lures contained filter paper and a plastic grid to provide mechanical stability. They were loaded with 5 or 10 μl (low dose) or 50 μl (high dose) of 3-MeBuOH and were placed in a hood with a 0.25 cm/sec airflow for 24 hr before use in a laboratory bioassay. Longevity was determined for lures loaded with 50 μl of 3-MeBuOH and with the 3-, 5- and 10-mm membrane release area. Release rates from two lures of each membrane release area were measured after four days and then every three to four days over a period of 21 days by using the same methods as those used for TSA plates. Lures were kept in a hood between measurements at ambient temperature and with an airflow of ~0.2 m/sec. Differences in release rates over time were compared with a heterogeneity of slopes model by using Proc GLM (SAS Institute, 1985). Mean release rates were used in linear regression analysis to determine the change in rate over time and the half-life of each lure.

Laboratory Bioassay of A. suspensa. All bioassays were conducted as two-choice bioassays by using 30.2- × 30.2- × 122-cm flight tunnels (Heath et al., 1993; Epsky et al., 1993). Tests were run in a greenhouse under natural light conditions. Liquid test substrates were placed in 500-ml narrow-mouth flasks. Solid test substrates were placed in 1.9-liter wide-mouth plastic jars (Anchor Hocking, St. Paul, Minnesota). Test substrates were vented into the tunnels for at least 1 hr before the addition of flies to stabilize the release of volatiles. New

plastic jars were used for each change in test substrate, unless a higher concentration of the same substrate was used in subsequent bioassays. Two horizontally mounted traps (140-ml clear plastic vial, BioQuip, Gardena, California) were suspended upwind inside the tunnel (Heath et al., 1993). A piece of fluorescent orange adhesive paper (6.5 × 6.5 cm; Atlantic Paste and Glue Co., Brooklyn, New York), was attached to the outside face of the trap with double-sided tape. This provided a visual cue (Greany et al., 1977; Sivinski, 1990) and a sticky surface to capture responding flies. Test substrate volatiles were introduced into the tunnel through the trap, and the trap face and the adhesive paper had 1.5-cm-diam. holes in the center to allow point source release of test substrate volatiles. The positions of the two test substrates were switched after each test to reduce position effects. Twenty females were released at the downwind end of the tunnel, and the number of flies captured on the adhesive paper trap face was recorded after approximately 20 hr. Flies were given water, but no food, during a bioassay.

In the first experiment, 1.7 and 17 ml of washed cells in 99.3 and 83 ml of tap water, respectively, were tested against a blank (100 ml of tap water). Washed cells were added to water to prevent desiccation during the bioassay. The experiment was replicated eight times. The second experiment was conducted to confirm the biological activity of 3-MeBuOH and to determine the concentration of 3-MeBuOH emitted from the washed cell preparation. Females were given the choice of 13 ml of washed cells in 87 ml of tap water and a low dose 3-MeBuOH lure at one of three release rates (lure with 3-mm membrane release area and loaded with 5 μl of 3-MeBuOH and one or two lures with 3-mm membrane release area and loaded with 10 μl of 3-MeBuOH). Each comparison was replicated six times. In subsequent testing, flies were given the choice of volatiles from *E. agglomerans*-inoculated and sterile TSA plates. Initial tests confirmed that volatiles from *E. agglomerans*-inoculated TSA plates captured more females than uninoculated plates. However, there was a large amount of variation in chemical release among the inoculated TSA plates and the inoculated TSA plates released a large amount of ammonia, which is known to be attractive to fruit flies, in comparison to sterile TSA plates tests (see Results). Therefore, the third experiment used synthetic lures in place of *E. agglomerans*-inoculated plates to standardize release rates and was designed to compare attraction due to ammonia with attraction due to 3-MeBuOH. Females were given the choice of an ammonia lure or a 3-MeBuOH lure loaded with 100 μl of 3-MeBuOH with either a 3-, 5- or 10-mm membrane release area. The test was replicated four times. The fourth experiment was conducted to determine if the combination of ammonia and 3-MeBuOH was more attractive than ammonia alone. The test was conducted five times and used the same lure formulations as experiment 3. Two-sample t tests (Proc TTEST; SAS Institute, 1985) were used for comparisons between the two choices offered together.

RESULTS

Chemical Analysis of E. agglomerans *Volatiles.* Analysis of volatiles from *E. agglomerans* found a single major peak with a retention time of 6.5 min in both washed cells and *E. agglomerans*-inoculated TSA plates. Integration of chemicals detected showed that this peak accounted for >85% of total chemicals detected. A library search (National Institute of Standards and Technology Library, Gaithersburg, Maryland) based on EI mass spectral data provided a significant match of the unknown with 3-MeBuOH. Confirmation was provided by comparison with synthetic 3-MeBuOH (Aldrich, St. Louis, Missouri). Chemical ionization mass spectra of natural and synthetic 3-MeBuOH were identical. This peak was also found in volatiles from the TSB supernatant from *E. agglomerans* culture; however, it was one of numerous peaks and was not the major peak. No 3-MeBuOH was detected among volatiles from sterile TSB media.

No ammonia was detected from washed cell preparations. The amount of 3-MeBuOH from these was variable, and the amounts observed ranged from 50 to 200 pg/hr ($N = 5$). Because of the low amounts observed, no attempt was made to quantify release of 3-MeBuOH from washed cells. Chemical analysis indicated little ammonia (16.0 μg/hr, $N = 2$) and no 3-MeBuOH released from sterile TSA plates. *E. agglomerans*-inoculated TSA plates, which contained actively growing colonies of bacteria, released 332.9 μg/hr ($N = 10$) ammonia and 1.48 μg/hr ($N = 4$) 3-MeBuOH after 24 hr of growth. There was large variability in release rates of both chemicals among inoculated plates, as ammonia and 3-MeBuOH release rates ranged from 54.8 to 684.4 and 0.80 to 2.28 μg/hr, respectively. Ammonia and 3-MeBuOH release rates from plates after 48 hr of growth averaged 895.0 and 2.48 μg/hr, respectively. No attempts were made to quantify bacterial growth on the TSA plates before chemical analysis, but variation in amount of growth that occurred before arrival in Gainesville apparently contributed to variation in release rates obtained from the inoculated plates.

Release Rates from 3-MeBuOH Lures. Initial release rates (mean ± SD) from the low-dose 3-MeBuOH lures (i.e., with 3-mm membrane release areas) were 0.05 ± 0.007, 0.08 ± 0.018, and 0.19 ± 0.018 μg/hr for 5-μl, 10-μl, and for two 10-μl-loaded lures, respectively. These lures were used in the laboratory bioassay comparisons with washed cells. Initial release rates from the high-dose lures (i.e., loaded with 50 μl) were 1.23 ± 0.30, 5.44 ± 0.78, and 12.16 ± 2.76 μg/hr for lures with 3-, 5- and 10-mm-diam. membrane release areas, respectively. There were significant differences in both the y intercepts ($F = 156.14$; $df = 2, 42$; $P = 0.0001$) and the slopes of the regressions ($F = 31.55$; $df = 2, 42$; $P = 0.0001$) of 3-MeBuOH release (y = μg/hr) versus days (x) for each lure type (Figure 1). Release rate decreased in all lures over time ($\beta_1 = -0.0429$, $r^2 = 0.96$; $\beta_1 = -0.19367$, $r^2 = 0.98$; and $\beta_1 = -0.34460$,

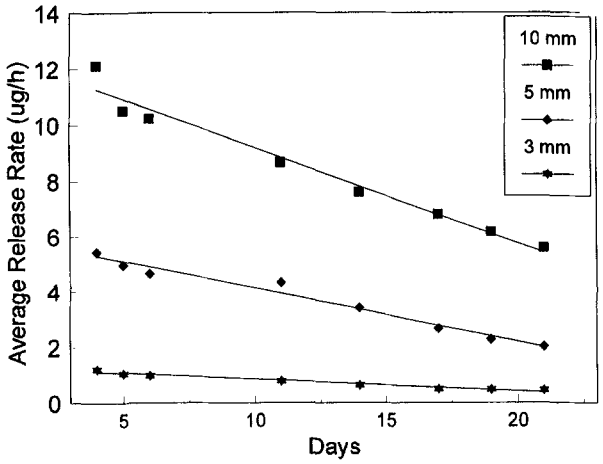

FIG. 1. Average release rates over time of 3-methyl-1-butanol formulated in membrane-based lures. Regressions were determined from lures (N = 2) containing 50 μl of 3-methyl-1-butanol and with membrane release areas of 3 (solid line, star), 5 (solid line, diamond), and 10 mm (solid line, square).

r^2 = 0.97 for 3-, 5- and 10-mm membrane release areas). Generally the half-life of the lures was 16 days.

Laboratory Bioassay of A. suspensa. More flies were captured in response to 17 ml of washed cells than to a water blank, although there was no difference when 1.7 ml of washed cells were tested (Table 1). The 3-MeBuOH lures that

TABLE 1. RESULTS OF TWO-CHOICE BIOASSAY TESTS: EXPERIMENTS 1 AND 2[a]

Choice 1		Choice 2				
Treatment	Response	Treatment	Response	t	df	P
Experiment 1						
1.7 ml cells	4.6 ± 3.58	water control	4.7 ± 1.67	0.0894	14	ns[b]
17 ml cells	8.1 ± 3.09	water control	2.0 ± 1.51	5.0349	14	0.0002
Experiment 2						
13 ml cells	4.8 ± 1.83	0.046 μg/hr	1.3 ± 1.21	3.8996	10	0.0030
13 ml cells	5.8 ± 2.92	0.082 μg/hr	2.2 ± 5.83	2.0117	10	0.0720
13 ml cells	6.7 ± 3.50	0.187 μg/hr	3.3 ± 3.56	1.6352	10	ns

[a] Response (mean ± SD) of 20 female *A. suspensa* per test to volatiles from washed *E. Agglomerans* cells versus a control (experiment 1) or several concentrations of 3-methyl-1-butanol synthetic lures (experiment 2).
[b] Not significant.

TABLE 2. RESULTS OF TWO-CHOICE BIOASSAY TESTS: EXPERIMENT 3[a]

Choice 1		Choice 2				
Treatment	Response	Treatment	Response	*t*	*df*	*P*
1.23 μg/hr lure	5.0 ± 2.16	ammonia	11.5 ± 3.70	3.0361	6	0.0229
5.44 μg/hr lure	5.3 ± 1.70	ammonia	12.3 ± 4.19	3.0921	6	0.0213
12.16 μg/hr lure	9.0 ± 2.16	ammonia	9.3 ± 3.86	0.1130	6	ns[b]

[a] Response (mean ± SD) of 20 female *A. suspensa* per test to volatiles from several concentrations of 3-methyl-1-butanol synthetic lures (average release rate) versus ammonia (100 μg/hr).
[b] Not significant.

released 0.187 μg/hr captured as many flies as washed cells (Table 1), confirming the biological activity of 3-MeBuOH and indicating the approximate release rate from this concentration of washed cells. Overall capture of flies in these tests, however, was low and only half the flies were captured in any of the bioassays. Therefore, tests were continued with higher release rates of 3-MeBuOH, and lures were formulated to mimic release from an *E. agglomerans*-inoculated TSA plate at the lowest concentration (i.e., lure with 3-mm membrane release area loaded with 50 μl 3-MeBuOH), and at 5- and 10-fold higher concentrations (i.e., lures with 5- and 10-mm membrane release areas, respectively, loaded with 50 μl 3-MeBuOH). In experiment 3, the three concentrations of 3-MeBuOH were tested against 100 μg/hr of ammonia, which is an optimal concentration for tests in our bioassay system (N.D.E., R.R.H., and B.D.D., unpublished data). The 3-MeBuOH at the two lower release rates captured fewer females than the ammonia, but 3-MeBuOH, at the highest release rate tested, captured as many flies as ammonia (Table 2). When tested in combination with ammonia in experiment 4, the highest concentration of 3-MeBuOH combined with ammonia captured more females than the ammonia alone (Table 3). In bioassays in which both ammonia and the highest dose 3-MeBuOH lure were offered, either alone (experiment 3) or in combination (experiment 4), an average of 18 of 20 flies in the bioassay were captured.

DISCUSSION

3-Methyl-1-butanol has been identified previously from fruit-fly-attractive substances. For example, 3-MeBuOH was one of 28 chemicals emitted from fermented host fruit of *A. ludens* (Robacker et al., 1990). It was not attractive by itself and was removed from further consideration as a host fruit attractant. A literature search indicated that 3-MeBuOH was the major volatile obtained from autoclaved supernatant of 8-day-old cultures of *Klebsiella pneumoniae*-

TABLE 3. RESULTS OF TWO-CHOICE BIOASSAY TESTS: EXPERIMENT 4[a]

Choice 1		Choice 2				
Treatment	Response	Treatment	Response	*t*	*df*	*P*
1.23 μg/hr lure + ammonia	8.6 ± 4.15	ammonia	9.2 ± 2.17	0.2860	8	ns[b]
5.44 μg/hr lure + ammonia	8.6 ± 4.03	ammonia	8.8 ± 1.30	0.1054	8	ns
12.16 μg/hr lure + ammonia	11.6 ± 2.41	ammonia	7.0 ± 3.31	2.5095	8	0.0364

[a] Response (mean ± SD) of 20 female *A. suspensa* per test to volatiles from several concentrations (average release rate) of 3-methyl-1-butanol synthetic lures plus ammonia versus ammonia alone. A 100 μg/hr ammonia release rate was used in all tests.
[b] Not significant.

inoculated TSB (Lee et al., 1995) and of 4- and 8-day-old cultures of *Citrobacter freundii*-inoculated TSB (DeMilo et al., 1996). There were 20 and 21 chemical components, respectively, identified in addition to this compound. However, there was no information regarding the attractiveness of the individual chemicals. In comparisons of autoclaved supernatant to nonautoclaved supernatant of 4-day-old *C. freundii*-inoculated TSB, the amount of 3-MeBuOH was greatly reduced in the autoclaved supernatant with no corresponding change in *A. ludens* attraction in laboratory bioassays (DeMilo et al., 1996). 3-MeBuOH was identified as a minor component in vacuum steam distillation extraction of corn protein hydrolyzate bait (Buttery et al., 1983).

3-MeBuOH production may be limited to certain species, as no 3-MeBuOH was identified among volatiles from 6-day-old cultures of *Staphylococcus* species-inoculated TSB (Robacker et al., 1993). MacCollom et al. (1992) found that more apple maggot flies were captured on traps baited with washed cells of *E. agglomerans* than with washed cells of *Klebsiella oxytoca*, *Enterobacter cloacae*, *Pseudomonas fluorescens*, or *Bacillus cereus*. It is not known if these species produce 3-MeBuOH. Variation in fruit fly attraction also may occur within species. *E. agglomerans* isolates from different sources varied in their ability to attract apple maggot flies and that attraction may be related to the substrates on which the isolates are growing (C.R.L., unpublished). Further studies are needed to determine if production of 3-MeBuOH is limited to particular bacterial species, specific bacterial isolates, and/or bacteria growing on certain substrates, and if attraction to bacteria is correlated with or enhanced by production of 3-MeBuOH.

The role of bacteria in the ecology of tephritid fruit flies is poorly under-

stood (reviewed in Drew and Lloyd, 1989). Several studies have noted that, among all the bacteria that fruit flies encounter during feeding, only a few are repeatedly recovered internally from fruit flies or their food. Vijaysegaran et al. (1997) found that structures on the mouthparts of *Bactrocera* species limited the size of bacteria that could be ingested by these flies. Thus, since only a subset of bacteria present in the environment could serve as a food source, they speculated that flies may be selectively attracted to fruit or leaf surfaces on which these bacterial species are growing. Attractiveness of fruit fly-associated *E. agglomerans* isolates was correlated with the presence of uricase, an enzyme that degrades uric acid, and *E. agglomerans* isolates that produce uricase constitutively have been isolated from bird dung (C.R.L., unpublished). Thus, bacteria in bird dung could degrade uric acid, which is excreted by birds, and make bird dung a more suitable nitrogen source for foraging. Adults of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), and the apple maggot feed on bird dung in the field (Hendrichs and Hendrichs, 1990; Hendrichs and Prokopy, 1990). Bird dung was attractive to adults of both species (Prokopy et al., 1992, 1993) and to adults of *A. suspensa* (Epsky et al., 1997). Thus, fruit flies attracted to bird dung on which an appropriate *E. agglomerans* isolate is growing may obtain protein by direct consumption of the bacteria themselves, as well as by feeding on substrate altered by microbial degradation.

Both ammonia and 3-MeBuOH were needed for optimal *A. suspensa* attraction in our studies. Synthetic 3-MeBuOH alone may be more attractive to apple maggot flies than to *A. suspensa*, because small amounts of washed cells (10 μl) enhanced capture of apple maggot flies in field trials (MacCollom et al., 1992), but 13 ml of a fourfold concentration of washed cells was needed for low-level attraction of *A. suspensa* in laboratory trials reported here. Release of ammonia alone may be responsible for *A. suspensa* attraction to *E. agglomerans*-inoculated TSA plates, because small amounts of 3-MeBuOH were released relative to ammonia. However, TSA represents an artificial substrate, and additional studies are needed to determine the amount of 3-MeBuOH released relative to ammonia from *E. agglomerans* growing on natural substrates.

Field studies of traps baited with 3-MeBuOH alone and in combination with ammonia and other previously identified fruit fly attractants, such as putrescine, acetic acid (Heath et al., 1995), and trimethylamine (Heath et al., 1997), are needed to test the effectiveness of these materials for use in traps for pest fruit flies. Such studies may lead to a better understanding of the role of bacteria in the ecology of fruit flies. In field tests of combinations of ammonia, acetic acid, putrescine, and trimethylamine, capture of other pest insects was observed, e.g., Lepidoptera and Blattodea. Availability of lures that can be formulated for a range of 3-MeBuOH release rates will facilitate field tests of various combinations of these synthetic attractants for pest fruit flies as well as for a variety of other pest insects.

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MODES OF ACTION OF ALLELOCHEMICAL ALKALOIDS: INTERACTION WITH NEURORECEPTORS, DNA, AND OTHER MOLECULAR TARGETS

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Abstract—Several alkaloids are toxic to insects and vertebrates and, in addition, can inhibit the growth of bacteria and plant seedlings. In vitro assays were established to elucidate their modes of action and to understand their allelochemical properties. Basic molecular targets studied, present in all cells, included DNA intercalation, protein biosynthesis, and membrane stability. The degree of DNA intercalation was positively correlated with inhibition of DNA polymerase I, reverse transcriptase, and translation at the molecular level and with toxicity against insects and vertebrates at an organismic level. Inhibition of protein biosynthesis was positively correlated with animal toxicity. Molecular targets studied, present only in animals, included neuroreceptors ($alpha_1$, $alpha_2$, serotonin, muscarinic, and nicotinic acetylcholine receptors) and enzymes related to acetylcholine (acetylcholine esterase and choline acetyltransferase). The degree of binding of alkaloids to adrenergic, serotonin, and muscarinic acetylcholine receptors was positively correlated in G-protein-coupled receptors. Receptor binding and toxicity was correlated in insects. The biochemical properties of alkaloids are discussed. It is postulated that their structures were shaped in a process termed "evolutionary molecular modeling" to interact with a single and, more often, with several molecular targets at the same time. Many alkaloids are compounds with a broad activity spectrum that apparently have evolved as "multipurpose" defense compounds. The evolution of allelochemicals affecting more than one target could be a strategy to counteract adaptations by specialists and to help fight off different groups of enemies.

Key Words—Alkaloids, molecular targets, neuroreceptors, DNA intercalation, DNA polymerase, reverse transcriptase, protein biosynthesis, membrane stability, nicotine receptor, muscarinic receptor, serotonin receptor, adrenergic receptors.

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INTRODUCTION

Alkaloids occur in approximately 20% of all plant species, and the number of identified structures exceeds 12,000 (Roberts and Wink, 1998). One of the main questions concerns their biological function. Are alkaloids just waste products, as was formerly believed (Mothes, 1955), or defense chemicals against insects, vertebrate herbivores, bacteria, fungi, viruses, and even other plants (Harborne, 1993; Wink, 1993)? A number of animals also produce alkaloids or acquire them from their food plants. Most agree that, similar to the situation in plants, they serve as protection against predators and microorganisms (Blum, 1981; Wink, 1993). Although the general defense concept appears acceptable and has been elaborated, for example, for quinolizidine and pyrrolizidine alkaloids (Boppré, 1990; Eisner and Meinwald, 1995; Hartmann, 1992; Hartmann and Witte, 1995; Meinwald, 1990; Rothschild et al., 1979; Schneider, 1992; Wink, 1987, 1988, 1992), we still need experimental data for most other classes of alkaloids (for reviews see Bernays and Chapman, 1994; Hartmann, 1992; Levin, 1976; Swain, 1977; Wink, 1987, 1993).

Whereas some natural products may be directed specifically towards a single group of organisms, others show a broad activity spectrum. We have found several alkaloids that have adverse effects on insect and vertebrate herbivores or inhibit the growth of bacteria and fungi and are phytotoxic as well (Tables 1–3).

In this comparative study, we have tried to elucidate the modes of action of about 70 alkaloids, which represent most structure types, in order to understand their various allelochemical activities. Compounds active against a wide range of organisms should affect basic molecular targets common to all of them. Those compounds that are toxic to animals exclusively should affect animal-specific targets.

We have developed bioassays (Latz-Brüning, 1994; Schmeller, 1995; Schmeller et al., 1994, 1995, 1997a,b; Wink and Twardowski, 1992) that can be used to determine interactions of alkaloids with basic molecular targets common to all cells. These include DNA intercalation, protein biosynthesis, and membrane stability. Others detect the effects of alkaloids on reverse transcriptase and DNA polymerase I, relevant targets in retroviruses or bacteria, respectively. In addition, we have established bioassays that detect the binding of alkaloids to neuroreceptors, targets that are present only in animals.

METHODS AND MATERIALS

Alkaloids

Alkaloids were either isolated or synthesized and purified in our laboratory (Schmeller et al., 1994, 1995, 1997a,b; El-Shazly et al., 1996a–d, 1997) or

purchased (Sigma, St. Louis, Missouri; Roth, Karlsruhe, Germany). Purity was checked by HPLC or GLC and was >95–98% in all cases.

Assays to Determine Alkaloid Interactions with Molecular Targets

Assays were optimized and standardized in terms of linearity, reproducibility, sensitivity, and specificity (Latz-Brüning, 1994; Schmeller, 1995).

Interaction of Alkaloids with DNA

Melting Point Determinations. If compounds intercalate with DNA, the melting temperature of DNA is shifted to higher values (Maiti and Chaudhuri, 1981; Nandi and Maiti, 1985) (Figure 1). In our experiments, 70 μM *Sinapis* DNA was incubated in 1 ml TE buffer (pH 7.4) with 70 μM alkaloid for 30 min at 22°C. The temperature was increased by 1°C/min to 90°C and the absorption was continuously determined in a spectrometer at 256 nm. Derivations of differences between two consecutive measurements were plotted to determine the kinetics (Latz-Brüning, 1994).

Methylgreen Assay. Methylgreen (MG) binds to DNA, and bound MG displays an absorption maximum at 642 nm, whereas free MG shows no absorption at this wavelength (Burres et al., 1992; Krey and Hahn, 1969). When alkaloids bind or intercalate with DNA, MG is released. This is measured as a decrease in optical density at 642 nm. DNA–methylgreen (Sigma) (70 μM) was incubated in the dark in 20 mM Tris HCl (pH 7.4) together with up to 5 mM alkaloids. After 24 hr, the OD_{642} of untreated controls and treated samples was determined (Latz-Brüning, 1994).

Gel Chromatography Assay. Herring sperm DNA (Serva, Heidelberg, Germany) (30 mg) was incubated for 15 min at 22°C together with 640 μM alkaloids in 2.5 ml water. This solution was applied to a Sephadex G-25 column (PD10 columns; Pharmacia, Freiburg, Germany), which was equilibrated with water. Elution was carried out with water, and effluents were monitored at 200–500 nm. DNA elutes first, and nonintercalated alkaloids follow later, so two well-established peaks are obtained. We tested, through UV spectroscopy and thin-layer chromatography, whether the DNA peak contained alkaloids. This was the case in all instances in which we found a significant effect in the DNA melting temperature assay (Latz-Brüning, 1994).

Inhibition of DNA Polymerase I. To determine the activity of DNA polymerase I, we modified a "nick translation assay" (Sambrook et al., 1989). The assay buffer contained 50 mM Tris HCl (pH 7.5), 10 mM $MgSO_4$, 0.1 mM DDT, 0.005% BSA, 500 ng of a linearized plasmid (pUC19), 625 μM dNTPs, 0.01 μCi [α-^{32}P]dCTP, 1 unit DNA polymerase I, 25 pg DNAse I, and up to 10 mM alkaloids. The reaction was started by adding DNAse I; after 15 min at 37°C, it was terminated by adding 100 mM EDTA (pH 8.0). Two variations

TABLE 1. ANTIBACTERIAL AND PHYTOTOXIC EFFECTS OF ALKALOIDS[a]

Alkaloid	Growth inhibition (%)				
				Lepidium sativum[b]	
	E. coli	*S. marcescens*	*B. subtilis*	1%	0.1%
13-Hydroxylupanine	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
17-Oxosparteine	na (10 mM)	na (10 mM)	25 (10 mM)	nd	nd
Aconitine	na (0.3 mM)	na (0.3 mM)	na (0.3 mM)	90	25
Ajmalicine	na (1 mM)	na (1 mM)	na (1 mM)	0	0
Ajmaline	100 (6 mM)	100 (6 mM)	100 (6 mM)	nd	nd
Angustifoline	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Arecoline	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Berberine	42 (1 mM)	40 (1 mM)	na (1 mM)	84	21
Boldine	96 (5 mM)	24 (5 mM)	28 (5 mM)	nd	nd
Brucine	88 (10 mM)	26 (10 mM)	26 (10 mM)	nd	nd
Caffeine	50 (10 mM)	41 (10 mM)	42 (10 mM)	100	83
Cinchonidine	100 (3 mM)	100 (10 mM)	100 (10 mM)	94	0
Cinchonine	100 (3 mM)	100 (10 mM)	100 (10 mM)	nd	nd
Colchicine	na (10 mM)	na (10 mM)	na (10 mM)	94	90
Cycloheximide	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Cytisine	na (10 mM)	na (10 mM)	na (10 mM)	54	21
Emetine	40 (10 mM)	27 (10 mM)	33 (10 mM)	nd	nd
Ergometrine	26 (10 mM)	44 (10 mM)	27 (10 mM)	71	32
Gramine	nd	nd	nd	54	30
Harmaline	100 (10 mM)	100 (10 mM)	100 (10 mM)	92	81
Harmine	100 (1 mM)	100 (1 mM)	100 (1 mM)	nd	nd
Hyoscyamine	31 (10 mM)	38 (10 mM)	38 (10 mM)	91	0
L-Ephedrine	na (10 mM)	na (10 mM)	na (10 mM)	36	13
Laudanosine	na (10 mM)	23 (10 mM)	na (10 mM)	nd	nd

Lobeline	100 (10 mM)	100 (10 mM)	100 (10 mM)	90	17
Lupanine	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Monocrotaline	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Narcotine	64 (5 mM)	50 (5 mM)	78 (5 mM)	94	0
Nicotine	na (10 mM)	na (10 mM)	na (10 mM)	40	20
Norharman	100 (1 mM)	100 (1 mM)	100 (1 mM)	nd	nd
Papaverine	22 (2.5 mM)	na (2.5 mM)	na (2.5 mM)	99	96
Physostigmine	53 (10 mM)	34 (10 mM)	32 (10 mM)	nd	nd
Pilocarpine	na (10 mM)	28 (10 mM)	44 (10 mM)	nd	nd
Piperine	na (0.3 mM)	na (0.3 mM)	na (0.3 mM)	84	47
Protoveratrine B	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Pseudopelletrine	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Quinidine	100 (3 mM)	100 (3 mM)	100 (3 mM)	89	24
Quinine	100 (5 mM)	100 (5 mM)	100 (5 mM)	100	93
Riddelline	33 (10 mM)	25 (10 mM)	27 (10 mM)	nd	nd
Salsoline	na (10 mM)	na (10 mM)	na (10 mM)	87	50
Sanguinarine	100 (0.1)	100 (0.1 mM)	100 (0.1 mM)	85	18
Scopine	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Scopolamine	22 (10 mM)	35 (10 mM)	48 (10 mM)	0	0
Senecionine	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Sparteine	na (10 mM)	na (10 mM)	na (10 mM)	65	60
Strychnine	91 (10 mM)	88 (10 mM)	85 (10 mM)	27	0
Theophylline	nd	nd	nd	100	73
Tropine	na (10 mM)	na (10 mM)	na (10 mM)	67	0
Vincamine	na (10 mM)	na (10 mM)	na (10 mM)	nd	nd
Yohimbine	68 (5 mM)	na (5 mM)	26 (5 mM)	nd	nd
Tetracycline	100 (0.1 mM)	100 (0.1 mM)	100 (0.1 mM)	nd	nd

[a] na = not active, i.e., inhibition < 20% at concentration given; nd = not determined; inhibitor concentrations in parentheses.
[b] Length of the roots was determined after 5 days; length of untreated controls was set at 0% inhibition (after Wink and Twardowski, 1992); 1% alkaloid is equivalent to approximately 30–50 mM and 0.1% to 3–5 mM.

TABLE 2. TOXICITY OF ALKALOIDS TO *Tubifex* WORMS (POLYCHAETA) AND INSECTS *Syntomis mogadorensis* (LEPIDOPTERA: ARCTIIDAE) AND *Apis mellifera* (HYMENOPTERA: APIDAE)

	Inhibitory or toxic concentrations (%)			
		Apis mellifera[b]		*Syntomis mogadorensis*
Alkaloid	*Tubifex* toxicity[a]	Toxicity (LD_{50})	Deterrency (ED_{50})	deterrency[3] >50%[c]
Aconitine	nd[d]	nd	nd	1
Anabasine	nd	nd	nd	0.1
Ajmalicine	nd	nd	nd	1
Ajmaline	0.002	nd	nd	0.01
Arecoline	0.002	nd	nd	0.1
Aristolochic acid	0.05	nd	nd	nd
Atropine	0.002	nd	nd	nd
Berberine	0.05	0.003	0.01	0.01
Boldine	0.002	nd	nd	0.01
Brucine	0.01	0.2	0.05	1
Caffeine	0.1	0.2	0.03	0.1
Canadine	0.01	nd	nd	0.1
Chelidonine	nd	nd	nd	0.1
Cinchonidine	0.002	nd	0.04	0.1
Cinchonine	0.002	nd	0.007	1
Colchicine	nd	0.03	0.2	0.01
Coniine	0.002	nd	nd	nd
Cytisine	nd	nd	nd	0.01
Emetine	0.002	nd	nd	0.1
Ergotamine	0.002	nd	nd	0.1
Glaucine	0.002	nd	nd	nd
Gramine	0.01	nd	0.2	1
Harmaline	nd	nd	nd	0.1
Harmine	nd	nd	0.008	0.1
Heliotrine	nd	0.1	0.09	nd
Hyoscyamine	0.01	0.1	0.005	0.01
L-Ephedrine	0.01	nd	0.09	0.01
Laudanosine	0.05	nd	nd	nd
Lobeline	nd	nd	0.008	0.01
Lupanine	nd	nd	nd	0.1
Narcotine	0.05	nd	nd	0.01
Nicotine	0.002	0.2	0.03	0.1
Norharman	0.002	nd	nd	nd
Papaverine	0.05	nd	nd	0.1
Physostigmine	0.002	nd	nd	0.01
Pilocarpine	nd	nd	nd	0.1
Piperine	nd	nd	nd	0.1
Protoveratrine B	nd	nd	nd	0.01
Pseudopelletierine	0.01	nd	nd	nd
Quinidine	0.002	0.02	0.02	0.01

TABLE 2. CONTINUED

Alkaloid	Inhibitory or toxic concentrations (%)			
	Tubifex toxicity[a]	*Apis mellifera*[b]		*Syntomis mogadorensis* deterrency >50%[c]
		Toxicity (LD_{50})	Deterrency (ED_{50})	
Quinine	nd	nd	0.04	0.01
Rescinnamine	1	nd	nd	nd
Salsoline	nd	nd	nd	0.1
Sanguinarine	nd	nd	nd	1
Scoulerine	0.01	nd	nd	nd
Scopolamine	nd	nd	0.03	0.01
Sparteine	0.05	0.05	0.03	1
Strychnine	0.002	0.2	0.02	1
Theobromine	0.1	nd	nd	nd
Theophylline	0.002	nd	nd	nd
Tropine	1	nd	0.2	1
Veratrine	0.002	nd	nd	nd
Vincamine	1	0.04	0.08	nd
Yohimbine	0.002	nd	0.008	1

[a]Concentration at which 80–100% of *Tubifex* were dead after 8–20 hr.
[b]Concentration of alkaloids toxic (ED_{50}) or deterrent (LD_{50}) for bees; after Detzel and Wink (1993).
[c]Concentration of alkaloid solutions applied to leaves of *Taraxacum* which prevented feeding on more than 50% of the leaf material. Untreated controls were usually 100% eaten under choice conditions (after Wink and Schneider, 1990).
[d]nd = not determined.

TABLE 3. VERTEBRATE TOXICITY OF ALKALOIDS: AFTER MERCK INDEX, (1996), DICTIONARY OF NATURAL COMPOUNDS, (1996), and WINK (1993)[a]

Alkaloid	LD_{50} (mg/kg)	
	Rat	Mouse
13-Hydroxylupanine	i.p. 199	i.p. 172
Aconitine	i.v. 0.08–0.14	i.p. 0.33
Ajmalicine		p.o. 400
Ajmaline	i.p. 94	
Arecoline		s.c. 100
Atropine	p.o. 500	i.v. 90
Berberine		i.p. 23
Boldine		p.o. 450
Brucine	i.p. 91	
Caffeine	p.o. 192	p.o. 127–137

TABLE 3. CONTINUED

Alkaloid	LD_{50} (mg/kg)	
	Rat	Mouse
Cinchonidine	i.p. 206	
Cinchonine	i.p. 152	
Cocaine	i.v. 17.5	
Colchicine	i.v. 1.6	i.v. 2
Coniine		i.v. 19
Cotinine		i.p. 930
Cycloheximide	p.o. 2	
Cytisine		i.p. 9.3
Echimidine	i.p. 200	
Emetine	i.v. 12.1	
Ephedrine	p.o. 400	
Ergometrine		i.v. 8.2
Ergotamine	i.v. 80	
Gramine		i.p. 122
Harmaline		i.p. 122
Harmalol	s.c. 120	
Harman		i.p. 50
Harmine		i.v. 38
Heliotrine	i.p. 300	
Hyoscyamine		i.v. 95
Lobeline	i.p. 107	i.p. 40
Lupanine	i.p. 180–192	i.p. 154–197
Monocrotaline	i.p. 175	i.p. 259
Morphine		i.v. 226–318
N-Methylcytisine		i.p. 51
Narcotine		p.o. 840
Nicotine		i.p. 95, i.v. 0.3
Papaverine		p.o. 325
Physostigmine		p.o. 2.5
Pilocarpine		p.o. 200
Protoveratrine		s.c. 0.2
Quinidine	i.v. 30	
Quinine		i.p. 115
Salsoline		i.v. 140
Sanguinarine	i.v. 29	i.v. 16
Scopolamine		i.v. 163
Senecionine	i.p. 50	i.v. 64
Seneciphylline	i.p. 77	i.v. 90
Solanine	p.o. 590	
Sparteine	i.p. 42–44;	i.p. 55–67
Strychnine	i.v. 0.9	
Vincamine		p.o. 1000
Yohimbine	i.p. 55	

[a] i.p. = intraperitoneal; i.v. = intravenous, p.o. = per os; s.c. = subcutaneous.

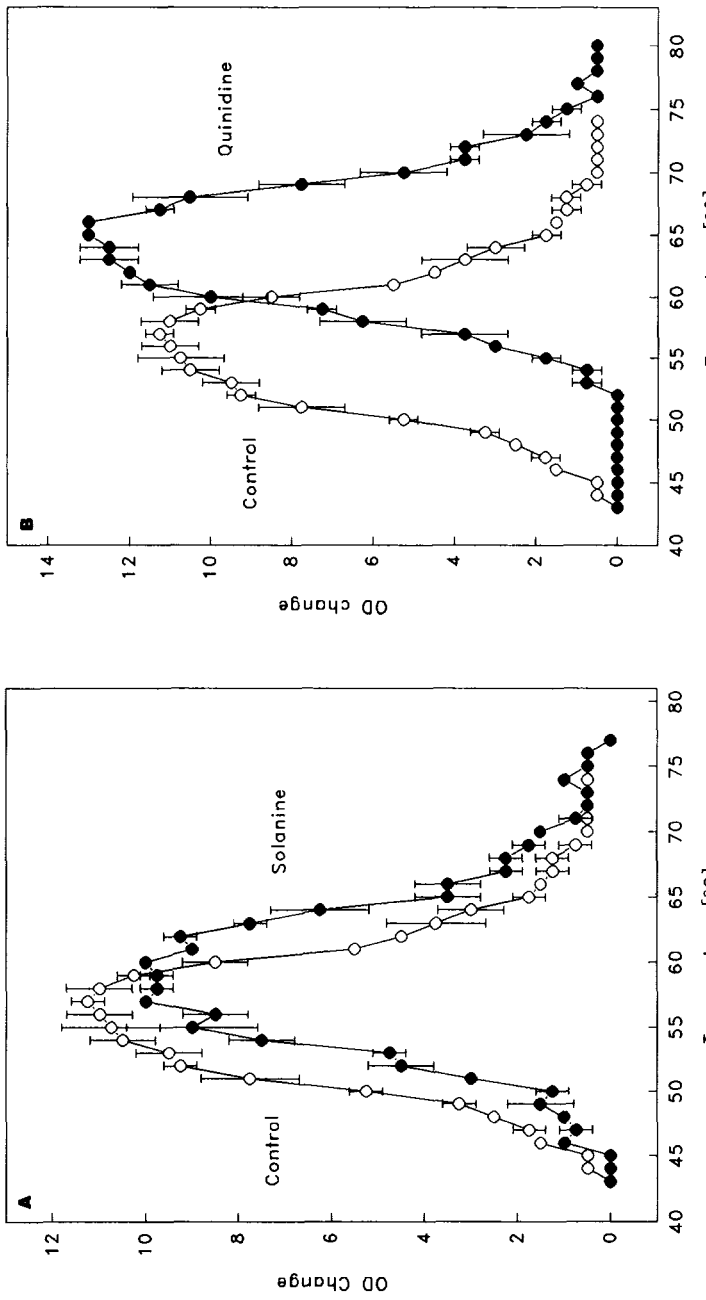


FIG. 1. Effect of alkaloids on the melting temperature of DNA: (A) solanine, (B) quinidine, (C) emetine, (D) harmaline.

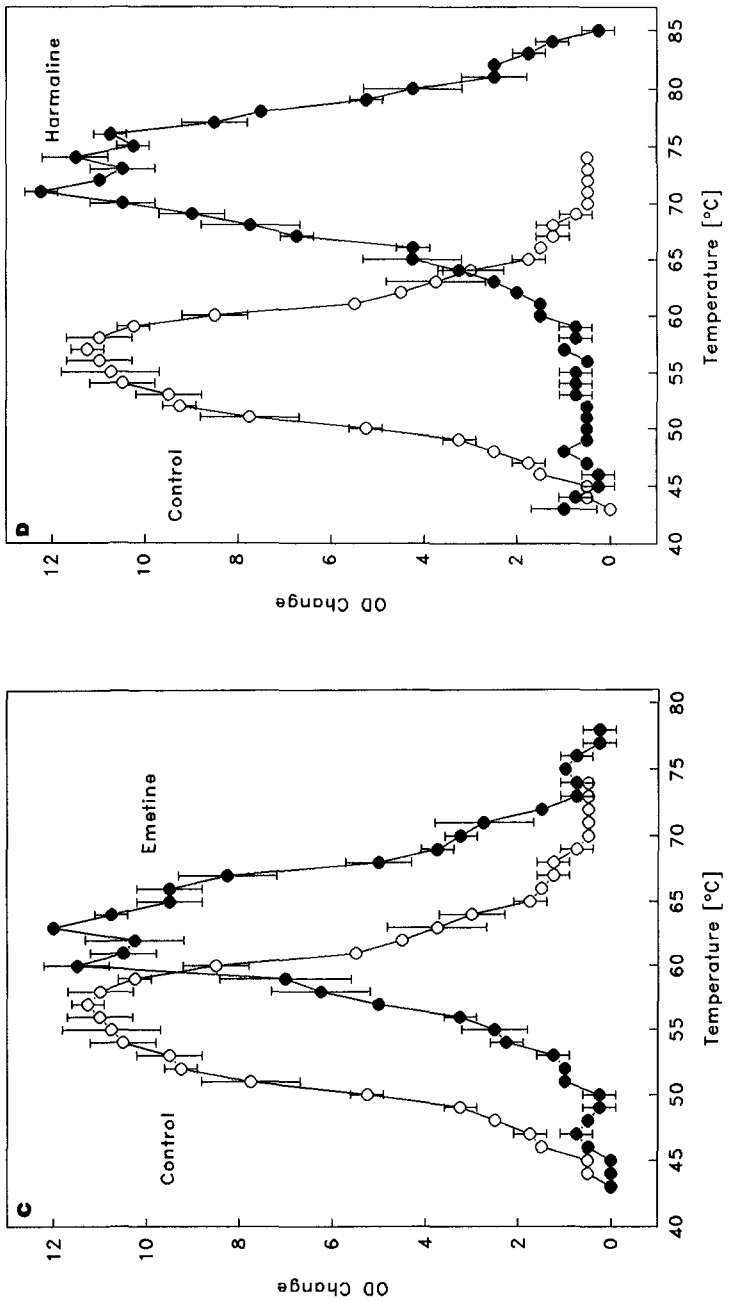


FIG. 1. Continued.

were carried out: (1) a 15-min preincubation of DNA polymerase I with alkaloids, prior to adding plasmid DNA, and (2) a 15-min preincubation of DNA and alkaloids before adding the enzymes in order to differentiate between alkaloidal effects on DNA polymerase I and on DNA. The incorporated radioactivity was removed from the nonincorporated [α-^{32}P]dCTP by gel filtration on Sephadex G-50 "Nick columns" (Pharmacia) and measured in a liquid scintillation counter (Latz-Brüning, 1994). Results for quinine are shown in Figure 2.

Inhibition of Reverse Transcriptase (RT). To measure the activity of reverse transcriptase, a protocol for the synthesis of cDNA was modified (Sambrook et al., 1989), and mRNA was isolated from rat liver according to standard protocol (Sambrook et al., 1989). mRNA (500 ng) and 500 ng random primer (Boehrin-

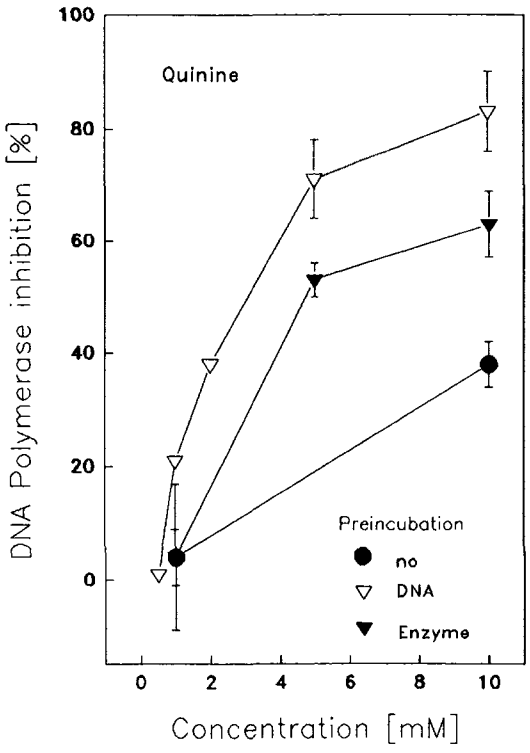


FIG. 2. Dose-response of the inhibition of DNA polymerase I by quinine. Three different assays were performed. no: the assay was started immediately after adding DNA, enzyme, and alkaloids (i.e., no preincubation). DNA: alkaloids and DNA were preincubated before adding the enzyme. Enzyme: alkaloids and DNA polymerase I were preincubated before adding the DNA.

ger, Mannheim, Germany) were denatured at 70°C for 5 min and immediately cooled in ice water. Then, 0.3 mM dNTPs, 0.01 μCi [α-^{32}P]dCTP, 6 units AMV reverse transcriptase (Promega) in RT buffer (50 mM Tris HCl, pH. 7.8, 10 mM $MgCl_2$, 80 mM KCl, 10 mM DTT) were added and incubated 30 min at 42°C. The reaction was terminated by adding 100 mM EDTA; the incorporation [α-^{32}P]dCTP was measured as described in the DNA polymerase assay. Two preincubation strategies as previously described for DNA polymerase I were employed (Latz-Brüning, 1994). Results for quinine and solanine are shown in Figure 3.

Inhibition of Protein Biosynthesis

An in vitro reticulocyte translation assay (Boehringer) was modified to determine inhibition of translation by alkaloids. An assay (total volume 25 μl) contained 2 μl of 12.5× translation mix, 10 μl reticulocyte lysate, 100 mM K acetate, 1.5 mM Mg acetate, 0.25 μCi L-[4,5-^{3}H(N)]leucine, 0.5 μg TMV RNA (Boehringer), and up to 5 mM alkaloids (buffered to pH 7). The mixture was incubated at 30°C; reactions were terminated after 0, 10, 20, 30, and 40 min. The radiolabeled protein was precipitated by adding 200 μl ice-cold trichloroacetic acid (TCA) (50%; w/v), and after 30 min, was filtered through GF 34 filters (Schleicher-Schüll), which bind proteins. After washing the filters three times with 50% TCA, they were dried at 85°C. Radioactivity of the filters was determined in a liquid scintillation counter (Latz-Brüning, 1994). Results for harmine and solanine are shown in Figure 4.

Influence of Alkaloids on Membrane Permeability

Sheep erythrocytes were purified and incubated in 50 μl PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 liter H_2O). They were incubated for 1 hr at 20°C together with 1 μM cytochalasin B and up to 5 mM alkaloids. Erythrocytes were precipitated by centrifugation (4 min at 2000*g*) and the hemoglobin released was determined photometrically at 543 nm (Latz-Brüning, 1994). A complete lysis by 1 mM saponin served as a positive 100% control. Results for solanine are shown in Figure 5.

Membrane Preparation for Neuroreceptor Binding Studies

Pig brains, which were obtained from a local slaughterhouse within 30 min after death of the animals, were used to prepare receptor-rich membranes. Brains were frozen immediately in liquid N_2; 50 g brain per 200 ml ice-cold buffer (0.32 M sucrose, 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA) were homogenized twice for 15 sec in a blender and then for 1 min with an ultraturrax. The homogenate was centrifuged three times for 15 min at 1400*g* and 4°C to separate cellular debris. The supernatant was spun at 100,000*g* for

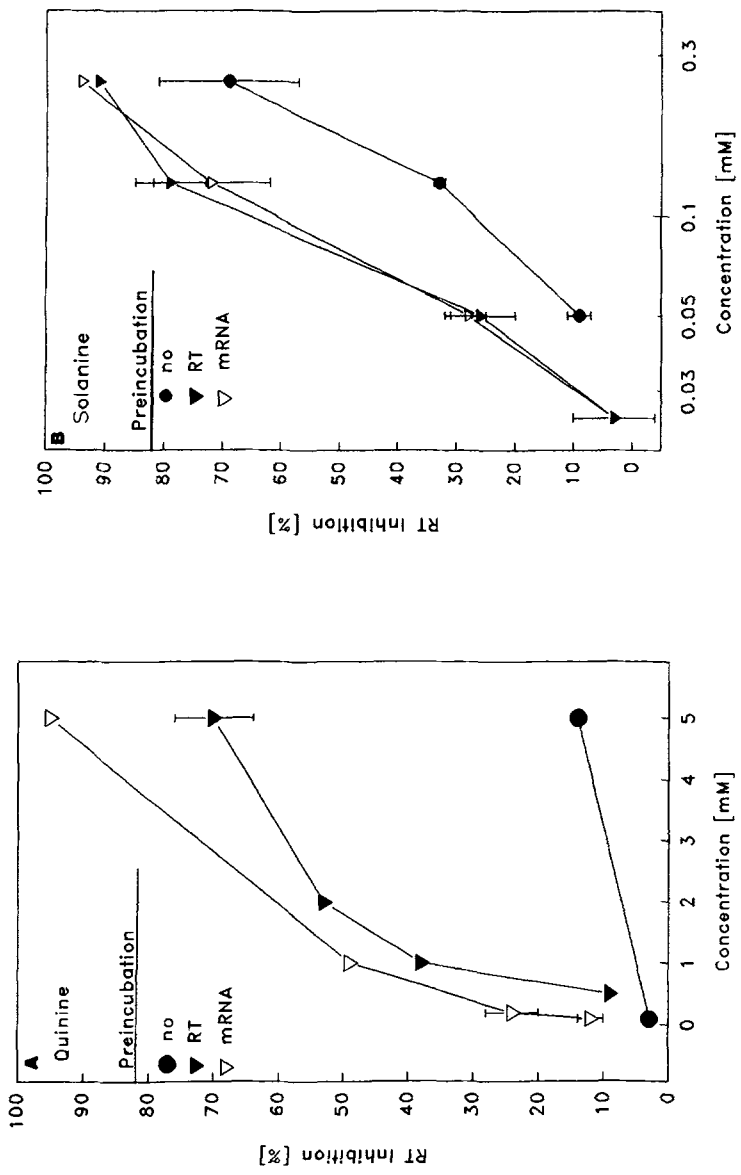


FIG. 3. Dose-response of the inhibition of reverse transcriptase (RT) by (A) quinine and (B) solanine. Preincubation strategies: three different assays were performed. no: the assay was started immediately after adding mRNA, RT, and alkaloids (i.e., no preincubation); RT: alkaloids and RT were preincubated before adding the mRNA (RT preincubation); and mRNA: alkaloids and mRNA were preincubated before adding the enzyme (mRNA preincubation). Preincubation of quinine with mRNA exhibited the most potent inhibitory effect.

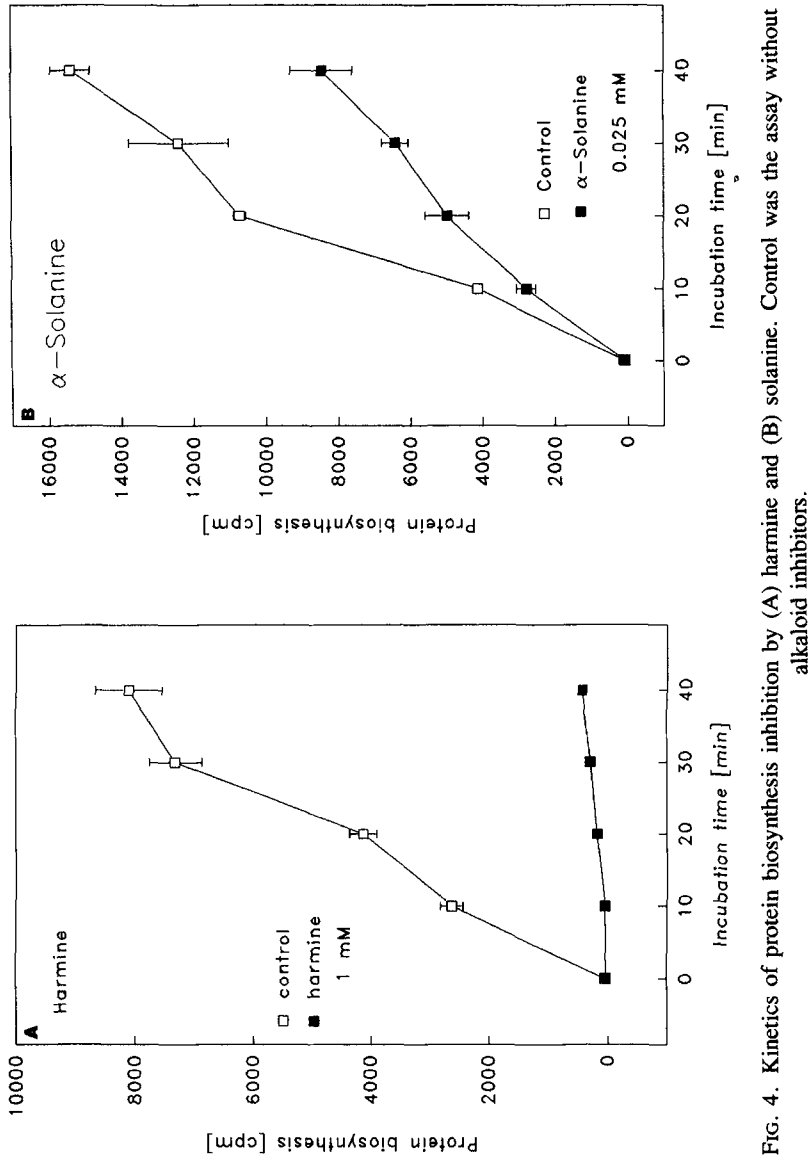


FIG. 4. Kinetics of protein biosynthesis inhibition by (A) harmine and (B) solanine. Control was the assay without alkaloid inhibitors.

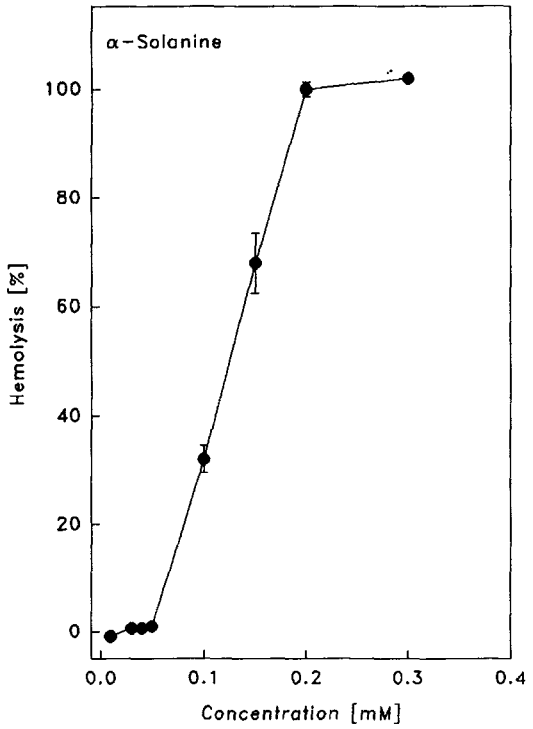


FIG. 5. Dose-response of the membrane disruption of erythrocytes by solanine.

60 min. The resulting pellet was resuspended in buffer (as above but without sucrose). Aliquots were stored frozen at −80°C. Protein content was determined by the Lowry method, with bovine serum albumin as a standard (Schmeller et al., 1994, 1995, 1997a,b).

Receptor Binding Assays

Binding assays (in triplicate) were performed with a rapid filtration technique, essentially as described by Schmeller et al. (1994, 1995, 1997a,b). The kinetics of binding, its dependence on protein content, the specific binding, saturation, a Scatchard plot, and the displacement of the tracer [^{3}H]quinuclidinyl benzilate (QNB) by atropine are shown in Figure 6; similar results were obtained for the other neuroreceptors.

Muscarinic Receptor (mAChR). Membrane preparations adjusted to 500 μg protein in a final volume of 500 μl buffer were incubated with [^{3}H]quinuclidinyl benzilate (QNB) (52.3 Ci/mmol; Dupont NEN) for 1 hr at 20°C in the absence and presence of alkaloids, with 20 μM atropine as a control. The incubation

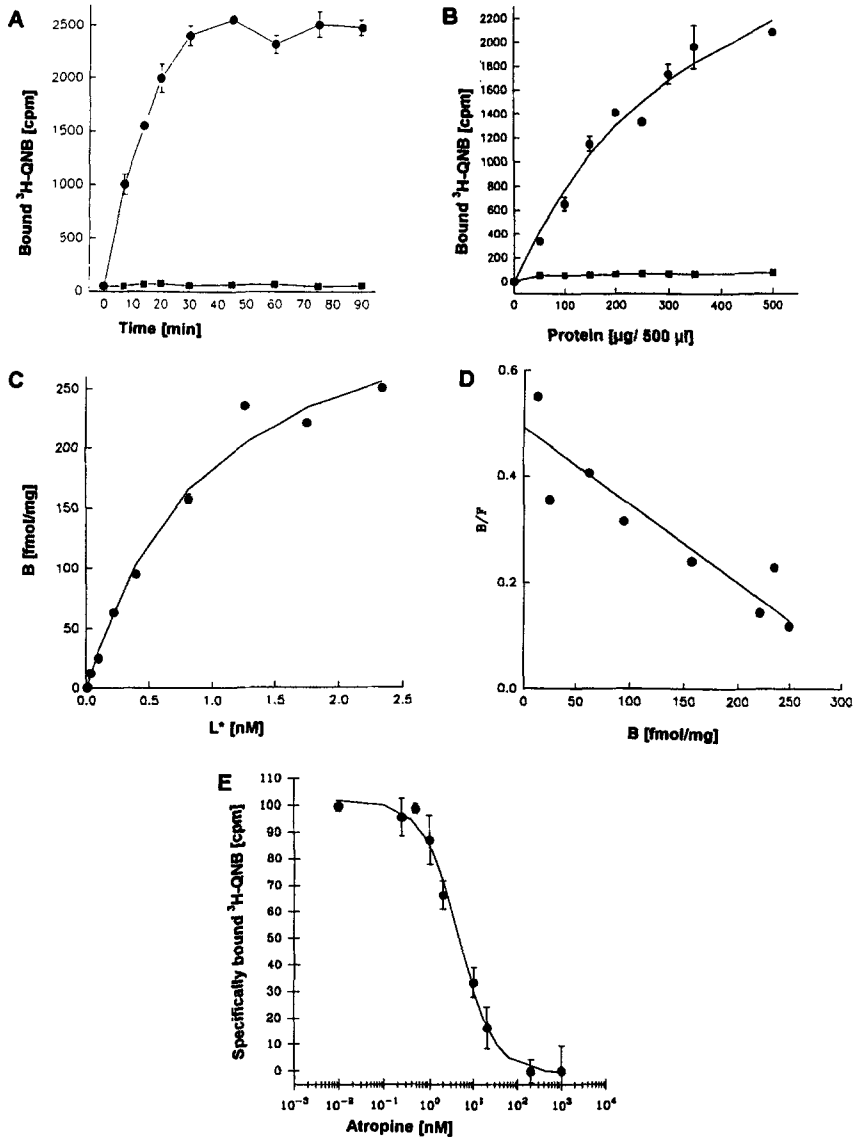


FIG. 6. Development of a radioactively labeled ligand receptor assay to determine the binding of alkaloids at muscarinic acetylcholine receptors: (A) kinetics of QNB binding; specific (upper curve) and unspecific binding (lower curve); (B) specific and nonspecific QNB binding in relation to protein content (i.e., concentration of receptors); (C) saturation of binding of QNB at mAChR; (D) Scatchard plot of saturation of mAChR by QNB; and (E) Replacement of specifically bound QNB by atropine.

was stopped with 3 ml ice-cold 0.9% NaCl solution and filtered (by suction) through Whatman GF/C glass fiber filters. Filters were washed three times with 3 ml 0.9% NaCl, placed in vials, and dried for 30 min at 60°C. Radioactivity was measured in a liquid scintillation counter (RackBeta, Pharmacia) by using Ultima-Gold (Packard) as scintillation cocktail.

Nicotinic Receptor. [^{3}H]Nicotine (85 Ci/mmol; Amersham) was used to assay specific binding of alkaloids to the nicotinic ACh receptor (nAChR). The membrane preparation was incubated for 40 min with differing concentrations of alkaloids or 1 mM nicotine as a positive control. The GF/C filters were presoaked with polyethylene glycol 8000 (5% in water) for 3 hr to reduce nonspecific binding of [^{3}H]nicotine. Further procedures were the same as described for mAChR.

Alpha$_1$ Receptor. [^{3}H]Prazosine (78 Ci/mmol; DuPont NEN) was used to assay specific binding of alkaloids to the alpha$_1$ receptor. The membrane preparation was adjusted to 400 μg in a final volume of 500 μl and incubated for 45 min at 20°C with differing concentrations of alkaloids or 400 μM phentolamine as a positive control. Further procedures were the same as described for mAChR.

Alpha$_2$ Receptor. [^{3}H]Yohimbine (81 Ci/mmol; DuPont NEN) was used instead of [^{3}H]prazosine; other conditions were the same as in the alpha$_1$ receptor assay.

Serotonin$_2$ Receptor. [^{3}H]Ketanserine (85,1 Ci/mmol; DuPont NEN) was used to assay specific binding of alkaloids to the serotonin$_2$ receptor (5-HT$_2$). The membrane preparation was adjusted to 400 μg in a final volume of 500 μl and incubated for 40 min at 20°C with differing concentrations of alkaloids or 100 μM mianserine as a positive control. Further procedures were as described for mAChR.

All experiments were reproduced at least twice and values in Figures 1–6 are arithmetic means $\pm$ SE (from at least three samples).

Other Bioassays

Bacterial Growth Inhibition. Gram-positive *Bacillus subtilis* (DSM 347) and gram-negative *Escherichia coli* (DSM 498) and *Serratia marcesens* (DSM 494) were obtained from a strain collection at ZMBH (Heidelberg). Bacteria were grown on LB medium (Sambrook et al., 1989). A turbidimetric test was used to determine growth effects of alkaloids. Bacteria (50 μl containing approx. 10^8 cells) were added to 5 ml LB medium (with up to 50 mM alkaloids) and incubated at 37°C (*E. coli*, *B. subtilis*) or 30°C (*S. marcescens*). After 6 hr, optical density was measured at 800 nm in a spectrophotometer. Assays were run in triplicate and compared to untreated controls. The data are presented in Table 1.

Inhibition of Radicle Growth. In order to determine phytotoxic effects, seeds of *Lepidium sativum* were incubated on water-soaked filter discs in Petri dishes to which different concentrations of alkaloids were added [as outlined in Wink and Twardowski (1992) and Wink and Latz-Brüning (1995)]. The lengths of the radicles, a good indicator of phytotoxic activity, (Table 1), were recorded after five days.

Animal Toxicity. Toxicity and deterrence of alkaloids towards bees (*Apis mellifera*) were reported previously by Detzel and Wink (1993). The data for feeding deterrence of alkaloids to larvae of the arctiid moth, *Syntomis mogadorensis* (choice experiments with alkaloids placed on food plants, such as *Taraxcum officinale*), derive from Wink and Schneider (1990). Polychaete worms (*Tubifex tubifex*) can easily be grown in the laboratory. They contain hemoglobin and normally live in colonies. If *Tubifex* are intoxicated, colonies disintegrate, and when they are dead, hemoglobin is released and can be determined photometrically after centrifugation of the samples. Worms were placed in 15 ml tap water in Petri dishes to which pH-adjusted alkaloids were added in concentrations between 0.002% and 1%. Behavior was recorded hourly, and free hemoglobin was measured at 415 nm after 16 hr (Table 2). Toxicity data for rats and mice (intraperitoneal and intravenous values when available) were taken from the literature (Merck Index 1996; Dictionary of Natural Compounds, 1996; Wink, 1993) (Table 3).

Construction of Dendrograms

The activities of alkaloids at the 14 molecular targets were converted in a 0/1 matrix and analyzed with maximum parsimony (MP) present in the program package PAUP 3.1.1 (Swofford, 1993). As outgroup, a data set was selected without activities, i.e., all sites were "0."

RESULTS AND DISCUSSION

The biological activity of more than 70 representative alkaloids from most structural types was assessed. Since results reported here were performed over a period of more than eight years, it was not possible to obtain data for each alkaloid in every test system used.

Antibacterial and Phytotoxic Properties

Alkaloids with marked antibacterial activity (growth reduction >50% at 5 mM or 100% at 10 mM) include ajmaline, berberine, boldine, cinchonidine, cinchonine, harmaline, harmine, lobeline, narcotine, norharman, quinidine, quinine, sanguinarine, strychnine, and yohimbine (Table 1). Several of these activities have been previously detected (see reviews: Verpoorte, 1998; Wink, 1993).

Only the benzophenanthridine alkaloid sanguinarine exhibits antibacterial activity similar to microbial antibiotics, such as tetracycline. Sanguinarine has been used accordingly as an antiplaque agent in mouthwash and toothpaste preparations (Martindale, 1993). The antibacterial activity of the other alkaloids is 10 times weaker, but still at a level at which it could be beneficial to a plant against microbial infections (see below).

Plants compete with other plants for light, water, and nutrients. The production, accumulation, and release of secondary compounds (including alkaloids) that inhibit germination of other plants (of the same or other species) or the development of seedlings is one of several complex strategies that have evolved to enhance the fitness of a plant (Rice, 1984; Rizvi and Rizvi, 1992; Wales, 1987; Roberts and Wink, 1998). Compounds are either actively secreted into the surrounding soil or are leached out by rain from aerial parts or detached leaves on the ground. Since compounds are often rapidly degraded by soil microorganisms, phytotoxic effects are not always clear-cut and appear to depend on several environmental factors (Rice, 1984; Rizvi and Rizvi, 1992; Waller, 1987).

Substantial phytotoxic properties (inhibition of radicle growth of *Lepidium sativum* $>50\%$ at 1% alkaloid concentrations, Table 1) were detected for the following alkaloids: aconitine, berberine, caffeine, cinchonidine, colchicine, cytisine, ergometrine, gramine, harmaline, hyoscyamine, lobeline, narcotine, papaverine, piperine, quinidine, quinine, salsoline, sanguinarine, sparteine, and theophylline. Although the inhibitory concentrations are usually quite high, a few are active at 3–5 mM concentrations, e.g., caffeine, colchicine, harmaline, papaverine, quinine, salsoline, and theophylline. These concentrations can be reached under natural conditions, suggesting that these alkaloids might exhibit phytotoxic effects in vivo.

Toxicity Towards Animals

Toxic effects against invertebrates were deduced from tests with *Tubifex tubifex* (Oligochaeta) and the hymenopteran *Apis mellifera* (Table 2). Apparent effects (toxicity at a concentration $<0.01\%$) were found for ajmaline, arecoline, aristolochic acid, atropine, berberine, boldine, brucine, canadine, cinchonidine, cinchonine, colchicine, coniine, emetine, ergotamine, glaucine, gramine, ephedrine, narcotine, nicotine, papaverine, physostigmine, pseudopelletierine, quinidine, scoulerine, sparteine, strychnine, theophylline, veratrine, and yohimbine. Even more alkaloids show substantial feeding deterrence to bees and a generalist herbivore *Syntomis mogadorensis* (Wink and Schneider, 1990) (Table 2).

Alkaloids are infamous for their vertebrate, especially mammalian, toxicity, and a large body of evidence has been published dealing with biochemical and pharmacological aspects (for reviews see Martindale, 1993; Robinson, 1981; Wink, 1993). Table 3 lists LD_{50} data for alkaloids used in our experiments.

LD_{50} data were determined with rats and mice; it has to be kept in mind that rats and mice are herbivores and, thus, adapted to dietary toxins. Other animals might be more sensitive. When possible, intraperitoneal and intravenous data were selected. When these data were not available, the corresponding per os values were taken.

Activity of Alkaloids at Basic Molecular Targets

Data from Tables 1–3 show that most alkaloids are toxic or inhibitory to more than one group of organisms; several affect bacteria, plants, insects, and mammals. Others show a more pronounced animal toxicity. The following experiments were devised to determine the potential modes of action underlying such allelochemical activities.

Interactions with DNA and Related Enzymes

We analyzed whether alkaloids affect basic molecular targets such as DNA intercalation, protein biosynthesis, or membrane stability. DNA intercalating alkaloids increase the melting temperature of DNA (Figure 1). Since the direct assay, however, is costly and time-consuming, we routinely used a more rapid assay that is based on the competitive release of the intercalating compound methylgreen from DNA (Burres et al., 1992). Several alkaloids increase the melting temperature (Table 4); strongest effects were detected for sanguinarine followed by harmine, berberine, ergotamine, berbamine, harmaline, quinidine, emetine, norharman, quinine, cinchonidine, boldine, cinchonine, solanine, canadine, chelidonine, lobeline, and ajmalicine (structures are given in Figure 14). A gel chromatography assay confirmed these findings: the intercalating alkaloids eluted with DNA as a single peak and were separated from free alkaloids (Latz-Brüning, 1994). The results were corroborated by the methylgreen assay (Table 4); the alkaloid-induced increase of melting temperature correlated with the release of methylgreen from DNA ($r = 0.857$; $P < 0.001$) (Figure 7A). The stronger the intercalation, the higher the melting temperature and the greater the release of methylgreen. DNA intercalation had been described previously for a few alkaloids, e.g., for sanguinarine, berberine, or harmaline (Krey and Hahn, 1969; Nandi and Maiti, 1985; Wink, 1993; Schmeller et al., 1997b). DNA intercalation can affect replication and transcription and can also lead to mutations, malformations, and even cancer.

Two related targets were studied: DNA polymerase I and reverse transcriptase (Table 4; Figures 2 and 3). Ajmaline, berbamine, berberine, boldine, cinchonine, cinchonidine, emetine, harmaline, harmine, lobeline, norharman, quinidine, quinine, and sanguinarine strongly affect both targets (Table 4). Inhibition of DNA polymerase I is correlated with bacterial growth inhibition caused by the alkaloids (Figure 8C). Although neither enzyme occurs in plants or animals, it is likely that DNA and RNA polymerases of plants and animals will

TABLE 4. INTERACTION OF ALKALOIDS WITH BASIC MOLECULAR TARGETS

	DNA melting temperature increase (°C)[a]	DNA methylgreen release IC_{50}[b]	DNA Pol I inhibition IC_{50}[c]	RNA RT inhibition IC_{50}[c]	Protein biosynthesis inhibition (%)[d]	Membrane permeability hemolysis (%)[e]
Imidazole alkaloids						
Pilocarpine	0	na[f]	na	na	na	na
Indole alkaloids						
Ajmalicine	1.0*	>1 mM	na (0.5 mM)	nd[f]	nd	na
Ajmaline	2.3*	>5 mM	>10 mM	7 mM	46 (4 mM)	na (1 mM)
Brucine	nd	>5 mM	na	5 mM	36	na
Ergometrine	nd	>5 mM	na	>10 mM	na	na
Ergotamine	13.7*	nd	nd	nd	20 (0.25 mM)	na
Gramine	nd	na	na	>10 mM	na	na
Harmaline	8.6*	0.3 mM	3.2 mM	2.4 mM	70	na
Harmine	16.1*	0.4 mM	0.9 mM	0.5 mM	95	2.3
Norharman	6.2*	<1 mM	8 mM	<0.2 mM	50 (0.2 mM)	3.3
Physostigmine	nd	na	na	>10 mM	15	na
Strychnine	nd	na	na	7 mM	20	na
Vincamine	nd	nd	na	na	54	2 (1 mM)
Yohimbine	nd	na	na	> 10 mM	62	na
Isoquinoline alkaloids						
Berbamine	13.2*	0.5 mM	0.7 mM	<0.25 mM	nd	2.3 (2 mM)
Berberine	15*	0.1 mM	0.4 mM	0.2 mM	100	na
Boldine	6*	<0.7 mM	5 mM	<1.5 mM	30	na
Canadine	1.8*	nd	nd	nd	15 (0.1 mM)	1.5 (0.2 mM)
Chelidonine	1.5*	(>2 mM)	(>2 mM)	(>2 mM)	28 (0.14 mM)	na
Emetine	7.4*	0.7 mM	5 mM	<0.5 mM	100 (0.01 mM)	na
Laudanosine	nd	na	na	4 mM	15	na
Narcotine	0	na	1 mM	na (0.5 mM)	na (0.1 mM)	4.5 (0.5 mM)

TABLE 4. CONTINUED

	DNA melting temperature increase (°C)[a]	DNA methylgreen release IC_{50}[b]	DNA Pol I inhibition IC_{50}[c]	RNA RT inhibition IC_{50}[c]	Protein biosynthesis inhibition (%)[d]	Membrane permeability hemolysis (%)[c]
Papaverine	0	na	na	nd	55 (0.8 mM)	1 (0.5 mM)
Salsoline	nd	na	na	na	30	na
Sanguinarine	24*	0.02 mM	<0.02 mM	0.03 mM	nd	5.2 (0.1 mM)
Phenylalkylamines						
L-Ephedrine	nd	na	na	na	na	na
Piperidine/pyridine alkaloids						
Anabasine	nd	na	na	na	na	na
Arecoline	nd	na	na	>10 mM	na	na
Cycloheximide	nd	na	na	na	90 (0.01 mM)	na
Lobeline	1.5*	>5 mM	>10 mM	<2 mM	45	na
Nicotine	nd	na	na	na	20	na
Piperine	nd	na	na	nd	35 (0.3 mM)	na
Pseudopelletierine	nd	na	na	na	na	na
Purine alkaloids						
Caffeine	nd	na	na	na	na	na
Pyrrolizidine alkaloids						
Heliotrine	nd	na	na	na	20	1
Monocrotaline	nd	na	na	na	25	na
Retronecine	nd	na	na	na	20	na
Riddelline	nd	na	na	na	20	na
Senecionine	nd	na	nda	na	50 (5 mM)	1.2
Quinoline alkaloids						
Cinchonine	5*	na	8 mM	6 mM	58 (5 mM)	na
Cinchonidine	6*	<5 mM	10 mM	<1 mM	90 (5 mM)	na

Quinidine	8*	1 mM	2.4 mM	< 1 mM	63	0.6 (1 mM)
Quinine	6*	2 mM	3.2 mM	< 2 mM	80	na
Quinolizidine alkaloids						
Anagyrine	nd	na	na	na	na	na
Angustifoline	nd	na	na	na	15 (5 mM)	na
Cytisine	nd	na	na	na	na	na
3β-Hydroxylupanine	na	na	na	na	na	91
13-Hydroxylupanine	na	na	na	na	38	3.5
Lupanine	na	na	na	na	15 (5 mM)	0.8
Lupinine	nd	na	na	na	31 (5 mM)	na
17-Oxosparteine	na	na	na	na	37	na
Sparteine	nd	na	na	na	na	na
Tetrahydrorhombifoline	nd	na	na	na	na	0.9
13-Tigloyloxylupanine	na	na	na	> 10 mM	20	na
Terpene alkaloids						
Aconitine	nd	na	na	na	na	na
Protoveratrine b	0	na	na	na	30	na
Solanine	3*	na (0.5 mM)	na (0.5 mM)	< 0.1 mM	50 (0.025 mM)	100 (0.2 mM)
Tropane alkaloids						
Hyoscyamine	nd	na	na	na	na	na
Scopolamine	0.3	na	na	na	na	na
Scopine	nd	na	na	na	na	na
Tropine	nd	na	na	na	15	na
Tropolone alkaloids						
Colchicine	nd	na	na	< 10 mM	na	0.7 (1 mM)

[a] 70 μM alkaloid solution and 70 μM *Sinapis* DNA; *alkaloid and DNA coelute when separated by gel chromatography on PD10 columns.
[b] Alkaloids were tested up to 5 mM; na = release < 25% at 5 mM.
[c] Alkaloids were preincubated with DNA or RNA; na = when inhibition at 10 mM < 25%.
[d] Alkaloids were tested at 1 mM; na = inhibition < 10%. If strong activities were discovered, then lower concentrations also were assayed.
[e] Alkaloids were tested at 5 mM; na = inhibition < 0.6%. If strong activities were discovered, then lower concentrations also were assayed.
[f] nd = not determined; na = not active.

be affected in a similar way. The compounds also showed substantial effects in the DNA intercalating assays; a significant ($P < 0.001$) correlation (Figure 7B) between the degree of alkaloid-induced increase of DNA melting temperature and the IC_{50} of DNA polymerase and RT inhibition suggests that binding of the corresponding alkaloids to DNA or RNA is the cause of enzyme inhibition in many instances. Effects were more strongly expressed when alkaloids and nucleic acids were preincubated (Figures 2 and 3) compared to alkaloids and enzymes preincubated in the absence of nucleic acids (Latz-Brüning, 1994). Solanine, which caused a weak increase of the melting temperature, showed a strong inhibitory effect on RT and a weaker one on DNA polymerase I. Narcotine and papaverine, which do not intercalate DNA, inhibit DNA (polymerase I and RT, respectively. We assume that mechanisms other than intercalation must be at work in the latter instances. Intercalation and related inhibitory effects are correlated with the degree of toxicity in bacteria, insects, and vertebrates. Significant correlations ($P < 0.001$) can be established with DNA melting temperatures, the release of methylgreen, and inhibition of bacterial growth

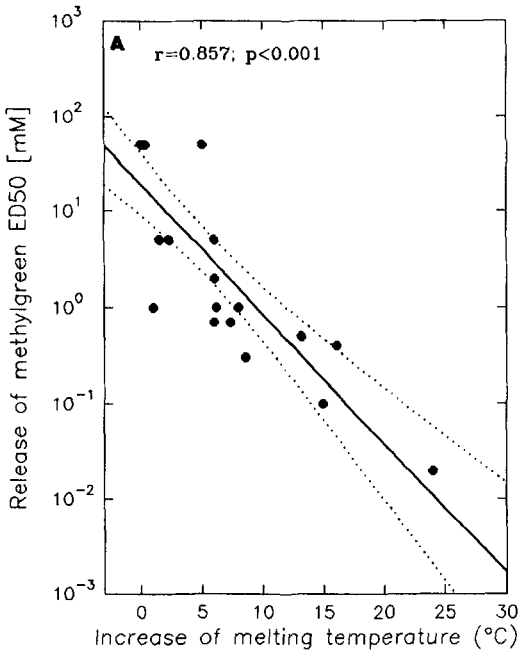


FIG. 7. Pairwise correlations between the degree of DNA intercalation (as measured by change in melting point) by an alkaloid and the corresponding release of methylgreen from DNA (A), inhibition (ED_{50}) of DNA polymerase I and reverse transcriptase (B), and inhibition of protein biosynthesis (C) (data from Table 4). The 95% confidence intervals are indicated by dotted lines.

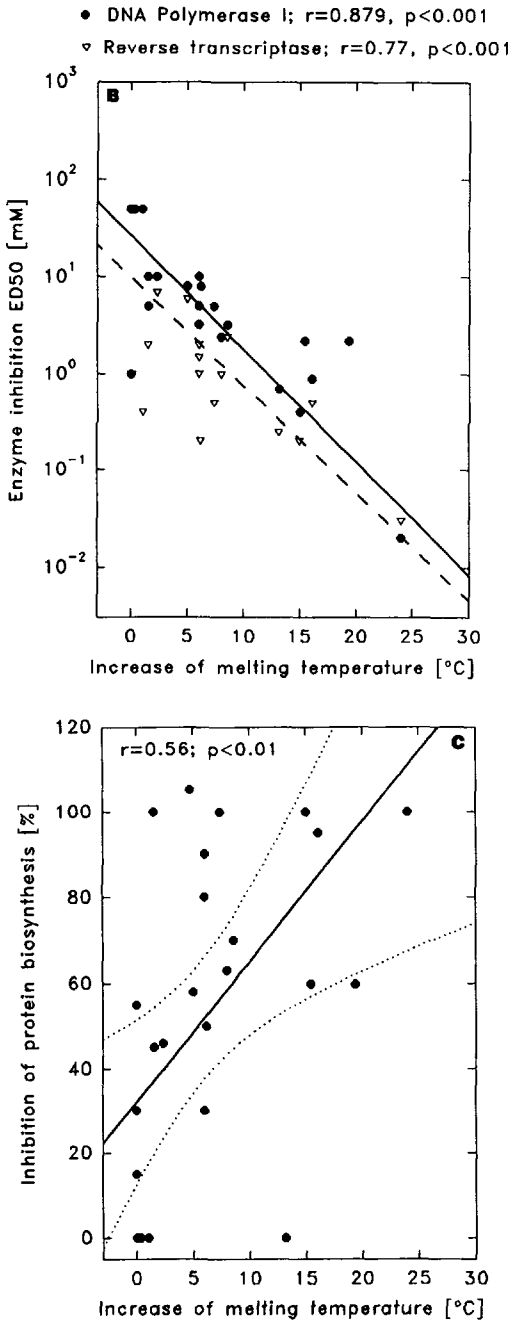


FIG. 7. Continued.

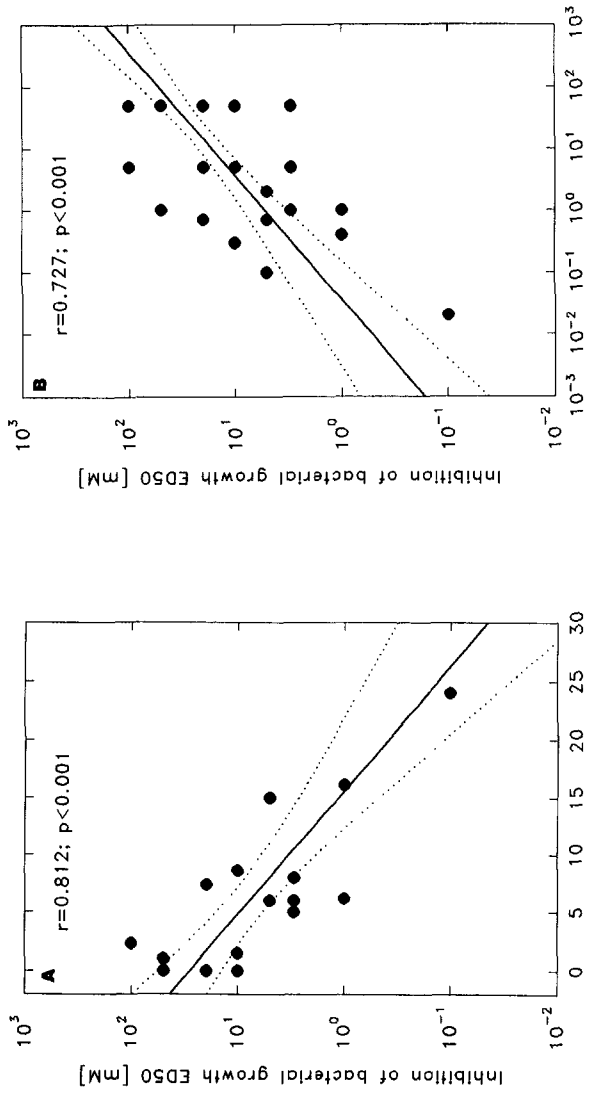


FIG. 8. Pairwise correlations between reduction of bacterial growth (ED_{50} of inhibition) and the effect of an alkaloid at basic molecular targets: (A) DNA intercalation (measured as increase of melting point) (B) release of methylgreen; (C) inhibition of DNA Pol I (ED_{50} values each); (D) inhibition of protein biosynthesis (in %) by alkaloids (data from Tables 1 and 4). Dotted lines indicate the 95% confidence intervals.

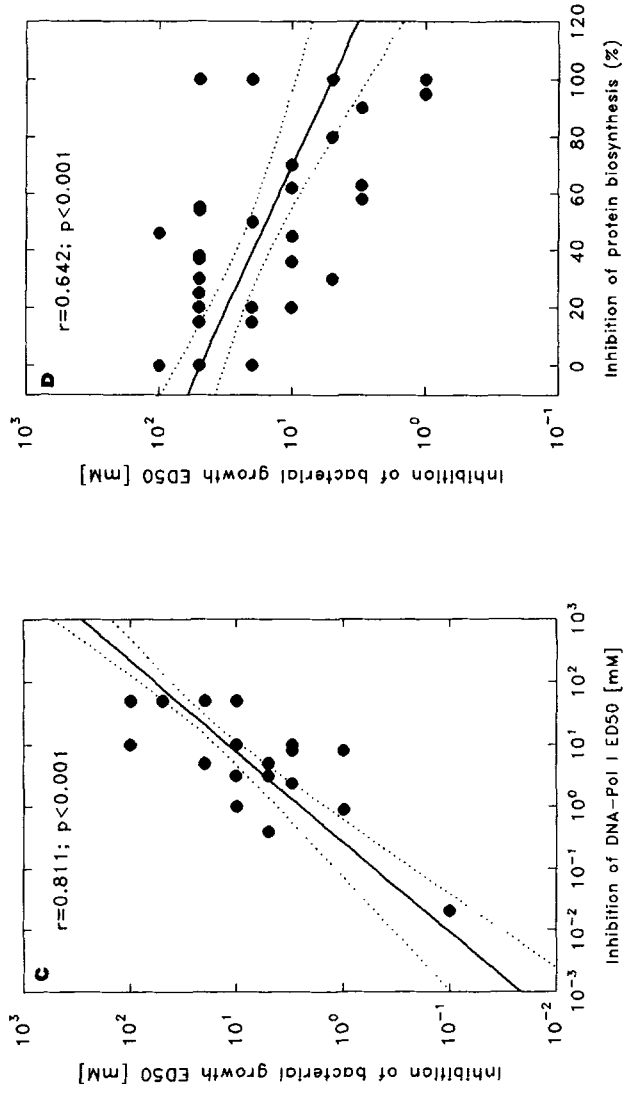


FIG. 8. Continued.

(Figure 8A,B), toxicity in bees (Figure 9G), and vertebrate toxicity (Figure 10A). Thus, intercalation of secondary metabolites with DNA is a strong indicator that they have inhibitory or toxic effects at the organismic level.

Inhibition of Protein Biosynthesis

Protein biosynthesis, which is a critical and important target in all cells, was strongly affected (inhibition >30% at 1 mM) by ajmaline, berberine, boldine, brucine, cinchonine, cinchonidine, emetine, harmaline, harmine, 13-hydroxylupanine, lobeline, monocrotaline, norharman, papaverine, piperine, quinidine, quinine, salsoline, sanguinarine, solanine, and yohimbine; a weaker inhibition (<20%) was observed for caffeine, lupanine, nicotine, sparteine, and strychnine (Table 4; Figure 4). Most compounds that substantially affected DNA, DNA polymerase I and RT, also were active as translation inhibitors; a weak but significant ($P < 0.01$ correlation is seen between the degrees of intercalation and inhibition of translation (Figure 7C). Interaction of alkaloids with ribosomal nucleic acids, for example, rRNA, tRNA, or mRNA, is likely, in addition to

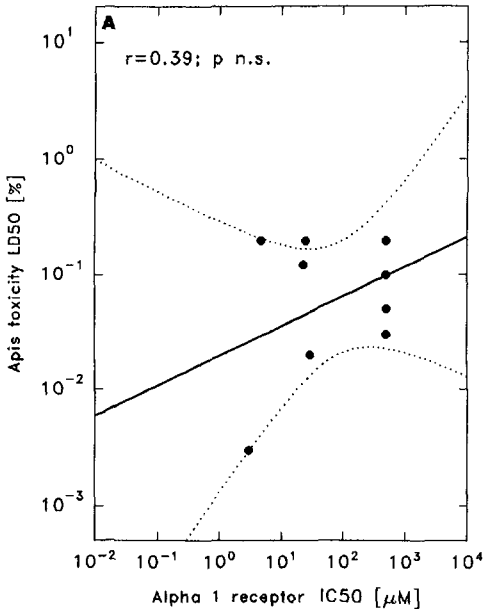


FIG. 9. Pairwise correlations between alkaloid-mediated toxicity in bees (LD_{50}) and the degree of neuroreceptor binding (IC_{50}) (A–E); inhibition of protein biosynthesis (F); and DNA intercalation (measured as release of methylgreen; ED_{50}) (G). Dotted lines indicate the 95% confidence intervals.

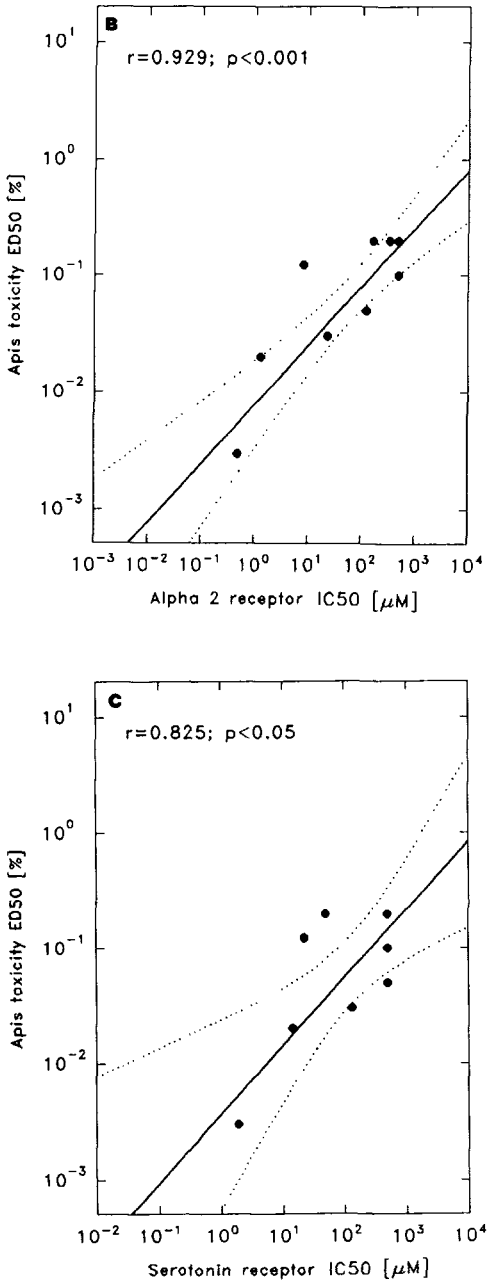


FIG. 9. Continued.

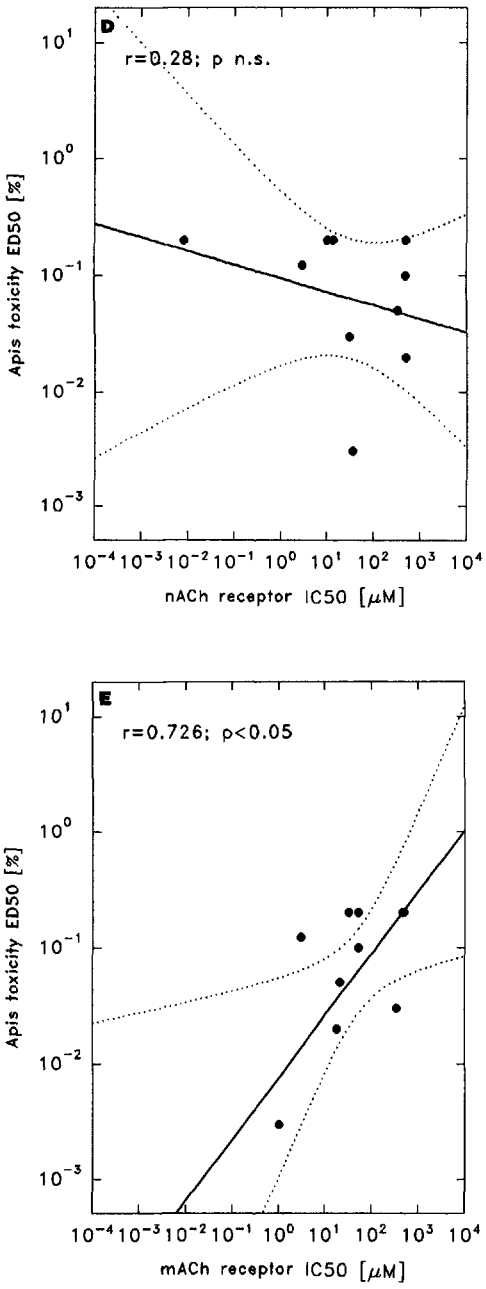


FIG. 9. Continued.

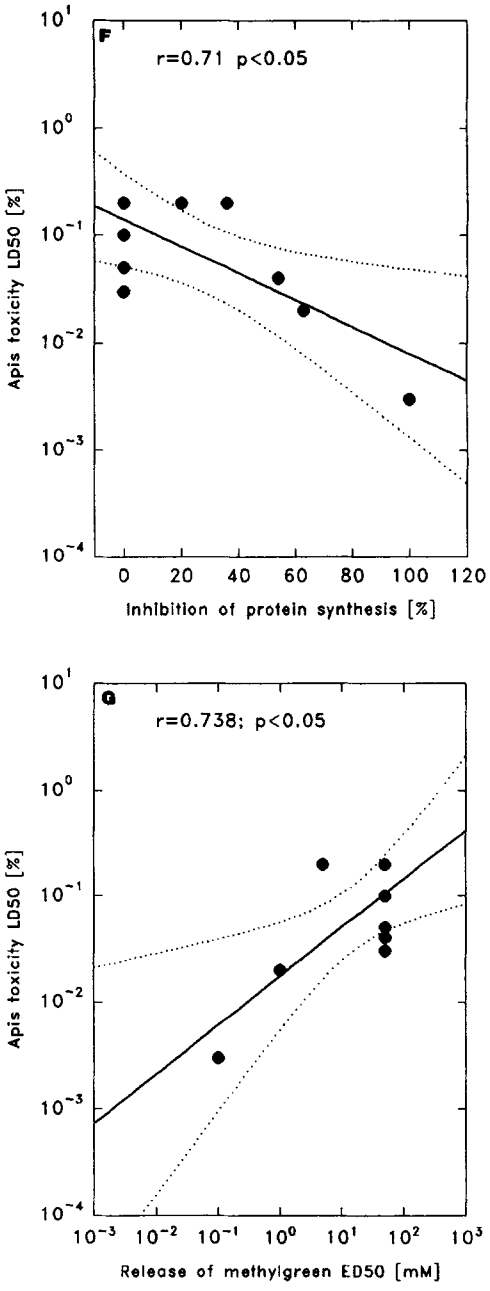


FIG. 9. Continued.

interactions with ribosomal proteins (Wink and Twardowski, 1992). A few intercalating compounds, such as berbamine or ergometrine, could not be established as protein biosynthesis inhibitors, since solubility problems prevented us from testing them at >0.1 mM concentrations. On the other hand, most compounds that do not intercalate (such as aconitine, caffeine, colchicine, cytisine, gramine, hyoscyamine, lupanine, narcotine, scopolamine, sparteine, or strychnine) do not influence translation substantially (inhibition < 20%); exceptions noted were papaverine, salsoline, and yohimbine. Inhibition of protein biosynthesis is significantly correlated with inhibition of bacterial (Figure 8D) and radicle growth (Figure 1C), and also with insect (Figure 9F) and vertebrate toxicity (Figure 10B), indicating the importance of this molecular target.

Permeability of Biomembranes

The integrity of biomembranes is of ultimate importance for the functioning of cells; compounds that disturb biomembranes and make cells leaky are usually

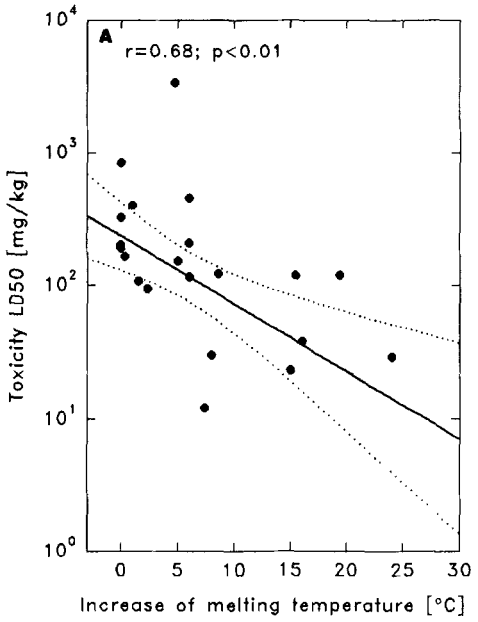
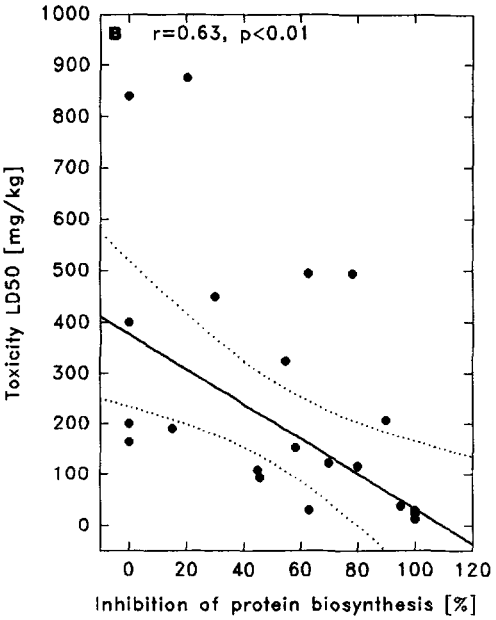


FIG. 10. Pairwise correlations between alkaloid-mediated vertebrate toxicity (LD_{50}) and alkaloidal effects at basic molecular targets: (A) DNA intercalation (increase of melting temperature); (B) inhibition of protein biosynthesis; and (C) IC_{50} values of alkaloid binding to different neuroreceptors ($alpha_1$, $alpha_2$, serotonin, nAChR, nAChR). Data taken from Tables 3–5. Dotted lines indicate 95% confidence intervals.



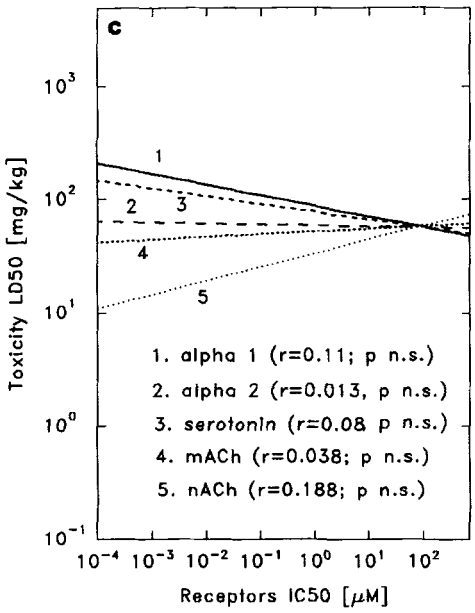


FIG. 10. Continued.

strong cell poisons. Natural products that exhibit these properties are either lipophilic or amphiphilic, such as mono-, sesqui-, and diterpenes or triterpenes and steroid saponins, respectively. These often exhibit allelochemical effects (Wink and Twardowski, 1992; Wink and Latz-Brüning, 1995). Hemolysis of erythrocytes offers a simple in vitro test system (Figure 5; Table 4) to study the effect of chemicals on membrane stability (Latz-Brüning, 1994). Of the series of alkaloids tested, only solanine and 3β-hydroxylupanine showed hemolytic activity. Since solanine has the structure of an amphiphilic saponin (see Figure 14 below), this activity is not surprising and consistent with previous results (Rice, 1994; Roddick et al., 1988). Weak hemolytic properties were detected for berbamine, harmine, 13-hydroxylupanine, narcotine, norharman, sanguinarine, and vincamine. Whether they contribute to phytotoxicity and other effects cannot be determined from these experiments.

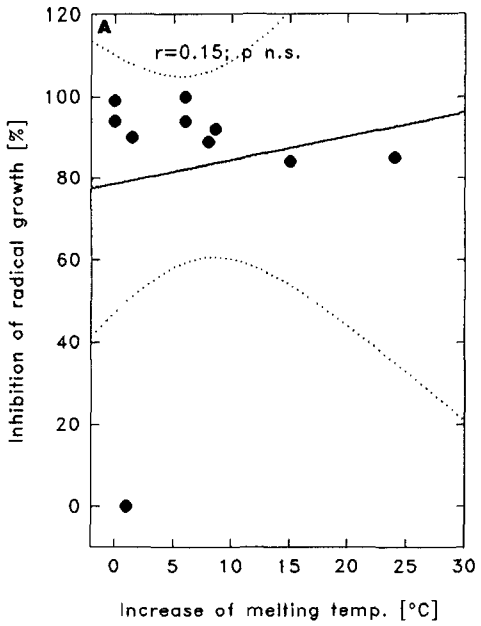


FIG. 11. Pairwise correlations between phytotoxicity and the effect of alkaloids at basic molecular targets. Phytotoxicity is expressed as inhibition of radicle growth in *Lepidium sativum* 1% alkaloid solutions (Table 1): (A) DNA intercalation (measured as increase of melting point); (B) release of methylgreen (ED_{50}); (C) inhibition of protein biosynthesis (data from Tables 1 and 4). Dotted lines indicate 95% confidence intervals.

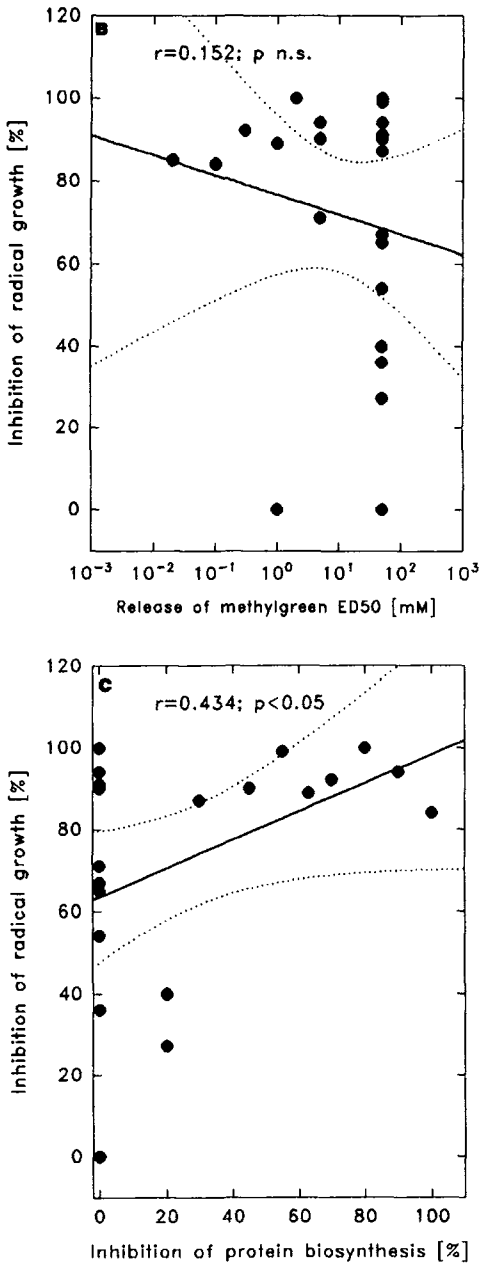


FIG. 11. Continued.

Other Molecular Targets

For a few antibacterial and phytotoxic alkaloids (Table 1), such as aconitine, caffeine, colchicine, cytisine, gramine, hyoscyamine, lupanine, nicotine, scopolamine, and sparteine, we could not detect a significant effect on the targets studied (Table 4). Interactions with other molecular targets must exist that mediate adverse effects in these and other instances.

It is well established that colchicine inhibits the polymerization of tubulin to microtubules, which are important for cellular transport and cell division. Since cell division takes place during germination, the colchicine–tubulin interactions could be relevant for phytotoxicity.

Caffeine inhibits phosphodiesterase in animals; cAMP and cGMP and the corresponding phosphodiesterase also exist in plants but their function is controversial. Whereas it is unlikely that cAMP/cGTP functions as part of the signal transduction (as in animals), the cyclic nucleotides might play a role in gene regulation (as in bacteria). Therefore, phosphodiesterase inhibition could be a relevant target; caffeine, theophylline (Wink and Twardowski, 1992), and papaverine, which are known phosphodiesterase inhibitors, also exhibited phytotoxic properties. Caffeine was shown to inhibit cell division in *Coffea arabica* (Friedman and Waller, 1983).

Aconitine, cytisine, lupanine, and sparteine strongly bind to acetylcholine receptors (Schmeller et al., 1994) and brucine/strychnine to glycine receptors (Table 5), but these targets should not be relevant in plants. However, aconitine, lupanine, and sparteine also affect Na^+ and K^+ channels in animals (also the phytotoxic ajmaline and quinidine inhibit this target) (Wink, 1993; Körper et al., 1998). Therefore, it needs to be established whether ion channels are affected in plant cells. Gramine might interact with the metabolism of plant growth factors since it shares structural similarities with auxins.

Arecoline, ammodendrine, anagyrine, and coniine are known mutagens and teratogens (Keeler and Dekker, 1992) leading to chromosome breakage and malformations in animals; whether they are active in a similar way in microbes or plants has not yet been established.

Binding of Alkaloids to Neuroreceptors

Many secondary metabolites affect neurotransmission and signal transduction (Harborne, 1993; Robinson, 1981; Wink, 1993, 1998). In animals, signal transduction in the peripheral and central nervous system (CNS) and at neuromuscular junctions, which govern the coordination and regulation of all organ and body functions, represents a crucial and important target that should be ideal for interference with defense chemicals. Since any substantial interference with an allelochemical at neuroreceptors (e.g., competitive inhibition of ligand bind-

ing by an antagonistic alkaloid or agonistic receptor activation by an alkaloid with structural similarity to the natural ligand) will influence neuronal signal transduction (including muscular activity and CNS activity), the intake of a larger dose of alkaloid-containing food should lead to short-term (within several hours after ingestion) physiological disturbances. These adverse effects and the bitter taste of most alkaloids should provide a clue to herbivores or predators to avoid alkaloid-producing organisms in the future (associative learning).

Molecular targets include the neuroreceptor itself, where agonists mimic the function of a signal compound by binding to its receptor and causing the normal response. Antagonists, in contrast, also bind to the receptor but do not activate the transmitter-induced effects. Thus, an antagonist acts as an inhibitor of the natural ligand (or agonist) by competing for binding sites on the receptor, thereby blocking the physiological response (antagonists are therefore often called blockers). Other targets are voltage-gated Na^+, K^+, and Ca^{2+} channels; enzymes that deactivate neurotransmitters after they have bound to a receptor, such as acetylcholine esterase, monoamine oxidase, and catechol-*O*-methyltransferase; transport processes, which are important for the uptake and release of the neurotransmitters in the presynapse or synaptic vesicles; and key enzymes of signal pathways {adenylyl cyclase (making cAMP), phosphodiesterase (inactivating cAMP), phospholipase C [releasing inositol phosphates such as IP_3 and diacylglycerol (DAG)], and several protein kinases, such as protein kinase C or tyrosine kinase (activating other regulatory proteins or ion channels)}.

We analyzed the binding of alkaloids at neuroreceptors as one of the prime targets of neuronal signal transduction. We determined whether an alkaloid can displace a specifically bound ligand from a neuroreceptor, such as $alpha_1$, $alpha_2$ adrenergic receptors, serotonin receptor, and nicotine and muscarinic acetylcholine receptors (Figure 6; Table 5). Almost every alkaloid can bind to at least one of the neuroreceptors studied and a number affect several (Table 5, Figure 12). Structure-function relationships are discussed in more detail in Wink (1998).

Affinity to one receptor is often significantly correlated with effects at other neuroreceptors. For example, alkaloids which affect $alpha_1$ receptors also affect $alpha_2$ and serotonin receptors (Figure 13A and B); those that affect $alpha_2$ are active at serotonin receptors (Figure 13E). Correlations between muscarinic and nicotinic acetylcholine receptors (Figure 13J) and between serotonin and adrenergic receptors are weaker (Figure 13C and H) or lack a clear correlation (Figure 13D,F,G,I,J). By studying synthetic antidepressants, it was shown recently that binding affinities at different G-protein-coupled neuroreceptors are usually correlated (Owens et al., 1997). Several interactions of alkaloids with neuroreceptors have been reported previously (Schmeller et al., 1994, 1995, 1997a,b; review in Wink, 1993) or can be deduced from corresponding pharmacological activities (Merck Index, 1996; Martindale, 1993).

TABLE 5. INTERACTION OF ALKALOIDS WITH NEURORECEPTORS AND ACETYLCHOLINE-RELATED ENZYMES[a]

	Adrenergic receptor		Serotonin receptor,	Acetylcholine receptor				
	$Alpha_1$	$Alpha_2$	5-HT_2	mACh	nACh	AChE	BChE	ChAT
Imidazole alkaloids								
Pilocarpine	na	na	na	11.0	1656	na	na	na
Indole alkaloids								
Brucine	4.7	347.3	na	51.3	13.6	na	na	na
Ergometrine	4.4	0.9	1.5	2.0	178.2	na	161.3	na
Gramine	26.3	8.7	8.5	677.1	30.7	1019	232.3	na
Harmaline	34.0	7.5	14.6	33.5	na	173.2	90.4	na
Harmalol	56.8	16.9	30.6	60.4	623.7	93.1	37.1	na
Harman	nd	nd	nd	nd	nd	na	72.0	nd
Harmine	nd	nd	nd	nd	nd	1005	175.3	nd
Harmol	nd	nd	nd	nd	nd	na	26.1	nd
Norharman	nd	nd	nd	nd	nd	na	63.5	nd
Physostigmine	na	73.6	394.2	66.2	1992	0.03	16.2	na
Strychnine	25.1	172.3	51.6	32.8	10.2	na	130.8	na
Isoquinoline alkaloids								
Berberine[b]	3.2	0.48	1.9	1.0	35.5	167.4	55.8	na
Boldine	0.53	0.09	0.67	118.1	11.1	na	na	na
Emetine	6.2	0.07	7.6	58.2	na	na	na	na
Laudanosine	18.4	0.82	8.2	67.1	1313	na	415.7	na
Morphine	na	na	na	na	na	na	na	na
Palmatine[b]	5.8	0.96	2.9	4.1	>100	124.5	425.6	>100
Salsoline	115.1	9.8	146.2	na	na	na	na	na
Sanguinarine[b]	33.6	6.4	91.7	2.4	11.8	10.9	17.4	0.3

Phenylalkylamines								
L-Ephedrine	186.4	14.6	60.0	2649	na	na	na	na
Piperidine/pyridine alkaloids								
Ammodendrine[c]	257.5	11.6	109.0	523.6	9.1	na	275.4	na
Anabasine	2374	47.6	na	na	0.58	na	na	na
Arecoline	na	29.7	na	32.1	5.7	na	na	na
Coniine	na	260.0	492.7	2071	19.0	na	327.5	na
Cotinine	na	na	na	1776	2.5	na	na	na
Nicotine	na	na	na	882.8	0.008	na	na	na
Pseudopelletierine	na	na	na	386.1	0.7	na	na	na
Purine alkaloids								
Caffeine	na	na	na	464.8	na	na	na	na
Pyrrolizidine alkaloids								
Acetylheliosupine[d]	39.1	2.9	23.2	71.3	159.7	na	na	na
Echihumiline[d]	na	358.8	549.0	89.2	na	na	314.4	na
Echihumuline N-oxide[d]	na	>50	182.0	8.7	na	na	na	na
Echimidine[d]	na	900	257.6	512.5	na	na	na	na
Heliosupine[d]	148.1	15.0	77.1	392.0	na	na	na	na
Heliosupine N-oxide[d]	na	na	na	350.0	na	na	na	na
Heliotrine[d]	na	na	535.4	52.2	na	na	na	na
Monocrotaline[d]	na	na	203.4	na	na	na	na	na
Pycnanthine[d]	na	na	407.6	177.2	na	na	462.6	na
Retronecine[d]	na	na	na	127.9	na	na	na	na
Riddeline[d]	na	na	na	208.7	na	na	na	na
Senecionine[d]	na	na	249.4	43.0	na	na	na	na
Seneciphylline[d]	na	341.4	608.6	52.6	na	na	na	na
Quinoline alkaloids								
Cinchonine	5.2	1.4	5.5	19.2	na	na	22.9	na
Cinchonidine	1.1	1.3	10.7	19.7	na	na	na	na
Quinine	5.7	2.5	6.4	4.5	na	na	109.0	na
Quinidine	29.7	1.3	14.4	18.4	na	na	na	na

TABLE 5. CONTINUED

	Adrenergic receptor		Serotonin receptor,	Acetylcholine receptor				
	$Alpha_1$	$Alpha_2$	$5\text{-}HT_2$	mACh	nACh	AChE	BChE	ChAT
Quinolizidine alkaloids								
Albine[c]	na	na	na	32.9	237.7	na	na	na
Anagyrine[c]	na	na	na	132.1	2096	na	na	na
Angustifoline[c]	na	na	na	25.3	na	na	na	na
Annotinine	na	na	na	260.5	na	na	na	na
Cytisine[c]	na	na	na	398.2	0.14	na	na	na
13-Hydroxylupanine[c]	na	na	na	139.7	467.2	na	na	na
13-Tigloyloxylupanine[c]	na	na	508	11.1	99.8	na	548.3	na
Lupanine[c]	na	na	na	118.0	5.3	na	na	na
Lupinine[c]	na	207.8	na	189.9	na	na	na	na
N-methylcytisine[c]	na	na	na	416.7	0.05	na	na	na
17-Oxosparteine[c]	na	180.7	na	117.9	155.0	na	na	na
3β-hydroxylupanine[c]	na	na	na	74.1	74.1	192.4	na	na
Multiflorine[c]	na	na	na	49.4	na	na	na	na
Sparteine[c]	na	127.7	na	21.3	330.8	na	165.5	na
Tetrahydrorhombifoline[c]	na	na	na	128.8	347.6	na	na	na
Terpene alkaloids								
Aconitine	na	331.6	na	1.3	na	na	na	na
Tropane alkaloids								
Atropine[c]	6.1	10.1	6.0	0.005	284.4	na	na	na
Cocaine[c]	na	506.7	317.3	56.7	371.4	na	274.3	na
6β-Hydroxyhyoscyamine[c]	12.6	42.0	nd	0.039	na	na	na	na
7β-Hydroxyhyoscyamine[c]	na	37.6	nd	0.008	na	na	na	na

Littorine[e]	66.3	72.3	68.9	0.003	909.8	na	na	na
Noratropine[e]	16.4	30.4	22.7	0.2	494.4	na	na	na
Scopolamine[e]	113.0	359.7	168.0	0.002	928.4	na	na	na
Tropine[e]	na	na	na	2631	na	na	na	na
Tropolone alkaloids								
Colchicine	na	23.8	133.2	347.3	30.0	na	na	na

[a] Concentrations are given as in μM that replace 50% of the specifically bound ligand (=IC_{50}) or that inhibit the enzymes by 50%; AChE = acetylcholine esterase, BChE = butylcholine esterase, ChAT = choline acetyltransferase; na = not active at 500 μM, nd = not determined.
[b] After Schmeller et al. (1997b).
[c] After Schmeller et al. (1994).
[d] After Schmeller et al. (1997).
[e] After Schmeller et al. (1995).

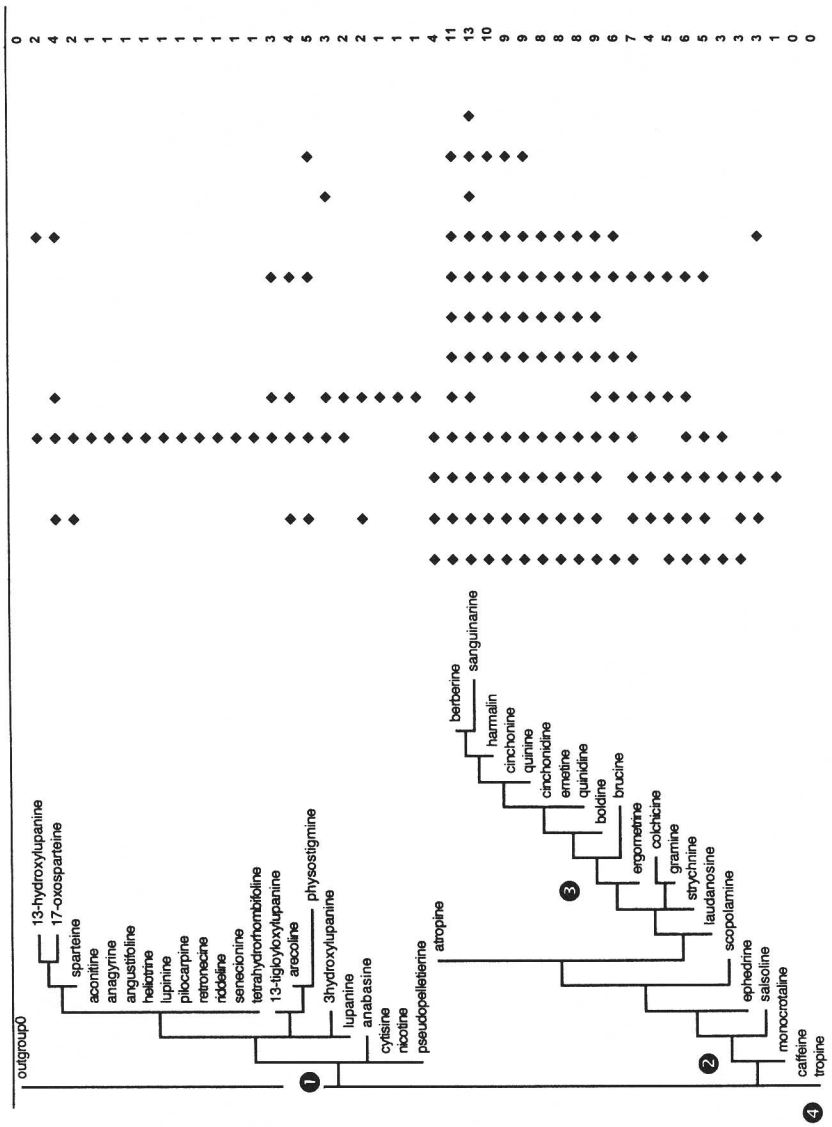

α1
α2
HT
mA
nA
DNA
Pol
RT
TR
M
AChE
ACT
N
outgroup0
13-hydroxylupanine
17-oxosparteine
sparteine
aconitine
anagyrine
angustifoline
heliotrine
lupinine
pilocarpine
retronecine
riddelline
senecionine
tetrahydrorhombifoline
13-tigloyloxylupanine
arecoline
physostigmine
3hydroxylupanine
lupanine
anabasine
cytisine
nicotine
pseudopelletierine
atropine
berberine
sanguinarine
harmalin
cinchonine
quinine
cinchonidine
emetine
quinidine
boldine
brucine
ergometrine
colchicine
gramine
strychnine
laudanosine
scopolamine
ephedrine
salsoline
monocrotaline
caffeine
tropine
1
2
3
4

FIG. 12. Dendrogram (established with maximum parsimony) of relationships between alkaloids and the molecular targets affected. Four main groups can be identified: (1) upper clade: alkaloids that affect only neuroreceptors (especially mAChR and nAChR), (2) upper part of clade 2 between monocrotaline and colchicine: alkaloids that bind to adrenergic and serotonin receptors, (3) lower part of clade 2 between ergometrine and berberine: alkaloids that affect concomitantly neuroreceptors and DNA and correlated targets, and (4) alkaloids that do not affect any target tested (i.e., caffeine and tropine). α_1 = alpha$_1$; α_2 = alpha$_2$ adrenergic receptors; HT = serotonin$_2$ receptor; mA = muscarinic acetylcholine receptor; nA = nicotonic acetylcholine receptor; DNA = DNA intercalation; Pol = DNA polymerase I; RT = reverse transcriptase; TR = protein biosynthesis; M = hemolysis; ACT = choline acetyl transferase; N = number of targets at which an alkaloid exhibits a significant effect. Consensus tree of a heuristic search; tree length: 36 steps; CI = 0.389; HI = 0.611; RI = 0.848.

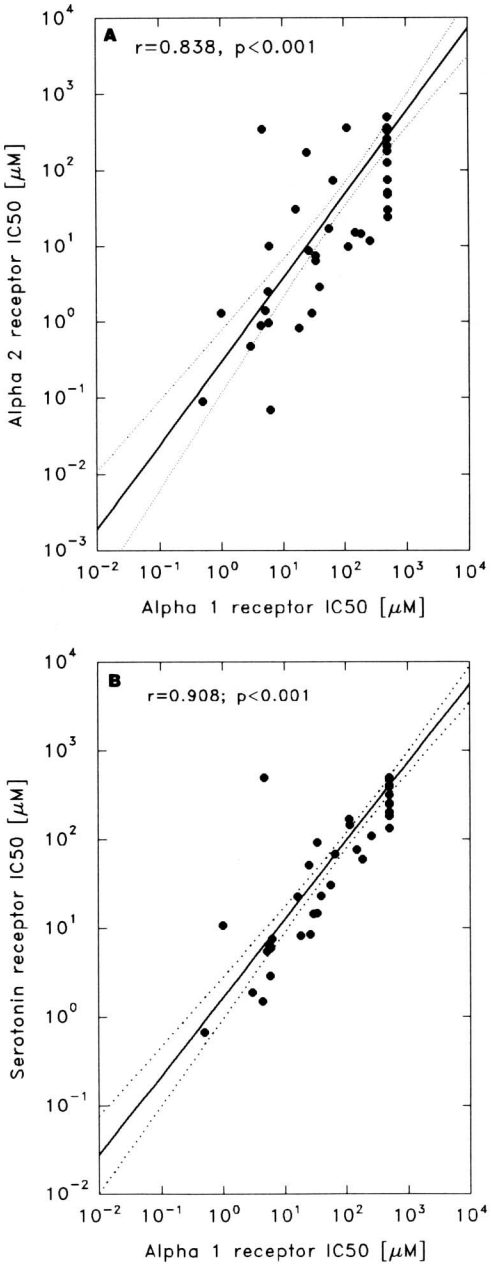


FIG. 13. Pairwise correlations between binding affinities of alkaloids (data from Table 5 as expressed as IC_{50} in μM) at different neuroreceptors. Dotted lines indicate the 95% confidence intervals.

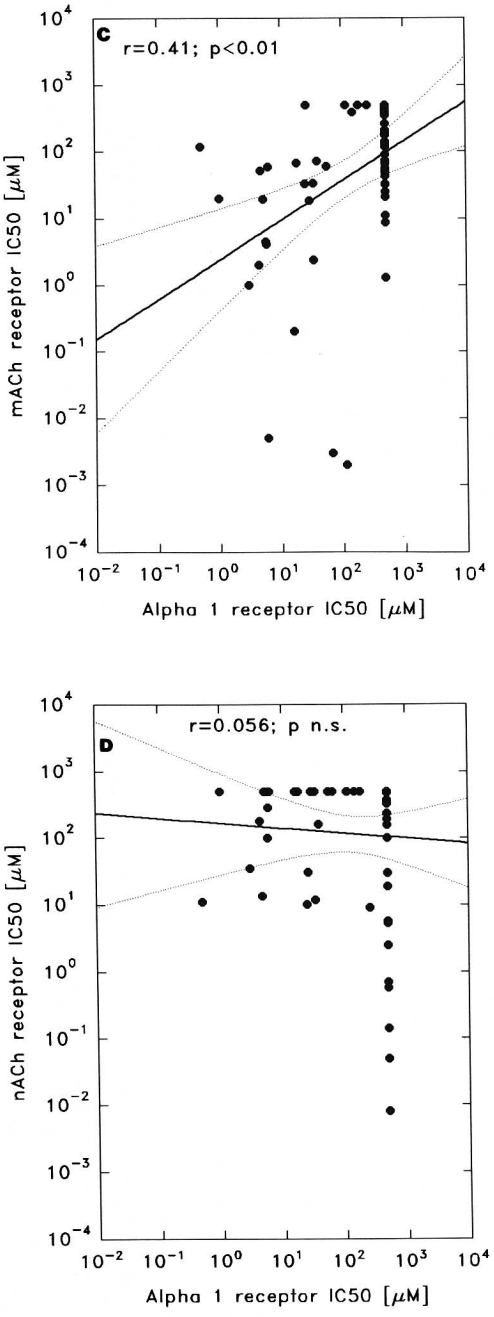


FIG. 13. Continued.

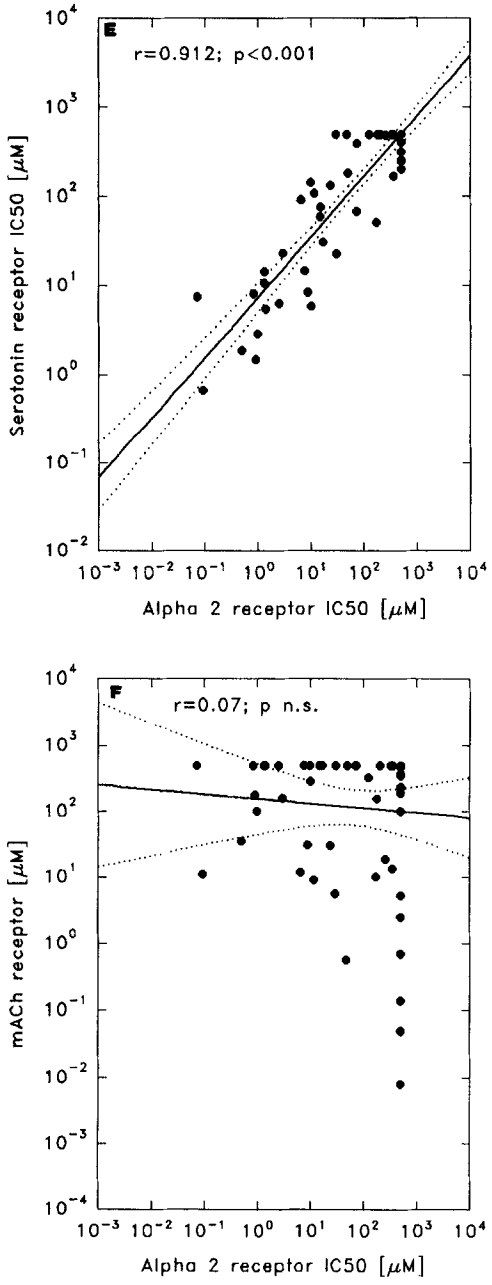


FIG. 13. Continued.

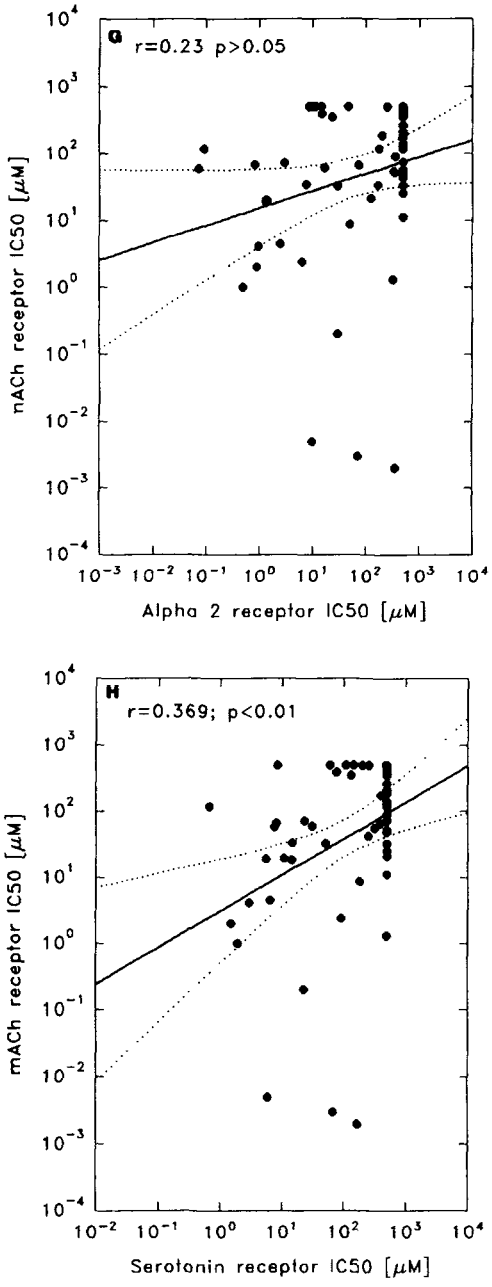


FIG. 13. Continued.

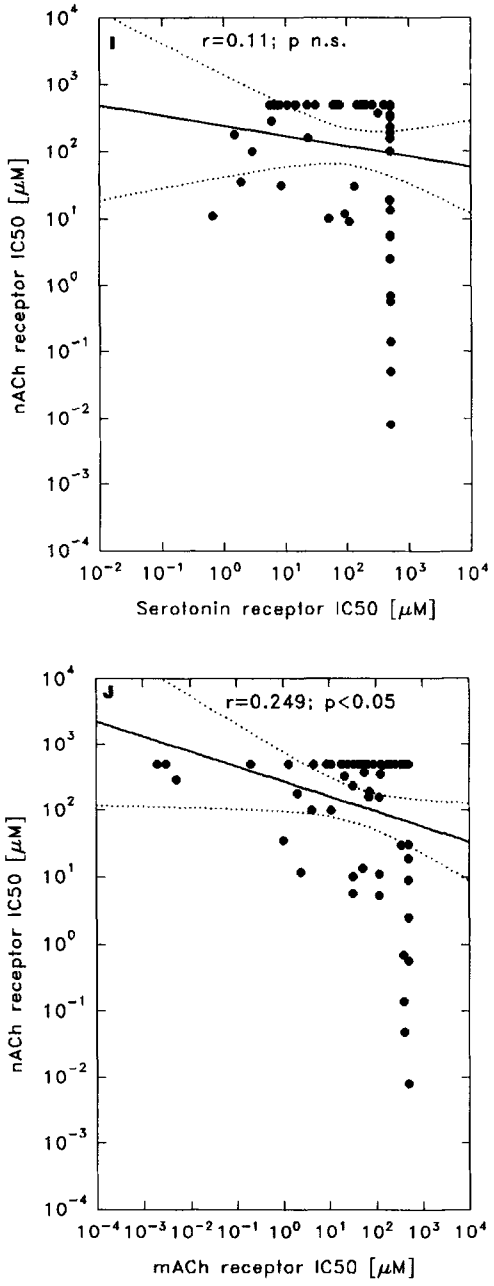


FIG. 13. Continued.

Are binding properties relevant for plant–herbivore interactions? Some caution is necessary in interpreting in vitro data. Lack of resorption and/or rapid detoxification of allelochemicals in animals may prevent the establishment of internal alkaloid concentrations high enough to activate or inhibit a given neuroreceptor. Despite this, significant correlations can be established between IC_{50} values of alkaloid binding at $alpha_2$, serotonin, and mACh receptors and bee toxicity (Figure 9B,C,E). However, corresponding correlations were not evident between IC_{50} binding values and vertebrate toxicity (Figure 10C), which is probably due to toxicity data used and the resorption/degradation problems mentioned. As expected, no significant correlation can be established between IC_{50} values of receptor binding and ED_{50} of feeding deterrence (Table 2).

A further question is whether the inhibitor concentration determined in in vitro experiments (Tables 4 and 5) relate in any way to the in vivo situation. A simple calculation may help to assess this problem: Alkaloids are usually stored in high concentrations at sites that are important for growth and reproduction and can reach 1–5% of the dry weight in seeds. We can assume an alkaloid concentration of 10 or 50 mg/g of seed and a small herbivore (such as a rabbit) with a body weight of 1000 g. If this animal ingested 100 g of these seeds, it would take up 1000–5000 mg of alkaloids. Assuming a mean molecular weight of 250, the alkaloid concentration in our herbivore would maximally be 4–20 mM. This concentration would be high enough to completely block the binding of acetylcholine, serotonin, or noradrenaline at their receptors (compare the IC_{50} values in Tables 4 and 5) or to interfere with DNA and related processes. Since resorption is usually never complete, however, and detoxification and/or elimination set in quickly after ingestion (depending greatly on the animal species in question), such a high internal concentration would not be reached in reality. Even a 10- to 20-fold lower concentration in the animal (i.e., 200–1000 μM), however, would be high enough to interfere with most receptors. Thus, the binding activities should matter in an ecological context.

CONCLUSIONS

Ajmaline, berbamine, berberine, boldine, cinchonine, cinchonidine, ergometrine/ergotamine, harmaline, harmine, lobeline, norharman, papaverine, quinidine, quinine, sanguinarine, and solanine all affect more than one of the basic molecular targets. It is likely that these interactions are responsible (at least in part) for phytotoxic and antibacterial effects and for animal toxicity caused by the alkaloids (Wink and Twardowski, 1992; Wink and Latz-Brüning, 1995). In addition, the alkaloids can bind to one or several neuroreceptors present in animals. Most of the allelochemical alkaloids are compounds with a broad spectrum of activity that can be reflected in a dendrogram (Figure 12).

DNA-intercalating alkaloids

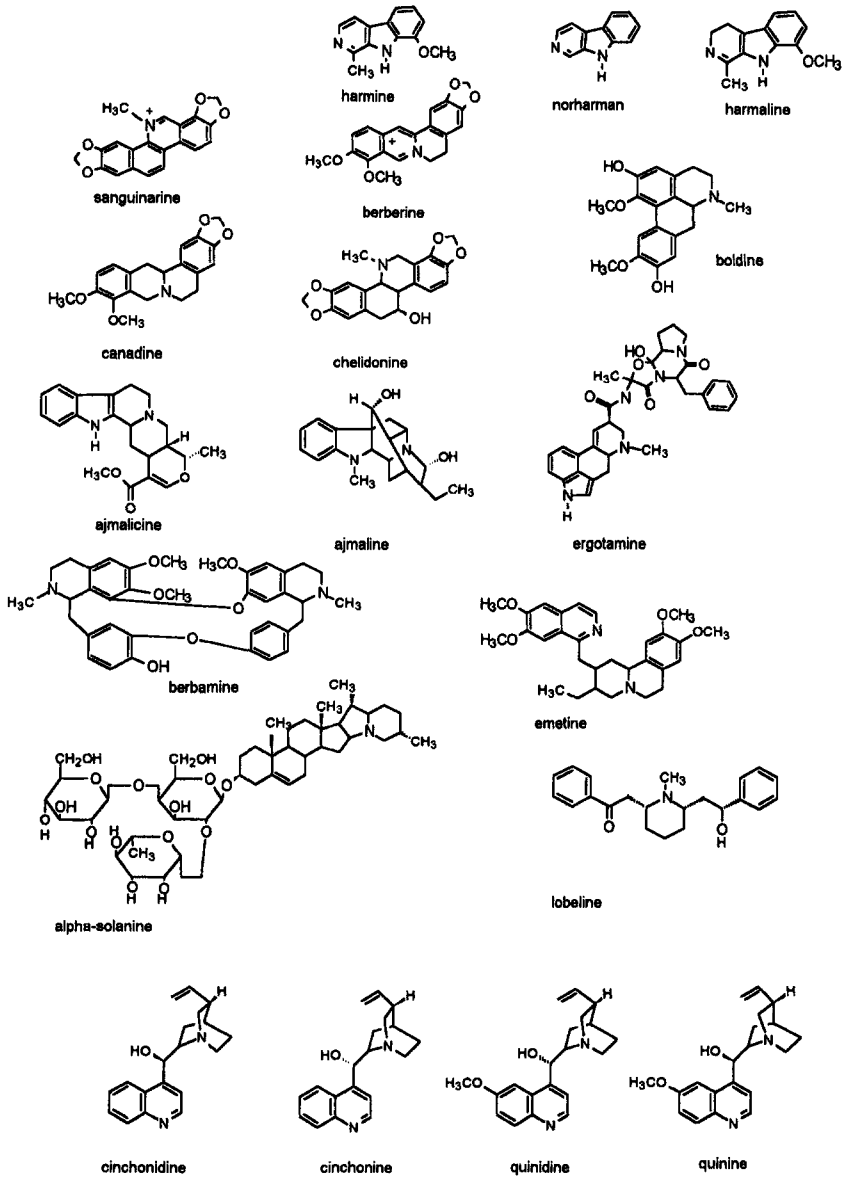


FIG. 14. Structures of DNA intercalating compounds.

Alkaloids apparently have evolved as defense compounds, and most plants that produce them are usually avoided by most herbivores (Wink, 1993). Because of their wide activities, we consider alkaloids as "multipurpose" defense substances. Since plants cannot predict or choose their competitors, infesting microorganisms, insects, or other herbivores, such multipurpose compounds are likely a means of being prepared for most situations.

In order to modulate their molecular targets, the molecules must interfere directly with the corresponding chemical structures. Thus, structure–activity relationships should exist. Intercalating alkaloids usually are lipophilic and planar molecules of a size that fits between the GC and AT stacks of the DNA double helix (Figure 14). Membrane active compounds are often amphiphilic. Many alkaloids derive from the same biogenic precursor as neurotransmitters and mimic them structurally (Figures 15–17). Alkaloids are charged and often quarternary N compounds at physiological pH and, thus, show similarities to acetylcholine, serotonin, dopamine, or noradrenaline which are also protonated under these conditions (Figure 15). Because of their structural similarities, alkaloids can bind to neuroreceptors (albeit with differing affinities) and can either block or diplace the endogenous neurotransmitters as agonists or antagonists. If chemists synthesize compounds that fit a known receptor site, they call the strategy molecular modeling. Analogously, we could speak of "evolutionary molecular modeling" if we consider the evolution of active alkaloids through natural selection. When a plant produces a compound that interferes with a molecular target in a herbivore, it gains a selective advantage, since this plant will probably not be eaten and can transfer its genes to the next generation. If mutations in the next generations improve the structure of such an inhibitor, the advantage will be higher, and eventually such a compound will be widespread in the respective taxon. For example, hyoscyamine and scopolamine, alkaloids found in *Hyoscyamus*, *Atropa*, *Scopolia*, *Mandragora*, or *Datura* inhibit mAChR with high affinity. Both alkaloids are the main ones in these taxa, whereas alkaloids with reduced binding affinities occur only as minor components (El-Shazly et al., 1998; Schmeller et al., 1995).

We hypothesize that plants eventually produce alkaloids that are structural analogs of neurotransmitters. But why have neurotransmitter mimics which also intercalate DNA? Plant–herbivore interactions are mutual processes. If a plant produces a nAChR inhibitor, it is likely that some insects will develop resistance in such a way that they modify the binding site of nAChR, so that the inhibitor can no longer bind. We have recently shown that *Danaus plexippus* has a modified binding domain of its Na^+, K^+-ATPase, which no longer interacts with cardiac glycosides, thus, providing insensitivity at this target (Holzinger et al., 1992; Holzinger and Wink, 1996). However, if the same compound affected other molecular targets simultaneously, an adaptation through target site modification would be more difficult and unlikely, since it would require several

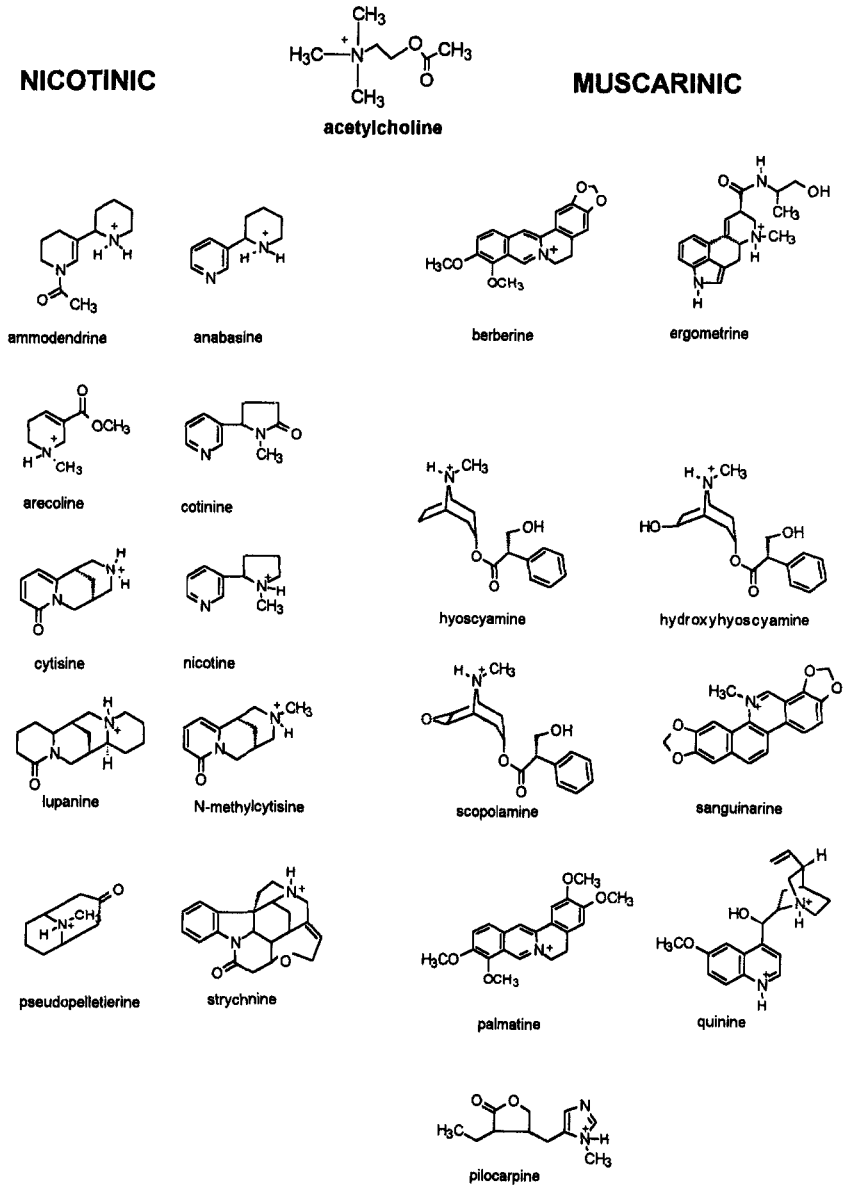


FIG. 15. Structures of acetylcholine and alkaloids that bind to muscarinic and nicotinic neuroreceptors.

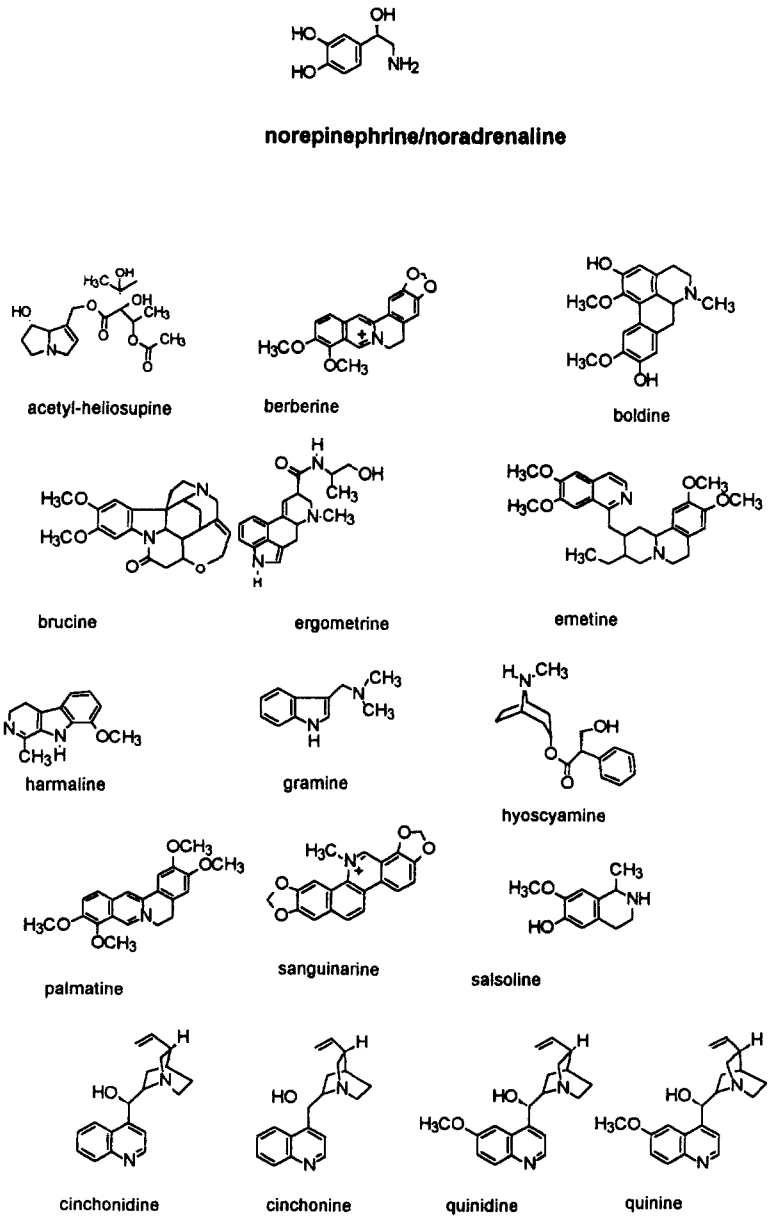


FIG. 16. Structures of noradrenaline and alkaloids that bind to adrenergic neuroreceptors.

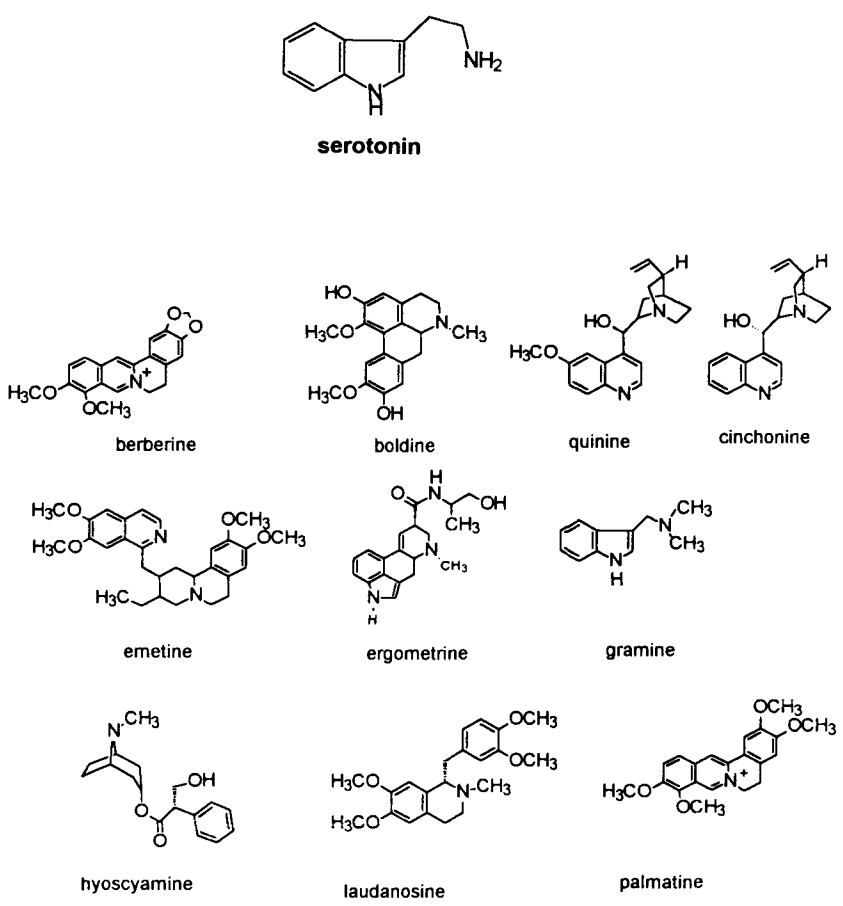


FIG. 17. Structures of serotonin and alkaloids that bind to serotonin neuroreceptors.

concomitant mutations. According to this model, the evolution of allelochemicals affecting more than one target would be a strategy to counteract adaptations by specialists and to fight off different groups of enemies.

As mentioned, the evolution of plant herbivore interactions is a dynamic process, and insect and vertebrate herbivores of today have developed strategies during evolution to overcome the defense chemistry of plants. Strategies that must be considered with regard to the underlying pharmacological mechanisms include: (1) selection of plants or plant parts low in secondary metabolites; (2) selection of plants or plant parts lacking the most potent toxins; (3) feeding on several food items instead of a single plant, thus avoiding high internal concen-

trations of a particular allelochemical; (4) modifications of receptors or other molecular targets in order to gain insensitivity; and most importantly, (5) the development of potent detoxification and elimination mechanisms that are typical for many insect and vertebrate herbivores.

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FIELD TESTS OF SYNTHETIC SEX PHEROMONE OF THE APPLE LEAFMINER MOTH, *Phyllonorycter ringoniella*

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Abstract—The apple leafminer moth, *Phyllonorycter ringoniella*, is becoming a more serious insect pest on apple trees with four to five generations a year in Korea. In order to devise a forecasting method for more accurate estimation of their numbers and development timing, the sex attractant was studied. Various ratios, from 10:0 to 0:10, of the two components, (*Z*)-10-tetradecenyl acetate (*Z*10–14:Ac) and (*E*,*Z*)-4,10-tetradecadienyl acetate (*E*4,*Z*10–14:Ac), identified from the sex pheromone gland (Jung and Boo, 1997), were tested for attractivity in terms of behavioral response (taxis, approach, and landing) against *P. ringoniella* males in a wind tunnel. The lure with *Z*10–14:Ac/*E*4,*Z*10–14:Ac in a ratio of 4:6 elicited the highest response in two (taxis and approach) measurement categories. For eliciting landing behavior, the two blends of 5:5 and 4:6 were best. The single component, *Z*10–14:Ac, elicited taxis behavior, but a combination of two chemicals was needed for eliciting all three behaviors. In the field, male attraction to various lure mixtures in Pherocon IC traps was usually greater than attraction to virgin females. The best field activity was in the lure baited with a 4:6 ratio of *Z*10–14:Ac and *E*4,*Z*10–14:Ac. Similar results were obtained from tests conducted in a net house. This optimum ratio for attracting *P. ringoniella* males in Korea is different from those reported in Japan (10:3) or China (7:3 to 6:4). The isomer *E*10–14:Ac neither improved nor depressed the number of catches when added at up to 10% of the total mixture to lures of the two components in the 4:6 ratio. The attractivity of the lures increased with higher amounts of the pheromones, up to 10 μg in the wind-tunnel experiment and 5 mg in the apple orchard. The number of males captured was not significantly different among traps installed at 0.3, 1.5, or 2 m above the ground, or among wing, delta, or water traps. A rubber septum dispenser

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impregnated with 1 mg of the 4:6 mixture maintained its field attractivity for up to eight weeks.

Key Words—*Phyllonorycter ringoniella*, apple leafminer, sex pheromone, (*Z*)-10-tetradecenyl acetate, (*E*,*Z*)-4,10-tetradecadienyl acetate, field trapping, geographical variation

INTRODUCTION

The apple leafminer moth, *Phyllonorycter ringoniella* Matsumura (Lepidoptera: Gracillariidae), is an economically important insect pest in apple orchards in Korea. Larvae injure leaves by making mines. There are four to five generations a year in Korea (Lee et al., 1985a). Each apple leaf may have only one mine from April to June, but two to four mines after July (Lee et al., 1985b). The mines hasten defoliation, decrease the photosynthetic area, which may eventually cause deterioration of fruits, and inhibit the growth of new buds (Lee et al., 1985b; Sugie et al., 1986). Because larvae live in the mines, insecticides often are not effective for their control.

Ujiye (1972) described calling and mating behavior of the apple leafminer moth, and its female sex pheromone was reported to be composed of (*Z*)-10-tetradecenyl acetate (*Z*10–14:Ac) and (*E*,*Z*)-4,10-tetradecadienyl acetate (*E*4,*Z*10–14:Ac) (Ujiye et al., 1986; Sugie et al., 1986). The most attractive ratio was 10:3 of *Z*10–14:Ac and *E*4,*Z*10–14:Ac in Japan (Oku, 1993), but 7:3 to 6:4 were the most attractive in China (Su and Liu, 1992).

Lures with synthetic pheromone of the apple leafminer moth are commercially available in Japan. Field attractivity of the product was tested in Korea, but moths were not strongly attracted to it (unpublished observation). Our preliminary GC analysis of *P. ringoniella* female abdominal tips (Jung and Boo, 1997) also gave a totally different picture from that reported in Japan. These results suggested that the composition of the female sex pheromone is different between populations in Korea and Japan. Therefore, we tested attraction of the two components of the pheromone in various combinations in the laboratory, net house, wind tunnel, and apple orchards in Korea to determine geographical variation of the species in pheromone composition.

METHODS AND MATERIALS

Laboratory Experiments. Male response (taxis, approach, and landing) of *P. ringoniella* to various combinations of the two pheromone components, (*Z*)-10-tetradecenyl acetate (*Z*10–14:Ac) (Chem Tech) and (*E*,*Z*)-4,10-tetradecadienyl acetate (*E*4,*Z*10–14:Ac) (Shin-Etsu Chem. Co.), was observed in a wind tunnel (0.6 × 0.6 × 2 m). The ratios tested were 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10 of *Z*10–14:Ac/*E*4,*Z*10–14:Ac, and 100 ng of the mixtures were applied to a filter paper (2 cm in diameter). Lures were

set up individually at the one end of the tunnel, after evaporation of the solvent, *n*-hexane, from the paper for 10 min. For male response to different amounts (0.1, 0.2, 1.0, and 10 μg), the two components were mixed at a fixed ratio of 4:6 of *Z*10–14:Ac/*E*4,*Z*10–14:Ac. Virgin males, 2–4 days old, were tested for their behavior during the first hour in the photophase. After 10 min, their behavior was classified as: taxis, when males flew beyond the half-way line of the wind tunnel; approach, when they approached within 30 cm from the source; or landing, when they landed at the filter paper having the pheromone. Five males were tested for response to different ratios, and 6–13 males to different amounts of the 4:6 mixture. The wind speed was adjusted to 0.1 m/sec.

Field Tests. Field tests were conducted with lures with the same ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10 blends of *Z*10–14:Ac and *E*4,*Z*10–14:Ac in apple orchards in Suwon, Korea. Wing traps were positioned in three blocks, with 15 m between traps within a block and at least 30 m between blocks. Traps were suspended 1.5 m above the ground. Red rubber septa (Daehan Scientific Co.) were loaded with 1 mg of a particular ratio of the pheromone components. Traps were randomly positioned. Blank traps and traps baited with one or two 2- to 3-day-old virgin females per trap were used for comparison. Moths trapped were counted every three days. After each counting, insects were removed, virgin females were changed, and trap positions were rotated. The lures were placed at the bottom of the traps.

To confirm the results of field bioassays, trapping experiments also were carried out with *Z*10–14:Ac/*E*4,*Z*10–14:Ac blends of 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10 in an outdoor net compartment (6 × 6 × 2 m). Initially, the four blends of 7:3, 6:4, 5:5, and 4:6 were placed at each corner of the net house, and then the other four blends of 3:7, 2:8, 1:9, and 0:10 were tested in the same way. The most attractive blends, one (4:6) from the first series and two more (3:7 and 2:8) from the second, were selected, and the three were subsequently compared for their male attractivity against one another. Thirty 2- to 4-day-old males were released per replicate, and males trapped were counted after two days. At every count, trap positions were rotated clockwise and new 30 virgin males introduced for the next test. (In no case did we have more than 30 males in any two-day period. Those males untrapped seemed to be unresponsive to pheromone lures). Wing traps were used, and the total amount of components in each rubber septum was 0.5 mg. The three tests were conducted for eight days each, from April 28 to May 7, 1995, for the first series, from May 8 to 16 for the second series, and from May 21 to 29 for the final series, with four replicates for each series. The data were expressed as a percentage of the total number trapped.

We further tested in the field: (1) the response of males to different lure doses (100, 500, 1000, 5000 μg/septum); (2) the trapping efficiency of different trap types (wing, delta, and water trap); (3) the effect of trap heights (0.3, 1.5,

and 2 m above the ground); and (4) the longevity of the synthetic pheromone in rubber septa. Field tests were replicated three to four times. Traps were spaced 15 m apart within a replicate and at least 30 m between replicates.

RESULTS

In wind-tunnel bioassays for mixtures of different ratios, most males started to fly to the source even with a single component, but approaching behavior was observed only with two-component lures (Figure 1). The best landing percentage was obtained with 5:5 and 4:6 blends, but this percentage was lower than with two virgin females. In the case of stimulation with different amounts of the 4:6 mixture, the level of male response tended to increase with the higher amount, but there was no clear relationship between the amount and percentage (unpublished).

In field tests of 11 blends in the fall of 1994, male attraction was not significantly different among five traps baited with the two pheromonal chemicals in ratios of 5:5, 4:6, 3:7, 2:8, and 1:9 (Table 1). Most of these blends,

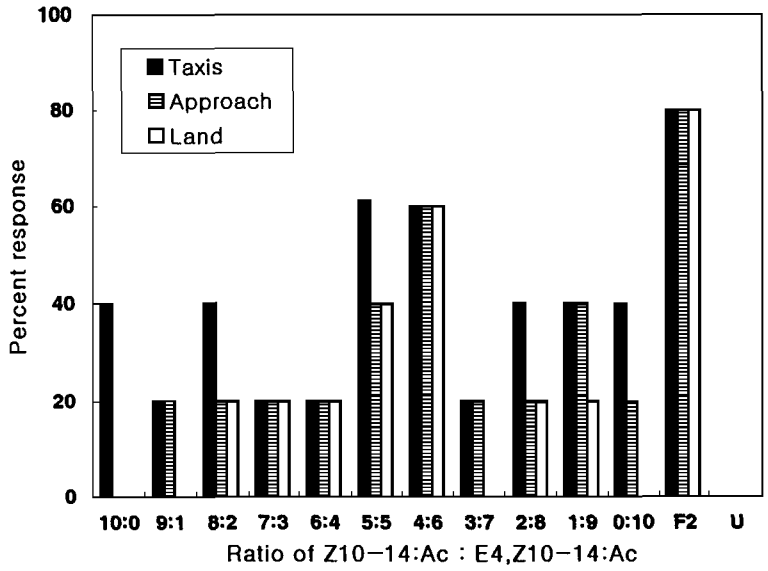

FIG. 1. Behavioral response (taxis, approach and landing) of *P. ringoniella* males to different blends of *Z*10-14:Ac and *E*4,*Z*10-14:Ac in a wind tunnel bioassay. Each blend had 100 ng impregnated on a piece of the filter paper. Five 2- to 4-day-old males were tested for each blend (F2: two virgin females; U: unbaited).

TABLE 1. MEAN NUMBER[a] OF *Phyllonorycter ringoniella* MALES CAUGHT IN PHEROCON 1C TRAPS IN APPLE ORCHARDS BAITED WITH 1 mg OF DIFFERENT BLENDS OF *Z*10–14:Ac AND *E*4,*Z*10–14:Ac (3 REPLICATES)

Ratio of *Z*10-14:Ac to *E*4,*Z*10-14:Ac	Fall trial (Oct. 2–18, 1994)	Spring trial (Apr. 20–May 11, 1995)
10:0	1.7 ± 1.5d	0.7 ± 1.2e
9:1	20.3 ± 7.6cd	8.3 ± 1.5de
8:2	128.0 ± 137.1bcd	16.7 ± 3.5d
7:3	157.0 ± 68.9bcd	40.3 ± 11.2bc
6:4	159.7 ± 19.2bcd	38.3 ± 7.4bc
5:5	242.3 ± 100.3abcd	44.3 ± 10.7bc
4:6	455.0 ± 343.6a	62.0 ± 4.4a
3:7	275.7 ± 156.9abc	32.3 ± 11.2c
2:8	249.0 ± 103.9abcd	49.7 ± 4.2b
1:9	305.7 ± 161.3ab	31.3 ± 10.3c
0:10	180.3 ± 109.6bcd	9.7 ± 4.2de
2 females	59.7 ± 45.0bcd	
Unbaited	1.7 ± 2.1d	0e

[a]Means followed by the same letter within each column are not significantly different by DMRT ($P > 0.05$).

however, were more attractive than virgin females to males. The 4:6 blend was the most effective, at least in the number of males caught. This was confirmed in a similar field test conducted in the spring of 1995 (Table 1). In subsequent outdoor tests inside the net compartment, the best attraction was again to the 4:6 blend (Table 2). In a dose-response test, increasing the amount of pheromone from 0.1 to 5 mg resulted in greater number of males captured (Table 3). The number of males trapped, however, was not significantly different among wing, delta, or water traps, capturing 33.0 ± 12.9, 21.6 ± 10.5, and 19.7 ± 8.0, respectively, during May 28–June 20, 1994. When traps were set at three different heights of 0.3, 1.5, and 2 m above ground, they captured 19.1 ± 11.3, 19.5 ± 11.9, and 7.8 ± 5.8 males, respectively (data not significant).

Attractivity of the synthetic sex pheromone was maintained for eight weeks (Figure 2), but, after eight weeks, attractivity of the old lure with 1 mg declined.

DISCUSSION

This study demonstrates that the Korean population of *P. ringoniella* is attracted best to the 4:6 blend of *Z*10–14:Ac and *E*4,*Z*10–14:Ac. This point

TABLE 2. RELATIVE RATE (%) OF *P. ringoniella* MALES CAUGHT IN PHEROCON 1C TRAPS BAITED WITH 500 μg OF DIFFERENT BLENDS OF Z10–14:Ac AND *E*4,Z10–14:Ac IN A NET HOUSE (APRIL 30–MAY 29, 1995, WITH 4 REPLICATES)[a]

Ratio of Z10–14:Ac to *E*4,Z10–14:Ac	First test (Apr. 28–May 7)	Second test (May 8–16)	Combined test (May 21–29)
7:3	4.5 ± 3.0c		
6:4	27.5 ± 12.1b		
5:5	22.0 ± 11.8b		
4:6	45.8 ± 13.9a		52.3 ± 23.9a
3:7		33.3 ± 8.1a	24.8 ± 17.0b
2:8		31.5 ± 5.8ab	22.9 ± 16.1b
1:9		20.3 ± 12.5ab	
0:10		15.0 ± 13.0b	
Unbaited			0b

[a]Thirty 2- to 4-day-old virgin males were tested every two days for a replicate. Means followed by the same letter within each column are not significantly different by DMRT ($P > 0.05$).

was not clear in the wind-tunnel experiment or in the first field experiment conducted in 1994. We think this was due to too low a level of chemicals and to too high a level of population density, respectively. The amount of lure, 100 ng, in the wind-tunnel bioassay elicited male behavior at a level lower than that to two virgin females. *P. ringoniella* has four or five generations per year in Korea and usually keeps building its population level with the season, showing a high level in latter generations, especially the last. This optimum ratio in Korea is clearly different from that reported in neighboring Japan and China.

TABLE 3. MEAN NUMBER[a] OF *P. ringoniella* MALES CAUGHT PER DAY IN AN APPLE ORCHARD WITH PHEROCON 1C TRAPS LOADED WITH DIFFERENT AMOUNTS OF 4:6 BLEND OF Z10–14:Ac and *E*4,Z10–14:Ac (MAY 11–25, 1995, WITH 3 REPLICATES)

	Amount loaded (μg)				
	100	500	1000	5000	1 female
Mean number	0.37 ± 0.25b	0.53 ± 0.12b	1.13 ± 0.87ab	1.93 ± 0.91a	0.40 ± 0.30b

[a]Means followed by the same letter are not significantly different by DMRT ($P > 0.05$).

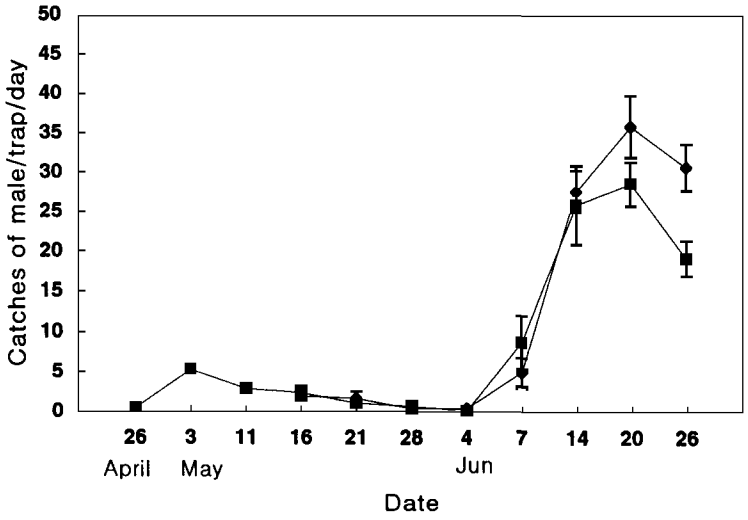

FIG. 2. Number (Mean ±SE) of *P. ringoniella* males caught in Pherocon 1C traps baited with synthetic sex pheromone lure in two separate field plots. In one plot (squares) the lure was installed on April 26, 1995, and kept until the end of the experiment (June 26, 1995), and in the other plot (diamonds) the lure was set up on May 16, 1995 and a fresh lure was placed on a new septum on June 10 of the same year. Synthetic sex pheromone was the 4:6 blend of *Z*10–14:Ac and *E*4,*Z*10–14:Ac and the total amount of components loaded in each rubber septum was 1 mg (4 replicates).

The optimal blend ratio was 10:3 (7.7:2.3) between *Z*10–14:Ac and *E*4,*Z*10–14:Ac in Japan (Oku, 1993) and 7:3 to 6:4 in China (Su and Liu, 1992). It is not yet clear why the best attractive ratios are different among populations in neighboring countries. Of course, we assume the populations belong to the same species. Such a variation in the optimum composition of sex pheromone has been reported in a few other insect species, such as *Amorbia cuneana* in southern California, USA (Bailey et al., 1986), *Ostrinia furnacalis* in northeastern Asia (Boo and Park, 1998), and *Dichocrocis punctiferalis* in northeastern Asia (Boo, 1996). Opposite ratios in the composition of two major sex pheromone components also were reported from *Helicoverpa assulta* in Korea among the *Heliothis/Helicoverpa* species (Cork et al., 1992); this latter situation concerns species level variation, not geographical variation of one species.

There is no continuity in apple-growing areas in China and Korea, even though the two countries have a common land border, and Japan is physically separated from both countries. This moth species is not a strong flier, which means that there is little possibility of mating between populations in the different

countries. Geographical isolation of populations may result in evolution into different species. This question will remain an interesting subject for a future study.

The 4 : 6 blend is attractive to *P. ringoniella* males. Therefore, we are using it with 1 mg of the total amount to monitor population size in apple orchards in Korea. More males were attracted to the lure with more of the sex pheromone, up to 5 mg. Similar results have been reported in *Phyllonorycter mespilella* (Gries et al., 1993b), *Lithocolletis blancardella* (Roelofs et al. , 1976), and *Lambdina fiscellaria lugubrosa* (Gries et al., 1993a). Amounts greater than 5 mg of the mixture were not tested in this study, excluding the possibility of obtaining the optimum dose to be loaded in a rubber septum. A 1-mg mixture in a rubber septum is large enough, however, to monitor the population size or the timing of leafminer appearance in the field.

For monitoring by farmers, we are setting up sticky traps at about 1 m above ground, although the efficiency of sticky traps, such as wing or delta traps, is interfered with by dust, other debris, or moisture (Howell, 1984). The high population density of *P. ringoniella* late in the season could easily cover the entire sticky surface, which means that sticky traps are not suitable for monitoring insect species such as the apple leafminer moth with wide change in its population density (Sanders, 1986). On the other hand, water traps are not saturated with moths and are easier to manipulate in the field, but they are inconvenient. This inconvenience factor has resulted in recommending that Korean farmers use sticky traps at present.

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INHIBITION OF BACULOVIRAL DISEASE BY PLANT-MEDIATED PEROXIDASE ACTIVITY AND FREE RADICAL GENERATION

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Abstract—The susceptibility of noctuid larvae to baculoviral infection is markedly affected by phytochemicals ingested during the acquisition of viral inoculum on foliage. We hypothesized that a major process causing phytochemical inhibition of viral disease is phenolic oxidation by phenolases, particularly peroxidase (POD), which subsequently generates free radicals. To test this hypothesis, we manipulated the chemical interactions in foliage of cotton, tomato, and lettuce by application of antioxidants, prooxidants, enriched extracts of phenolases, and/or phenolic substrates. Larvae of *Heliothis virescens* or *Helicoverpa zea* that received viral inoculum on treated foliage were less likely to die from viral infection the higher the POD activity of this foliage. Furthermore, the higher the POD activity, the more free radicals were generated in crushed foliage, and the more free radicals generated, the lower the incidence of viral disease. We present a series of reactions hypothesized to lead to inhibition of viral disease by free radicals, the generation of which is mediated, at least in part, by POD. Phenolic redox cycling catalyzed by POD involving clastogenesis (generation of H_2O_2) appeared to be a critical driver of phytochemical reactions leading to free radical generation and inhibition of baculoviral disease in their noctuid hosts. We also report application of an assay for the detection of free radicals by using methemoglobin as a new modification of this method for detecting radicals in plant foliage in the immediate aftermath of an oxidative burst.

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Key Words—Baculovirus, nucleopolyhedrovirus, free radicals, phenolic redox cycling, clastogenesis, peroxidase, polyphenol oxidase, antioxidants.

INTRODUCTION

The course and severity of baculoviral disease in insects is strongly influenced by the host plant (Hayashiya et al., 1968; Keating and Yendol, 1987; Felton and Duffey, 1990; Schultz and Keating, 1991; Sosa-Gomez et al., 1991; Forschler et al., 1992; Hunter and Schultz, 1993; Duffey et al., 1995; Farrar et al., 1996; Hoover et al., 1998a,b). Simultaneous ingestion of viral inoculum with certain phytochemicals profoundly affects whether or not the insect acquires a lethal inection and/or how quickly the insect succumbs to infection. Unfortunately, despite a plethora of information on the phenomenon of inhibition of viral disease, there are few reports on the mechanism(s) by which inhibition of disease occurs, primarily because of the experimental difficulty of establishing cause and effect (Duffey et al., 1995). Of the numerous phytochemicals that have been implicated in the inhibition of baculoviruses, plant phenolics and/or plant-derived oxidative enzymes are perhaps the most potent modulators of disease by baculovirus (Felton et al., 1987; Keating et al., 1989, 1990; Fazal et al., 1990; Felton and Duffey, 1990; Duffey et al., 1995; Young et al., 1995). For example, the oxidation of catecholic phenolics by plant phenolases is strongly correlated with inhibition of baculoviral disease in tomato [Felton and Duffey, 1990; (perioxidase-induced tomato) Hoover et al., 1998b], cotton (Forschler et al., 1992; Hoover et al., 1998a,b), and romaine lettuce (Hoover et al., 1998a). We found, for example, that the higher the constitutive peroxidase (POD) activity of foliage, the lower the mortality of larval *H. virescens* that ingested baculoviruses on this foliage (Hoover et al., 1998a). Furthermore, induction of higher levels of POD by damaging plants was correlated with decreased mortality of larval *H. virescens* and *H. zea* treated with baculoviruses on damaged cotton and tomato, respectively (Hoover et al., 1998b). In contrast, neither induced nor constitutive levels of another plant phenolase, polyphenol oxidase (PPO), were consistently related to larval mortality by virus (Hoover et al., 1998a,b).

We suspect that the mechanism(s) whereby baculoviral disease is inhibited by phytochemicals involves the formation of free radicals, be they active oxygen species (AOS), ferryl radicals, or carbon-based radicals. Furthermore, we hypothesize that free radical generation leading to inhibition of viral disease is mediated, at least in part, by POD. There are numerous studies demonstrating that phenolic oxidation catalyzed by POD produces, directly or indirectly, a variety of radical species. First, it is well established that POD is a source of free radicals in plants (Halliwell, 1978; Sutherland, 1991). Plant cell wall PODs

are induced under conditions where lignification of cell walls is employed as a defense against phytopathogens with the generation of AOS (Halliwell, 1978; Sutherland, 1991). Second, PODs in insects are known to use H_2O_2 and a number of phenolic substrates, *o*-catechols in particular, to form semiquinone, aryl, and aryloxy free radicals producing a variety of cross-linked aromatic products (see insect cuticle, Hasson and Sugumaran, 1987). Third, phenolic oxidation catalyzed by POD in wines produces semiquinone free radicals (Singleton, 1987). In wines, semiquinones are also strongly suspected to undergo autoxidation, which produces superoxide anion free radicals ($O_2^{\cdot -}$) (Singleton, 1987). Generation of $O_2^{\cdot -}$ can lead to propagation of a chain of oxidations of phenols and/or subsequent generation of AOS. For example, dismutation of $O_2^{\cdot -}$ to H_2O_2 may participate in Fenton-type hydroxylations involving H_2O_2 and Fe^{2+}, producing the highly reactive hydroxyl radical ($OH\cdot$) (Singleton, 1987).

We hypothesized that a major process responsible for the attenuation of baculoviral disease on plants is the oxidation of phenolics, catalyzed by phenolases, with the subsequent generation of free radicals as the mechanism (aromatic radicals and/or AOS) (Figure 1, reaction numbers in brackets). Despite the fact that both PPO and POD catalyze the oxidation of catecholic phenolics to *o*-quinones, we suspected that POD is a more important factor in the inhibition of viral disease than PPO because POD activity produces a larger variety of reactive products from both catecholic phenolics and monophenols, including the direct production of free radicals (Figure 1, equations 2–4, 7) (Butt, 1981; Butt and Lamb, 1981; Pierpoint, 1983; Hasson and Sugumaran, 1987; Singleton, 1987). When plant foliage is crushed, POD oxidizes catecholic phenolics to quinones by a one-electron transfer mechanism employing H_2O_2 as a cosubstrate, producing highly reactive semiquinone free radical intermediates (Figure 1, equation 2), which can then initiate further propagation of free radicals (Figure 1, equations 3–5) (Butt, 1981; Butt and Lamb, 1981; Singleton, 1987; Ahmad, 1995; Bi and Felton, 1995; Bi et al., 1997). Furthermore, POD oxidizes a variety of monophenols to aryloxy radical intermediates, which may lead to propagation of free radicals (Figure 1, equation 7). PPO, in contrast, directly produces the quinone using molecular oxygen as a cosubstrate (Figure 1, equation 2) (Mayer, 1987), which, if our hypothesis is correct, assumes that the quinone is much less detrimental to the virus because it is less effective at secondarily producing free radicals by redox cycling. However, PPO may be indirectly involved in free radical generation via the production of quinones that go on to participate in redox cycling, which secondarily produces free radicals (Figure 1, equations 4 and 5).

We propose several reactions for the generation of free radicals during the ingestion of foliage by larval noctuids, mediated by POD and/or PPO, which may subsequently attenuate viral disease (Figure 1); however, we do not suggest that these reactions are mutually exclusive. These reactions include the oxidation

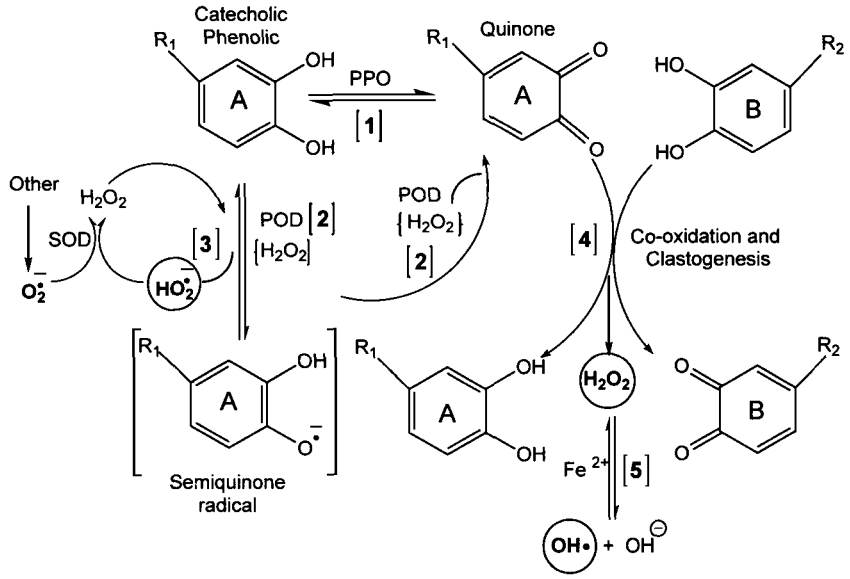

FIG. 1. Reactions hypothesized to generate free radicals during phenolic oxidation catalyzed by phenolases resulting in inhibition of viral disease. Equations are in numbered brackets; active oxygen species are circled. Reactions are for illustrative purposes only; they are not meant to show stoichiometry. Equation 1: Oxidation of a catecholic phenolic (A) catalyzed by polyphenol oxidase (PPO) forms the quinone. Equation 2: Oxidation of a catecholic phenolic (A) by peroxidase (POD) forms a semiquinone free radical intermediate with H_2O_2 as a cosubstrate. The semiquinone may be further oxidized to the quinone by POD (equation 2 continued), or it may react with another phenolic species (B) forming a second free radical species regenerating the reduced catecholic phenolic (A) (see equation 6). Equation 3: The reaction depicted in equation 2 also produces superoxide anion free radical ($O_2^{\cdot -}$) (produced as $HO_2^{\cdot}$ which immediately loses a proton to become $O_2^{\cdot -}$). Under the influence of SOD, H_2O_2 produced from $O_2^{\cdot -}$ may increase POD activity. Equation 4: Oxidation of phenolic (A) catalyzed by PPO or POD forms the quinone which in turn cooxidizes phenolic (B), thereby regenerating the reduced form of (A). Cooxidation (redox cycling) of phenolic species produces H_2O_2 (clastogenesis) and other AOS. Equation 5: Clastogenesis subsequently produces other AOS, such as the OH·, by the Fenton reaction (and other reactions). This reaction may also be catalyzed by other transition metal ions. Equation 6: Oxidation of a catecholic phenolic (A) catalyzed by POD in equation 2 produces a semiquinone free radical intermediate. The semiquinone cooxidizes a second catecholic phenolic (B), producing the reduced form of (A) and the semiquinone or aryl radical of (B). Equation 7: Oxidation of a monophenol (C) catalyzed by POD/H_2O_2 produces the aryloxy radical intermediate. Equation 8: Redox cycling between catecholic phenolics (B) and monohydroxyphenolics (C) may produce a variety of organic radical species.

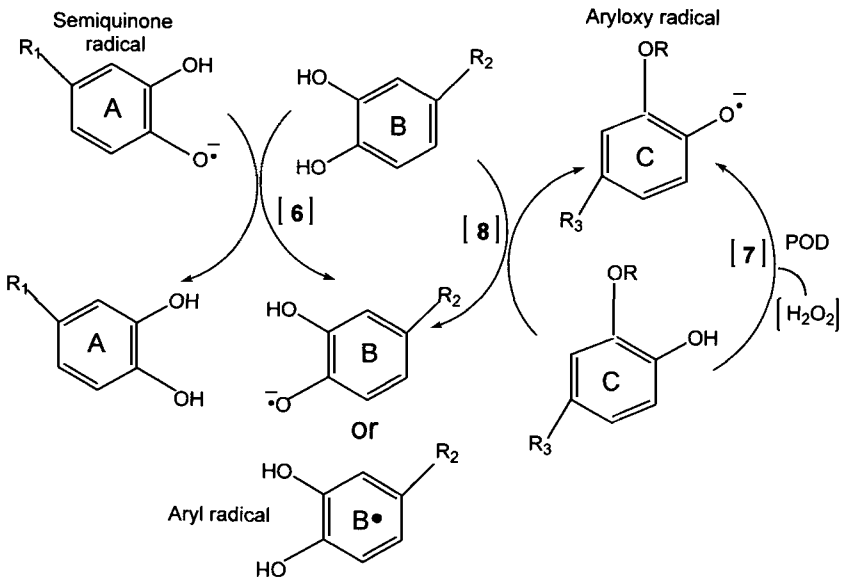

FIG. 1. Continued.

of catecholic phenolics to *o*-quinones catalyzed by PPO (Figure 1, equation 1) or POD (equation 2) semiquinone free radical intermediate producing $O_2^{\cdot -}$ (equation 3); cooxidation between catecholic phenolic redox couples catalyzed by either PPO or POD involving generation of H_2O_2 (clastogenesis) (equation 4); production of OH· from H_2O_2 catalyzed by transition metal ions (Fenton reaction) (equation 5); and finally, the generation of aryl, aryloxy, and semiquinone free radicals by redox cycling between mono- and diphenols, catalyzed in part by POD (equations 6, 7).

Based on the reactions described above (Figure 1, Table 1), we designed our experiments to address three questions concerning processes that may explain inhibition of viral disease by phytochemicals: (1) Is quinone formation catalyzed by PPO sufficient to explain this effect? (2) Is POD activity, with the production of free radical intermediates, capable of causing this effect? (3) Is redox cycling among phenolic species, involving clastogenesis with subsequent generation of AOS, capable of causing this effect?

To address these questions, and in line with our proposed reactions for free radical generation described above (Figure 1), we tested the impact of a series of chemical interventions to cotton, lettuce, and tomato foliage on viral disease (Table 1). Specifically, we manipulated the chemical interactions in foliage of

TABLE 1. HYPOTHETICAL INFLUENCES ON VIRAL DISEASE BY APPLICATION OF PHENOLASES, PROOXIDANTS, AND/OR ANTIOXIDANTS TO PLANT FOLIAGE OF COTTON, TOMATO, AND LETTUCE

Class of chemical	Name of chemicals tested[a]	Mechanism of action	Biochemical steps involved (Figure 1)[b]	Presumed effect on POD activity	Presumed effect on baculoviral disease
Phenolases	Peroxidase (POD)	Catalyze one-e^- oxidation of catecholic phenolics or monophenols using H_2O_2 as cosubstrate	Eqs. 2, 4, 7	Add more POD to foliage = ↑ POD activity	↑ FR = ↓ viral disease
	Polyphenol oxidase (PPO)	Catalyze two-e^- oxidation of catecholic phenolics to *o*-quinones	Eq. 1	No effect	No effect
Prooxidants	Quercetin, ferulic acid, *p*-coumaric acid quebracho tannin	Promote free radical generation or oxidation of other materials	Eqs. 3–8	↑ POD activity	↑ FR = ↓ viral disease
	Redox couples (CHA plus rutin or catechin)	Generate H_2O_2 by redox cycling	Eqs. 4 and 8	↑ POD activity	↑ FR = ↓ viral disease

Antioxidants					
Reducing agents	Ascorbic acid catecholic phenolics	Reduce quinones to the diphenol. Retard propagation of free radical chains	Eqs. 1–4	↓ POD activity	↓ FR = ↑ viral activity
Free radical scavengers	BHT, ascorbic acid, mannitol, lutein	Serve as FR sink, may form covalent bonds	Eqs. 2–8	↓ POD activity	↓ FR = ↑ viral disease
Enzymatic antioxidants	Catalase	Catalyzes H_2O_2 to H_2O	Eqs. 2–7	↓ POD activity	↓ FR = ↑ viral disease
	SOD	Catalyzes $O_2^{\cdot-}$ to H_2O_2	Eq. 3	May ↑ POD activity	
Chelators	Sodium borate	Forms chelate with catecholic phenolics	Eqs. 1–4, 6, 8	↓ POD activity	↓ FR = ↑ viral disease
	Some catecholic phenolics	Inhibit transition metal-containing enzymes (e.g., PPO and POD)	Eqs. 1, 2, 4, 6–8	↓ POD activity	

[a] Chemicals used to test the ability of a specific mechanism as outlined in Figure 1 to inhibit viral disease. Abbreviations: PPO = polyphenol oxidase, POD = peroxidase, BHT = butylated hydroxytoluene, SOD = superoxide dismutase. CHA = chlorogenic acid.

[b] Refers to the hypothetical mechanisms (specific equation is listed) that may result in inhibition of viral disease as shown in Figure 1.

these three plants by applying one or more of the following to foliage: (1) enriched extracts of cotton POD or tomato PPO, respectively, (2) additional catecholic phenolic substrates, (3) one of a variety of prooxidants, or (4) one of a variety of antioxidants (Table 1).

METHODS AND MATERIALS

Chemicals and Commercially Purified Enzymes

Catalase (from bovine liver, EC 1.11.1.6, 11,000 units/mg), superoxide dismutase [MnSOD from *E. coli* (not inhibited by H_2O_2), EC 1.15.1.1, 4,000 units/mg], horseradish peroxidase (HRP; type I, EC 1.11.1.7, 120 purpurogalin units/mg), and PPO (mushroom tyrosinase, EC 1.14.18.1, 4,400 units/mg) were obtained from Sigma (St. Louis, Missouri). All chemicals tested for their prooxidant or antioxidant effects [sodium borate, mannitol, butylated hydroxytoluene (BHT), galvinoxyl, lutein (a xanthophyll), sodium ascorbate, chlorogenic acid (CHA), caffeic acid, (±) catechin, rutin, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, quercetin, and $FeSO_4$] were obtained from Sigma. Rabbit hemoglobin was also obtained from Sigma. Quebracho tannins were purchased from Van Dyke Supply Co. (Woonsocket, South Dakota). All reagents, buffers, and enzymes were prepared by using milliQ H_2O.

Enzymes

Bulk Extraction of Enzymes from Plants. To obtain an enriched, semipurified extract of tomato PPO (*Lycopersicum esculentum* cv. Bonnie Best), 150 g of greenhouse-grown tomato foliage were macerated in 6 vol of ice-cold 0.1 M K phosphate buffer, pH 7, containing 7% (w/v) polyvinyl polypyrrolidone to adsorb phenolics. This homogenate was vacuum filtered over a thin bed of Hyflo Supercell (Fisher, St. Louis, Missouri). The filtrate was brought to 35% $(NH_4)_2SO_4$ to precipitate PPO, but not POD (Gentile et al., 1988; Felton and Duffey, 1991). Each step of the extraction and concentration of the enzyme was followed by monitoring enzymatic activity in 3 mM CHA (Ryan et al., 1982). The mixture was stirred for 2 hr, followed by centrifugation at 20,000*g* for 15 min. The pellet was resuspended in 0.01 M K phosphate buffer, pH 7, and dialyzed at 4°C against two changes of the same buffer by using 10,000 MWCO cellulose dialysis tubing. The dialysate was flash frozen in an ethanol–Dry Ice bath and lyophilized to yield 400 mg of partially purified protein. This lyophilized protein had an activity of 250 units/mg (1 unit = 0.001 ΔOD/min) and no activity in 5 mM guaiacol–35 μM H_2O_2 (no POD contamination). This represents a threefold purification with a yield of 30%.

Cotton POD was partially purified from greenhouse-grown cotton foliage (*Gossypium hirsutum* cv. Acala SJ2), in the same manner as described for tomato PPO above, except we used the precipitate at 55–75% $(NH_4)_2SO_4$ saturation. Gentile et al. (1988) reported on this procedure with tomato. The lyophilized protein had no PPO activity when assayed with CHA, caffeic acid, or catechin, i.e., there was no activity without the addition of H_2O_2. The lyophilized enzyme had an activity of 2500 units/mg in 5 mM guaiacol–35 μM H_2O_2. This represents a fourfold purification and a yield of 66%. Both tomato PPO and cotton POD were stored desiccated at −20°C. After four months, both enzymes lost approximately half of their activity. For all experiments that used semipurified enzymes, PPO and POD were solubilized in 0.1 M K phosphate buffer, pH 7, at 25 and 10 mg/ml, respectively.

Crude Extracts of Enzymes from Individual Leaves or Leaflets. Crude enzyme extracts from foliage of greenhouse-grown cotton, tomato, and/or romaine lettuce (*Latuca sativa* L. cv. Valmaine) were prepared as described in Felton et al. (1989). Crude enzymes extracts were kept on ice until used.

Assays for Foliar Levels of Phenolases and Phenolics

Enzyme Assays. The substrate used to determine PPO and POD activities varied among experiments and will be described within the specific experimental system. Enzyme activities were calculated as ΔOD_{470}/per gram per minute with 1 unit = 0.001 unless otherwise indicated (Ryan et al., 1982; Felton et al., 1989). Although the ΔOD_{470} actually measures melanin production (Ryan et al., 1982), it is customary for this assay method to be used as an index of the rate of quinone formation (Ryan et al., 1982; Felton et al., 1987; Stout et al., 1994); thus, we used the same index throughout this study.

Phenolic Assays. Catecholic phenolic content of fresh foliage was determined colorimetrically by using a 0.5% diphenylborinic acid–ethanolamine complex. CHA and rutin were used as standards at OD_{390} and OD_{440}, respectively (Broadway et al., 1986). Total phenolics were measured with the Folin-Ciocalteau reagent and CHA as the standard (Singleton and Rossi, 1965).

Extraction Efficiency of Phenolases and Phenolics in a Leaf Press. Because we measured free radical generation in crushed foliage with a leaf press and we correlated these measurements with the extent of viral disease, we evaluated whether the leaf press used to crush foliage for measuring the free radicals was in fact releasing sufficient phenolases and phenolics to warrant this relationship. To determine the efficiency of the leaf press in the extraction of phenolases and phenolics from foliage described in the section below entitled "Measurement of AOS/Free Radicals in Crushed Foliage," we weighed six leaf or leaflet samples of cotton, tomato, and lettuce. Three leaves or leaflets each were used to assess the enzyme and phenolic extraction efficiency of the leaf press machine.

Leaf samples were first crushed by the leaf press as described, except the solvent used at the same time of extraction for enzymes and phenolics was 0.1 M K phosphate buffer, pH 7, and 50% methanol, respectively. After leaf samples passed through the leaf press, they were further processed to determine how much enzyme or phenolics remained. To extract the remaining enzymes from the samples that had been crushed by the leaf press, we used the methanol described above. Thus, we also added 10% Triton-X 100 at the same final concentration to the pressed leaf extracts to minimize confounding enzyme activities with detergent effects. PPO and POD activities were assayed with CHA and guaiacol–H_2O_2 as substrates, respectively (Ryan et al., 1982; Felton et al., 1989). For the second extraction, we used the method described above in the section "Phenolic Assays."

By comparing values from the first and second processing, we calculated the mean percent recovery of POD, PPO, and phenolics after using the leaf press. The leaf press extracted 23 ± 3.7% of the POD from cotton, 37 ± 6.4% from lettuce, and 45 ± 2.0% from tomato foliage, respectively. The leaf press released 31 ± 5.9% and 86 ± 2.9% of the PPO from lettuce and tomato, respectively. Recovery of phenolics with the leaf press was fairly complete for each plant species. For cotton, lettuce, and tomato, 51 ± 0.08, 90 ± 0.03, and 94 ± 0.01% of the total phenolics and 65 ± 0.1, 86 ± 0.05, and 88 ± 0.02% of the catecholic phenolics were released from foliage by the leaf press.

AOS/Free Radical Generation in Foliage

Premise for Heme Assay for Free Radicals. Heme proteins represent a unique class of compounds that can be used as markers for oxidative processes (North et al., 1996). Heme proteins contain an iron–porphyrin complex, and the oxidation state is dependent upon the particular heme protein that is capable of accepting unpaired electrons from various sources (e.g., AOS, semiquinone, ferryl, and ascorbyl radicals). Oxidation of the iron affects the energy state of the porphyrin ring and ultimately results in distinctly different spectra of the reduced and oxidized states, which allows measurement of reversible and irreversible damage to heme proteins by free radicals. Thus, heme has been used to develop simple, reproducible methods for quantitating oxidative damage in biological samples (North et al., 1996).

Spectrum Characterization and Preparation of a Standard Curve. We first characterized the spectrum from 300 to 700 nm of a 0.02% solution of rabbit hemoglobin dissolved in 0.1 M K phosphate buffer. The hemoglobin solution had a characteristic peak at 406 nm, which is consistent with methemoglobin, the partially oxidized form of hemoglobin (Fe^{3+}) (North et al., 1996). We then prepared a standard curve with Fenton reagent to generate free radicals (e.g., $OH\cdot$ and $OH_2^{\cdot-}$) (Haber and Weiss, 1934). For the standard, a 0.04% stock

solution of methemoglobin was prepared in the same buffer. This solution was mixed 1 : 1 with 0.1 mM $FeSO_4$. We then added H_2O_2 to obtain a series of final concentrations of H_2O_2 of 5–400 μM in a 2.5-ml volume. After taking the initial absorbance for each sample at 406 nm, absorbances were read again at 1 and 2 hr. A standard curve was generated based on the decrease in absorbance at each time point, which was used to calculate free radical generation (based on micromoles of added H_2O_2, reported as micromoles of free radical/AOS equivalents). The curve was linear up to 200 μmol; after 200 μmol the curve gradually became asymptotic (Figure 2). We obtained the same equation for the standard curve whether samples were incubated for 1 or 2 hr; thus, we chose to use a 1-hr incubation period for evaluating free radical generation in crushed foliage samples.

Measurement of AOS/Free Radicals in Crushed Foliage. Using a homemade leaf press that crushes a moistened leaf between two solid metal cylinders turning in unison (designed by T. L. German, University of Wisconsin), we collected the pressed leaf extract from each preweighed leaf sample as it was washed with 2.5 ml of a 0.02% solution of methemoglobin pH 7 buffer into a

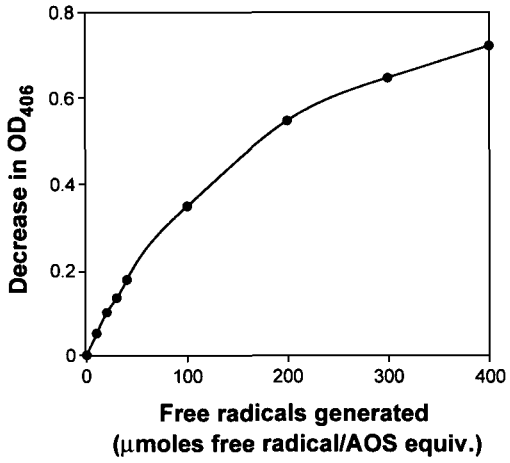

FIG. 2. Standard curve used to quantify free radical generation in crushed foliage. The standard curve was prepared by incubating a 0.02% solution of methemoglobin with Fenton reagent as the free radical generator followed by measurement of the decrease in initial absorbance after 1 hr. The x axis represents increasing micromoles of H_2O_2. Equation: free radical generation (μmol H_2O_2 equiv) = ΔAbs $-$ 0.02/(0.003 $-$ 3.5 $\times$ 10^{-6}); Linear regression F = 643; df = 2,8; $P < 0.0001$, R^2 = 0.99. The curve is linear up to 200 μmol H_2O_2. The coefficient of variation was less than 0.6% for replicates on the same day and 1.1% for replicates run on different days.

20 ml scintillation vial. The cylinders of the leaf press were washed with distilled water between samples. Immediately after collecting the pressed leaf extract, the sample was placed in an Eppendorf tube followed by centrifugation at 14,000 rpm for 3–5 sec to precipitate the remaining leaf material. The supernatant was immediately transferred to a 1.5-ml cuvette and the absorbance read at 406 nm against a buffer blank. Samples were incubated for 1 hr in the dark. After 1 hr, the absorbance was read again. Because oxidation of the phenolic compounds from the pressed-leaf-extract occurs during the incubation period by the enzymes released by crushing, we also read the initial and final absorbances of samples in the absence of heme. The increase in absorbance due to oxidation was subtracted from the decrease in absorbance obtained with heme.

To determine the amount of AOS/free radicals generated in each sample, the difference between the initial absorbance and the final absorbance was corrected for leaf weight to obtain the change in absorbance per gram of leaf tissue. This value was entered into the equation for the standard curve to quantify free radical generation in micromoles of free radical/AOS equivalents.

Impact of Prooxidants, Antioxidants, Phenolases, and/or Phenolic Substrates on Free Radical Generation. To determine the impact of a variety of prooxidants, antioxidants, phenolases, and/or catecholic phenolic substrates on free radical generation in cotton, tomato, and lettuce, four leaves of cotton and lettuce were divided into equal pieces. Each chemical treatment was applied to a piece of the same leaf. Because tomato has five to seven leaflets per leaf, rather than cutting the leaf, one leaflet from each leaf was used as a control. All leaf pieces and leaflets were weighed prior to crushing. For each chemical treatment applied to foliage in bioassays (described below), 25 μl of a test chemical solution (phenolase, phenolic, antioxidant, or prooxidant) was first added to the scintillation vial used to collect the pressed leaf extract. Test chemicals and their final concentrations are given in Tables 2–5. Samples were also run in the absence of heme to permit correction for oxidation as described above. Free radical generation in crushed cotton, lettuce, and tomato foliage is reported as the mean $\pm$ SE of four replicates.

Free Radical and AOS Generation In Vitro

Redox Potentials of Catecholic Phenolics. To establish a basis for examining the relative reactivities of a given phenolase in a variety of substrates (single phenolic species vs. mixtures), we measured the redox potential of caffeic acid, catechin, CHA, rutin, and quercetin buffered solutions at four different pHs. We prepared 0.4 mM solutions of each chemical in 0.1 M K phosphate buffer at pH 7, 8, 9, and 10. Redox potentials of these solutions were estimated to the nearest millivolt with a Corning redox combination electrode with a platinum electrode, a Ag–AgCl reference electrode, and a Chemtrix 40E pH

TABLE 2. EFFECTS OF APPLYING PHENOLASES AND/OR PHENOLIC SUBSTRATES TO COTTON, TOMATO, AND LETTUCE FOLIAGE ON PLANT PHENOLASE ACTIVITY, FREE RADICAL GENERATION, AND VIRAL DISEASE IN *H. virescens* THAT INGESTED VIRUS ON TREATED FOLIAGE[a]

	Chemicals applied to foliage								
Measured variable	Gelatin only control	POD (100 μg/ml)	PPO (250 μg/ml)	POD + φ (100 μg/ml + 2 mM)	PPO + φ (250 μg/ml +2 mM)	HRP (0.1 μg/ml)	Boiled POD (100 μg/ml)	Boiled PPO (250 μg/ml)	Boiled HRP (0.1 μg/ml)
Cotton									
POD	143 ± 8.9	318 ± 4.3	NT	428 ± 4.9	NT	206 ± 13	110 ± 2.0	NT	153 ± 11
FRs	359 ± 25	578 ± 105	NT	618 ± 34	NT	452 ± 125	NT	NT	NT
%Δ in FRs vs. control[b]		↑61		↑72		↑26			
% mortality	13	4.4		2.3*		7.6	9.0		10.1
Tomato									
POD	61 ± 30	211 ± 120	58 ± 25	385 ± 49	33 ± 13	135 ± 23	97 ± 27	33 ± 7.5	69 ± 2.8
PPO	48 ± 2.4	64 ± 12	58 ± 13	51 ± 2.2	55 ± 13	54 ± 12	46 ± 12	33 ± 12	46 ± 2.2
FRs	131 ± 20	396 ± 176	222 ± 30	570 ± 25	38 ± 2.6	312 ± 114	NT	NT	NT
%Δ in FRs vs. control		↑202	↑69	↑335	↓71	↑138			
% mortality	23	6.3*	23	0*	32	6.3*	13	10.3	9.1
Lettuce									
POD	40 ± 3.0	68 ± 3.8	68 ± 1.5	27 ± 33	63 ± 27	300 ± 87	NT	NT	NT
PPO	162 ± 8.3	142 ± 72	277 ± 124	160 ± 120	315 ± 185	40 ± 12	NT	NT	NT
FRs	293 ± 32	339 ± 106	450 ± 45	63 ± 34	399 ± 10	425 ± 88	NT	NT	NT
%Δ in FRs vs. control		↑16	↑53	↓79	↑36	↑45			
% mortality	11	7.9	1.0*	23	1.4*	2.5*			

[a]Mean ± SE (total %) mortalities of larvae that received virus on diet as controls for bioassays on cotton, tomato, and lettuce were 30 ± 17 (30), 22 ± 8.1 (22), 15 ± 2.2% (15.3), respectively. Asterisks indicate treatments significantly different from gelatin control. Percent mortality is the combined percentage of insects that died in both replicates. N = 40–48 larvae/replicate. Bioassays were replicated twice. Units for enzyme activities and free radical (FR) generation are ΔOD/g/min (1 unit = 0.001) and μmol free radical/AOS equivalents, respectively.

[b]Percent change vs. control is the change in free radical generation of the treatment relative to untreated control foliage.

Top row: φ = chlorogenic acid (CHA) + rutin at 20 μM each, POD = semipurified cotton peroxidase, PPO = semipurified tomato PPO, HRP = horseradish peroxidase. NT = not tested. For treatments involving enzymes plus phenolic substrates, enzymes were applied to opposite sides of the leaf disk from the phenolic. Cotton POD was assayed in guaiacol; tomato and lettuce enzymes were assayed in CHA–rutin. Enzyme activities and free radicals are the mean ± SE of two and four replicates, respectively.

TABLE 3. EFFECTS OF APPLYING SELECTED PROOXIDANTS TO COTTON, TOMATO, AND LETTUCE FOLIAGE ON PLANT PHENOLASE ACTIVITY, FREE RADICAL GENERATION, AND VIRAL DISEASE IN *H. virescens* THAT INGESTED VIRUS ON TREATED FOLIAGE[a]

Measured variable	Chemicals applied to foliage[b]						
	Gelatin only control	Quercetin (2 μM)	Ferulic acid (20 μM)	*p*-Coumaric acid (20 μM)	H_2O_2 (88 μM)	Quebracho tannin (0.01%)	Tannin + lysolecithin (0.01%)
Cotton							
POD	143 ± 8.9	160 ± 7.5	262 ± 13	135 ± 6.8	150 ± 5.6	222 ± 12	NT[c]
FRs	359 ± 25	508 ± 71	450 ± 60	448 ± 237	460 ± 130	464 ± 150	NT
%Δ in FRs vs. control[d]		↑42	↑25	↑25	↑28	↑30	NT
% mortality	13	4.3*	8.3	9.5	10	0*	0*
Tomato							
POD	61 ± 30	0 ± 0	12 ± 28	0 ± 0	61 ± 24	567 ± 152	NT
PPO	49 + 2.4	8.1 ± 2.5	24 ± 1.9	18 ± 3.7	39 ± 3.4	NT	
FRs	131 ± 20	97 ± 16	112 ± 27	41 ± 24	96 ± 49	454 ± 164	
%Δ in FRs vs. control		↓26	↓15	↓69	↓27	↑247	
% mortality	23	19.4	25	38	15	NT	NT
Lettuce							
POD	40 ± 3.0	NT	NT	NT	NT	301 ± 38	NT
PPO	162 + 83	NT	NT	NT	NT	99 ± 13	
FRs	293 ± 32	471 ± 69	144 ± 40	49 ± 24	184 ± 29	416 ± 88	NT
%Δ in FRs vs. control		↑61	↓51	↓83	↓37	↑42	
% mortality	11	NT	NT	NT	NT	5.3*	2.6*

[a] Cotton POD was assayed in guaiacol, tomato, and lettuce enzymes in CHA–rutin. Enzyme activities and free radicals are the mean ± SE of two and four replicates, respectively. Asterisks indicate treatments significantly different from gelatin control. Percent mortality is the combined percentage of insects that died in both replicates. N = 40–48 larvae/replicate. Bioassays were replicated twice. NT = not tested.

[b] Units for enzyme activities and free radical (FR) generation are ΔOD/g/min (1 unit = 0.001) and μmol free radical/AOS equivalents, respectively.

[c] Enzyme activities could not be obtained because addition of lysolecithin created too much turbidity in the assay mixture.

[d] Percent change vs. control = the change in free radical generation of the treatment relative to untreated control foliage.

TABLE 4. EFFECTS OF APPLYING SELECTED ANTIOXIDANTS TO COTTON, TOMATO, AND LETTUCE FOLIAGE ON PLANT PHENOLASE ACTIVITY, FREE RADICAL GENERATION, AND VIRAL DISEASE IN *H. virescens* THAT INGESTED VIRUS ON TREATED FOLIAGE[a]

Measured variable	Chemicals applied to foliage[b]						
	Gelatin only control	Ascorbate (100 mM)	BHT (20 μM)	Mannitol (20 μM)	Lutein (0.4 mM)	Borate (80 μM)	CHA/rutin (20 μM each)
Cotton							
POD	143 ± 8.9	0 ± 0	120 ± 6.1	145 ± 15	114 ± 5.2	147 ± 18	15 ± 3.8
FRs	359 ± 25	487 ± 195	104 ± 35	99 ± 43	183 ± 30	411 ± 116	151 ± 8.6
%Δ in FRs[c] vs. control		↑36	↓71	↓74	↓49	↑14	↓58
% mortality	13	21	38*	44*	25*	41*	19
Tomato[c]							
POD	61 ± 30	0 ± 0	32 ± 6.6	NT	21 ± 3.5	NT	NT[c]
PPO	49 ± 2.4	0 ± 0	37 ± 3.7		28 ± 8.2		NT
FRs	131 ± 20	919 ± 375	42 ± 9.9	25 ± 11	111 ± 9.5	127 ± 22	87 ± 66
%Δ in FRs vs. control		↑602	↓69	↓81	↓15	↓3	↓34
% mortality	23	33*	33*	NT	23	NT	38*
Lettuce							
POD	40 ± 3.0	NT	5.0 ± 3.5	NT	7.7 ± 3.4	14 ± 2.8	NT
PPO	162 ± 13	NT	190 ± 18	NT	44 ± 7.4	90 ± 13	NT
FRs	293 ± 32	709 ± 256	99 ± 10	199 ± 68	470 ± 44	118 ± 61	166 ± 135
%Δ in FRs vs. control		↑142	↓66	↓32	↑60	↓60	↓43
% mortality	11	NT	16	NT	16	18	5.5

[a]NT = not tested. Cotton POD was assayed in guaiacol; tomato and lettuce enzymes were assayed in CHA/rutin. Thus, the effect of application of CHA plus rutin to tomato or lettuce on enzyme activities could not be assessed. Enzyme activities and free radicals are the mean ±SE of two and four replicates, respectively. Percent mortality is the combined percentage of insects that died in both replicates. Asterisks indicate treatments significantly different from gelatin control. N = 40–48 larvae/replicate. Bioassays were replicated twice.

[b]Units for enzyme activities and free radical (FR) generation are ΔOD/g/min (1 unit = 0.001) and μmol free radical/AOS equivalents, respectively. On tomato, all antioxidants except phenolics were added in combination with POD.

[c]Percent change vs. control is the change in FR generation of the treatment relative to untreated control foliage.

TABLE 5. EFFECTS OF APPLYING SELECTED ENZYMATIC ANTIOXIDANTS TO COTTON, TOMATO, AND LETTUCE FOLIAGE ON PLANT PHENOLASE ACTIVITY, FREE RADICAL GENERATION, AND VIRAL DISEASE IN *H. virescens* THAT INGESTED VIRUS ON TREATED FOLIAGE

	Chemicals applied to foliage[a]		
Measured variable	Gelatin only control	Catalase (1 unit/ml)[b]	SOD (5 units/ml)
Cotton			
POD	143 ± 8.9	0 ± 0	148 ± 12
FRs	359 ± 25	81 ± 31	158 ± 25
%Δ in FRs vs. control[c]		↓77	↓56
% mortality	13	22*	15
Tomato			
POD	61 ± 30	0 ± 0	
PPO	49 ± 2.4	46 ± 5.7	
FRs	131 ± 20	32 ± 17	NT
%Δ in FRs vs. control		↓76	
% mortality	23	21	NT
Lettuce			
POD	40 ± 3.0	NT	NT
PPO	162 ± 83	NT	NT
FRs	293 ± 32	77 ± 66	NT
%Δ in FRs vs. control		↓74	
% mortality	11	NT	NT

[a] NT = not tested. Units for enzyme activities and free radical (FR) generation are ΔOD/g/min (1 unit = 0.001) and μmol free radical/AOS equivalents, respectively. Cotton POD was assayed in guaiacol; tomato and lettuce enzymes were assayed in CHA/rutin. Enzyme activities and FRs are the mean ± SE of two and four replicates, respectively. Percent mortality is the combined percentage of insects that died in both replicates. Asterisks indicate treatments significantly different from gelatin control. N = 40–48 larvae/replicate. Bioassays were replicated twice.

[b] Catalase was tested on tomato for bioassay in combination with POD.

[c] Percent change vs. control is the change in free radical (FR) generation of the treatment relative to untreated control foliage.

meter. Accuracy of this apparatus was verified by testing our equipment on ZoBell's solution, which has a known redox potential (Nordstrom, 1977). Fresh solutions of each chemical at the four pH values were prepared daily. Readings were allowed to stabilize for 2–5 min before they were recorded at ambient temperature (23–24°C). Duplicate readings, separated by a period of 20–30 min, were taken of each solution. This experiment was repeated six times on three consecutive days.

We estimated E_h for each chemical, which is the redox potential relative to the standard hydrogen electrode potential E_0. To convert readings to E_h, the difference between the Ag–AgCl reference electrode and the standard hydrogen electrode (+197 mV) was added to the observed potential. Because we used buffered solutions for these measurements, the reported E_h values represent a "mixed potential," consisting of the redox potentials contributed by the test chemical and by the phosphate ions in the buffer. Our reported measurements should, therefore, not be taken as precise measurements of the E_h values, but instead should be viewed as useful measurements for comparing redox potentials of these phenolic species to each other under the conditions utilized in the experiments throughout. One-way ANOVA was used to compare E_h values for each phenolic separately at each pH (Steel and Torrie, 1980).

Cooxidation Between Catecholic Phenolic Species. Quinones may indirectly lead to free radical generation by redox cycling among phenolic species (Figure 1, equation 4). The *o*-quinone produced via catalysis by phenolases may subsequently serve as the oxidizing agent of a second catecholic phenolic species. Redox cycling among catecholic phenolics may thus lead to generation of free radicals. We examined whether CHA and rutin were capable of cooxidation and whether co- or autooxidation produces free radicals, which may subsequently influence viral disease.

Spectrophotometric Evidence of Cooxidation Between Rutin and CHA Involving Clastogenesis. We examined cooxidation between rutin and CHA under the influence of clastogenesis by using semipurified PPO and POD. We also tested for cooxidation with crude enzyme extracts from cotton, tomato, and romaine lettuce. Crude enzyme extracts from cotton have no detectable PPO activity, but tomato and lettuce extracts contain both PPO and POD (Hoover et al., 1998a,b). Three leaf samples were used for each plant species; each assay for each sample was done in duplicate.

As an index of cooxidation, we examined the ΔOD_{470} per minute catalyzed by PPO or POD by using each phenolic species alone versus the two in mixture. Thus, assays of PPO and POD were performed in the following substrates dissolved in 0.1 M K phosphate buffer, pH 8: 2 mM CHA, 2 mM CHA–35 μM H_2O_2, 2 mM rutin, 2 mM rutin–35 μM H_2O_2, 2 mM CHA + 2 mM rutin, 2 mM CHA + 2 mM rutin + 35 μM H_2O_2, 2 mM guaiacol–35 μM H_2O_2 (as another control). All assays contained 1 ml of substrate solution and 10 μl of enzyme solution. To determine whether clastogenesis during cooxidation occurred, we added catalase (1 unit/ml final concentration) to a set of reaction mixtures. We interpreted a decrease in the activity of PPO in the CHA–rutin mixture in the presence of catalase, but not in CHA alone, as evidence of clastogenesis because tomato PPO has no activity against rutin alone. Evidence of POD activity in the CHA/rutin mixture without addition of H_2O_2, and a

decrease in apparent activity with the addition of catalase, also suggests that cooxidation involves clastogenesis.

We hypothesized that if redox cycling between CHA and rutin produces free radicals, then free radical scavengers added to the phenolic mixture should decrease the apparent phenolase activity. We tested the impact of the following free radical scavengers alone or in combination on cooxidation: BHT (20 μM final concentration) and/or mannitol (20 μM final concentration), lutein (4 μM final concentration), or ascorbate (10 mM final concentration). Repeated-measures ANOVA was performed to determine if a particular scavenger significantly affected phenolase activity within each substrate group relative to the uninhibited controls (Steel and Torrie, 1980).

Quantitation of Cooxidation of Rutin by CHA. To semiquantitate the cooxidation of rutin by CHA under the influence of phenolases, we examined the disappearance of these two compounds in the presence of semipurified PPO and POD in vitro. Three milliliters of each of the following six treatments were prepared in 0.1 M K phosphate buffer, pH 8, in test tubes as follows: (1) 2 mM CHA; (2) 2 mM rutin; (3) a mixture of 2 mM CHA and 2 mM rutin; (4) same as (3) except for the addition of 40 μl of catalase at 1 mg/ml; (5) same as (3) except for the addition of 250 μl of a mixture of 2 mM BHT and 2 mM mannitol; and, (6) same as (3) except no enzyme was added (control). The volume of each treatment was equalized (to 3.25 ml) by the addition of buffer.

To start the reaction (time zero), 40 μl of semipurified tomato PPO solution were added to each treatment (except the control). At 30, 60, and 120 min, a 1-ml aliquot was removed from each treatment and the reaction was stopped by lowering the pH to 5.0 with the addition of 2 N HCl. To partition the phenolics into an organic solvent, we added 500 mg of $(NH_4)_2SO_4$ and 500 μl of isopropanol–ethyl acetate (1 : 1 v/v). After vortexing the samples for 30 sec, we spotted 10 μl of the upper organic layer of each treatment on microcrystalline cellulose TLC plates (Kodak). TLC plates were run in 2% aqueous formic acid. The R_f values were calculated and the positions compared to standards.

We semiquantified the amount of rutin and CHA remaining in each treatment by comparing the treated lanes to CHA and rutin standards run under the same conditions in the absence of enzyme. The experiment was replicated and plates were scored independently by three different researchers. The percent of the substrate remaining at each time point was determined by comparing treatments to standards of known concentrations with a level of discrimination of 2 nmol.

Free Radical Generation During In Vitro Oxidation of Monophenols by POD. Indirect measurement of the generation of free radicals during oxidation of a monophenolic substrate (5 mM guaiacol–35 μM H_2O_2), catalyzed by cotton POD, was examined by recording the effect of a variety of antioxidants and prooxidants on POD activity. Ten microliters of a prooxidant and/or antioxidant

solution was added to a 1.5-ml cuvette containing 1 ml of the substrate solution. Then 10 μl of crude cotton POD extract was added. The ΔOD_{470} per gram per minute was recorded and used as an index of free radical generation. We interpreted an increase in the rate of the reaction in the presence of an added prooxidant as evidence of free radical generation, whereas a decrease in the rate of the reaction in the presence of an added antioxidant was interpreted as free radical scavenging. We tested the ability of the prooxidants quercetin, quebracho tannin, and *p*-coumaric and/or ferulic acids and the free radical scavengers BHT or mannitol to enhance or inhibit oxidation of guaiacol, respectively (see Table 12 below for final concentrations). We further tested the ability of these scavengers to quench the activity produced by the addition of a prooxidant. Reaction volumes were kept constant by adding the same volume of buffer to controls as used for the test chemicals. The experiment was replicated three times. Treatments were compared by one-way ANOVA followed by Fisher's PLSD (Steel and Torrie, 1980).

Free Radical Generation During Redox Cycling Among Catecholic Phenolics and Monophenols In Vitro. We indirectly measured free radical generation by monitoring the ΔOD_{470} per gram per minute during oxidation of catecholic phenolics catalyzed by cotton POD. We tested the ability of catalytic concentrations of quercetin (1 μM final concentration) or quebracho tannin (0.01%) to act as prooxidants (or antioxidants) by using 2 mM CHA–35 μM H_2O_2 or 2 mM CHA–2 mM rutin–35 μM H_2O_2 mixtures as substrates. Crude enzyme extracts were prepared from cotton as described previously. To a 1-ml volume of substrate solution, we added 10 μl each of enzyme and test chemical solution. Reaction volumes were kept constant by adding the same volume of buffer to controls instead of the test chemicals. The experiment was replicated twice. Treatments were compared by one-way ANOVA followed by Fisher's PLSD (Steel and Torrie, 1980).

Bioassay Methods

To determine the nature of the influence of phenolase activity and/or free radical generation on viral disease, we designed bioassays such that the complexity of the phytochemical mixture used to treat the virus increased with each set of bioassays. Thus, our series of bioassays progressed from in vitro (single vs. mixtures of phenolic species) to in vivo treatment of polyhedral occlusion bodies (OBs) of baculoviruses.

Insects. Eggs of tobacco budworm (*H. virescens*) and corn earworm (*H. zea*) were obtained from the USDA/ARS (Stoneville, Mississippi). Neonate larvae were reared to third instar on semisynthetic diet (Southland Products, Lake Village, Arkansas) in 24-well tissue culture plates (Fisher) at 26 $\pm$ 1°C and 16L : 8D. Within 6 hr after molting to the third instar, larvae were transferred to empty 24-well tissue culture plates for 24 hr to allow them to void the gut.

Viruses. Autographa californica M nucleopolyhedrovirus (AcMNPV, C6 clone) (Ayers et al., 1994) and *Helicoverpa zea* SNPV (HzSNPV, original isolate plaque purified from Elcar, Sandoz-Wander, Wasco, California) were amplified in larvae of *H. virescens* and *H. zea*, respectively. These viruses were extracted, partially purified, and stored until use as described by Hoover et al. (1995).

Influence of Phenolic Oxidation Catalyzed by Phenolases on Subsequent Infectivity of Treated Virus In Vitro. We evaluated direct effects of phenolic oxidation by POD and PPO on the infectivity of baculoviruses in vitro by using single vs. mixtures of phenolic substrates. A series of preliminary experiments were conducted to develop appropriate methodology to control as many variables as possible (pH, substrate concentration, enzyme concentration, detergent, inhibitor, and time effects; data not shown).

All in vitro bioassays used similar methodology as follows: phenolic substrates and enzymes were solubilized in 0.1 M K phosphate buffer at pH 8 and pH 7, respectively. All other reagents were prepared in the same buffer at pH 8, except the virus and 2 N HCl, which were prepared in milliQ H_2O. Treatments were as follows: 2 ml of a 3 mM substrate solution were placed in a 7-dram glass vial to which 20 μl of virus were added at a final concentration of 30 OBs/μl (AcMNPV) or 7 OBs/μl (HzSNPV). Two different enzyme concentrations were used for PPO and two to three for POD (enzyme sources varied and are presented in the relevant experimental section below). PPO and POD were prepared at concentrations that produced 50 and 100 units of activity in the experimental substrate (1 unit = 0.001 ΔOD/min). POD was also tested at 300 units. For all treatments involving phenolic oxidation catalyzed by POD, 35 μM H_2O_2 was in mixture with the substrate(s). A small stir bar was added to each reaction mixture. To start the reaction, 20 μl of solubilized PPO or POD was added at time 0 (treatment only) and vials were placed on a stir plate for 15 min. After 15 min, 20 μl of inhibitor solution was added to stop the reaction followed by addition of 20 μl of 2 N HCl to lower the pH to 6.5. Because every added chemical affected viral infectivity to some extent, paired treatments were required. Every treatment had a control that received all the same components, except the inhibitor was added to the beginning of the incubation period instead of the end. All reaction mixtures were of equal volume; thus, each contained an equal concentration of virus. One microliter of each reaction mixture was applied to 40 8-mm^3 cubes of artificial diet in 24-well tissue culture plates. A starved third instar of *H. virescens* was placed in each well. After 24 hr, larvae that consumed an entire dose were transferred to excess diet, one larva per diet cup, and maintained at 26 $\pm$ 1°C. Mortality was scored at eight to nine days after infection. Each treatment was replicated three times with 35–40 larvae per treatment. Mean percent mortality was compared among treatments and enzymes by two-way ANOVA (Steel and Torrie, 1980), except the experiment with the

Fenton reagent, where treatments were replicated two times and compared by Student's unpaired *t* test (Steel and Torrie, 1980).

In Vitro Exposure of Virus to Oxidation of a Single Phenolic Species. The ability of quinone formation alone to attenuate viral disease was examined by incubating OBs in a single species of catecholic phenolic in the presence of PPO (Figure 1, equation 1). We also examined the effect of oxidation of a single phenolic species by POD on the infectivity of treated OBS. For these experiments, OBs of AcMNPV were treated with PPO (mushroom tyrosinase) and/or HRP using CHA, (±)-catechin, or caffeic acid as substrates. Sodium ascorbate was used as the inhibitor at a final concentration of 10 mM.

In Vitro Exposure of Virus to Redox Cycling Among Phenolic Species. We examined the effect of cooxidation of catecholic phenolic redox couples, catalyzed by semipurified tomato PPO, mushroom tyrosinase or HRP, on infectivity of treated OBS of AcMNPV (Figure 1, equation 4). The redox couples tested were CHA plus catechin and CHA plus rutin. For the CHA plus catechin mixture, 10 mM ascorbic acid, 4 mM borate, 1×10^{-5} M galvinoxyl (solubilized first in acetone at 10^{-2} M and then further diluted in buffer), and catalase at 1 unit/ml were each tested as inhibitors to determine the nature of the effect on the virus. For example, if quinone formation leads to viral inactivation, then borate, because it forms a chelate with *o*-dihydroxyphenolics (Pitzer and Babock, 1977), may inhibit quinone formation and protect the virus. For the CHA plus rutin mixture, 10 mM ascorbate was used as the inhibitor. Lysolecithin (0.001%) was added in combination with PPO or POD to the CHA plus rutin mixture to examine detergent effects on phenolase activities and subsequent effects on viral infectivity.

In Vitro Exposure of Virus to Hydroxyl Radicals. To examine the effect of OH· formation on viral infectivity (Figure 1, equation 5), we incubated polyhedra of HzSNPV with Fenton reagent (0.1 mM $FeSO_4$ and 35 μM H_2O_2) (Haber and Weiss, 1934). The treated mixture was subsequently fed to larvae of *H. virescens*. A OH· scavenger (200 μM mannitol) and H_2O_2 in the absence of $FeSO_4$ were used as controls.

Effects of Complex Phenolic Mixture Incorporated into Artificial Diet on Viral Disease. We tested the effect of oxidation of a complex mixture of phenolics on viral disease by incorporating a phenolic mixture into artificial diet. Tobacco budworm diet (BioServ, Inc.) was formulated with the addition of 3 mM CHA, 3 mM rutin, 30 μM ferulic acid, and 3 μM quercetin without the addition of the vitamin pack, which contains ascorbate. A control diet was also prepared that was identical except for the absence of phenolics. A 1:1 mixture of virus (15 OBs/μl of AcMNPV, final concentration) and a semipurified phenolase solubilized in 0.1 M K phosphate buffer, pH 7, were applied in 2-μl aliquots to a small cube of diet (16 mm^3) in 24-well tissue culture plates. Virus–enzyme treatments included the following: (1) semi-purified tomato PPO

(100 units), (2) semipurified cotton POD (100 and 300 units), (3) boiled PPO (100 units), (4) boiled POD (300 units), or (5) pH 7 buffer. These treatment mixtures were applied to 40 diet cubes each of the phenolic diet and the phenolic-free diet. A starved third instar of *H. virescens* was placed in each well, one larva per well, until the next day. Larvae that consumed their entire dose were transferred to excess diet containing vitamins but no phenolics until they died or pupated. Enzyme activities were determined as the ΔOD_{470} at pH 8 by using the same mixture and concentration of phenolics as in the test diet.

In Vivo Treatment of Viral Inoculum: Effects of Chemical Manipulations to Foliage on POD Activity, Free Radical Generation, and Viral Disease. To determine the degree of the influence of POD and free radical generation on viral disease, we tested the effects of a series of chemical manipulations to cotton, lettuce, and tomato foliage on (1) the POD and PPO activities of treated foliage (Tables 2–5); (2) the level of free radicals generated in each of these treatments (Tables 2–5); and, (3) disease of noctuid larvae that received virus on treated foliage (Tables 2–8). For these in vivo bioassays, we used different approaches on different plant species, depending upon the degree to which the untreated foliage inhibited baculoviral disease. Because cotton has no detectable PPO activity, high POD activity, and markedly inhibited baculoviral disease in previous studies (Forschler et al., 1992; Hoover et al., 1998a), we primarily examined the ability of antioxidants applied to cotton foliage to protect the virus. We did, however, also evaluate whether attenuation of viral disease on cotton could be enhanced further by applying a prooxidant or additional semipurified POD to foliage. Because tomato and lettuce have both PPO and POD activity and do not inhibit viral disease as much as does cotton (if at all) (Forschler et

TABLE 6. MODEL OF INFLUENCE OF APPLYING SELECTED CHEMICALS TO COTTON FOLIAGE ON MORTALITY OF *H. virescens* DOSED WITH THE BACULOVIRUS AcMNPV AT 30 OBs/LARVA[a]

Variable	Parameter coeff.	Effect on larval mortality	*t*	*df*	*P*
Viral dose	+0.04	↑	+6.2	49	<0.0001
$\log_e$(POD)	−0.17	↓	−3.7	56	0.0005

[a]Model chi-square = 51, $df = 2$, $P < 0.0001$; probability of dying = $\exp(\beta X)/[1 + \exp(\beta X)]$, where $\beta X = [-2.9 - 0.17(\log_e \text{POD}) + 0.04(\text{viral dose})]$. Array of POD activities was obtained by applying semipurified cotton POD and/or a variety of prooxidants or antioxidants to leaf disks cut from cotton foliage that were subsequently treated with the baculovirus AcMNPV. POD activity was determined by assaying foliage in 5 mM guaiacol–35 μM H_2O_2 (see Tables 2–5 for a list of treatments). Viral dose was determined by infecting insects on artificial diet.

TABLE 7. MODELS OF INFLUENCE OF APPLYING SELECTED CHEMICALS TO TOMATO FOLIAGE ON MORTALITY OF *H. virescens* OR *H. zea* DOSED WITH BACULOVIRUS AcMNPV OR HzSNPV AT 30 AND 3 OBs/LARVA, RESPECTIVELY

Variable	Parameter coeff.	Effect on larval mortality	*t*	*df*	*P*
H. virescens[a]					
$\log_e$(POD)	−0.37	↓	−3.7	50	0.0005
PPO	+0.02	↑	+2.6	50	0.0111
Dose	0.07	↑	+5.0	50	<0.0001
H. zea[b]					
$\log_e$(POD)	−2.2	↓	−2.5	6	0.0453

[a] For *H. virescens*, the array of POD activities was obtained by applying semipurified POD, PPO, and/or various antioxidants or prooxidants to leaf disks cut from tomato foliage that were subsequently treated with the baculovirus (see Tables 2–5 for list of treatments). Model chi-square = 58, $df = 3$, $P < 0.0001$; probability of dying = $\exp(\beta X)/[(1 + \exp(\beta X)]$, where $\beta X = [-2.9 - 0.37(\log_e \text{POD}) + 0.02(\text{PPO}) + 0.07(\text{Dose})]$.

[b] For *H. zea*, array of POD activities was obtained by applying semi-purified POD, PPO, and/or 2 mM each of chlorogenic acid and rutin to leaf disks cut from tomato that were subsequently treated with the baculovirus HzSNPV. Model chi-square = 9.4, $df = 1$, $P < 0.0001$; probability of dying = $\exp(\beta X)/[1 + \exp(\beta X)]$, where $\beta X = [6.1 - 2.2(\log_e \text{POD})]$. PPO had no effect on mortality by virus ($t = -0.55$, $df = 5$, $P = 0.6039$).

TABLE 8. MODEL OF INFLUENCE OF APPLYING ENZYMES AND PHENOLIC SUBSTRATES TO LETTUCE FOLIAGE ON MORTALITY OF *H. virescens* DOSED WITH BACULOVIRUS AcMNPV AT 30 OBs/LARVA[a]

Variable	Parameter coeff.	Effect on larval mortality	*t*	*df*	*P*
$\log_e$(PPO)	+0.60	↑	+4.2	64	<0.0001
$\log_e$(POD)	−0.28	↓	−3.1	64	0.0029
Viral dose	+0.17	↑	+5.9	64	<0.0001

[a] Array of PPO or POD activities was obtained by applying four different concentrations of PPO or POD, additional phenolic substrate (mixture of 2 mM chlorogenic acid and 2 mM rutin), or antioxidants to leaf disks cut from lettuce foliage (see Tables 2–5 for a list of treatments). Leaf disks were subsequently treated with the baculovirus AcMNPV. There were 40–48 larvae in each replicate and the experiment was replicated three times. Viral dose was entered in the model as the percent mortality of insects fed virus on artificial diet. Model chi-square = 60, $df = 3$, $P < 0.0001$; probability of dying = $\exp(\beta X)/[1 + \exp(\beta X)]$, where $\beta X = [-2.3 + 0.60(\log_e \text{PPO}) - 0.28(\log_e \text{POD}) + 0.17(\text{viral dose})]$.

al., 1992; Hoover et al., 1998a,b), our primary objective was to examine the effects of applying an additional phenolase and/or a prooxidant to foliage on viral disease.

Plants were used at the four to five-leaf stage. A leaf was randomly removed from each of 16 plants. Using a No. 3 cork borer, we cut 0.65 cm^2 leaf disks from each leaf and placed them in Petri dishes partially filled with 2.4% agar. Leaf disks were distributed such that each chemical treatment would be applied to three disks from each plant. Gelatin–chemical mixtures were prepared by dissolving Knox gelatin in 0.1 M potassium phosphate buffer, pH 7, at 50°C at a concentration of 4% for cotton and 6% for lettuce and tomato. After cooling to 37°C, test chemicals were added to the gelatin as described in Wolfson and Murdock (1987) (see Tables 2–5 for specific treatments at their final concentrations). For treatments involving application of HRP or semipurified POD or PPO to foliage, an additional control included boiling the enzyme for 15 min before mixing with gelatin. On lettuce, semipurified PPO and POD were applied at 125, 62.5, and 31.25 μg/ml and 100, 62.5, 31.25, and 15.6 μg/ml, respectively, to evaluate whether inhibition of disease responded in a dose-dependent manner. Ten microliters of each gelatin–chemical mixture at 35–37°C was applied to each leaf disk with a pipet and distributed evenly with a fine paint brush. Each treatment mixture was applied to a group of 48 leaf disks. After drying at ambient temperature ($\approx$30 min), a viral formulation was applied to each leaf disk.

AcMNPV was tested on cotton and lettuce against *H. virescens*, whereas HzSNPV was tested on tomato against *H. virescens* and *H. zea*. Polyhedral OBs were suspended at a single concentration (30 OBs/μl of AcMNPV and 3 OBs/μl for HzSNPV) in milliQ H_2O for application in 1-μl aliquots to each leaf disk. We used a low dose because we suspect that lower viral doses are probably more biologically relevant (Duffey et al., 1995). Furthermore, in previous studies, the greatest inhibition of disease occurred at lower viral doses (Felton et al., 1987; Hoover et al., 1998a,b). The dose for each replicate was determined by treating a group of insects on small cubes (8 mm^3) of artificial diet. Viral inoculum was allowed to dry at ambient temperature. Once the viral solution was dry, leaf disks were placed one per well in 24-well tissue culture plates partially filled with 2.4% agar. A starved larva was then transferred, one to a well, so that each larva received a single leaf disk. After 18 hr, insects that had consumed an entire treated leaf disk or artificial diet were transferred individually to excess diet in 35-ml cups and maintained until death or pupation at 26 $\pm$ 1°C and 16L:8D. A group of control insects was treated with 80 μM sodium borate in the absence of virus to ensure that this chemical was not toxic to the larvae at this concentration. Mortality was scored at eight to nine days after infection.

Foliar Chemical Assays in Conjunction with Bioassays. The effect of chem-

ical applications to foliage on subsequent phenolase activities was examined by using a portion of eight of the 16 leaves used for bioassay. POD and PPO activities were determined colorimetrically as the ΔOD_{470} per gram per minute as described above. The substrate used for enzyme assays depended upon which plant species was being tested, in an effort to achieve a more realistic estimation of phenolase activities at the time of ingestion by the insect. Because CHA and rutin are the major phenolic compounds in lettuce (Sharples, 1964) and tomato (Waiss et al., 1981; Isman and Duffey, 1982a,b), POD and PPO activities for these plants were determined by using a mixture of 2 mMCHA–2 mM rutin with or without 35 μM H_2O_2, respectively, as substrates. Because cotton foliage contains a complex mixture of mono-, di-, and polyphenols (Hedin et al., 1992; Bi et al., 1997), 5 mM guaiacol–35 μM H_2O was used to assay POD activity in cotton. Cotton POD activity in this substrate is not significantly different from the activity obtained in CHA/rutin (Table 11 below). To 1 ml of substrate solution, 10 μl of a test chemical(s) solution (final concentrations given in Tables 2–5) were added before the addition of the crude enzyme extract.

Final concentrations of test chemicals prepared in gelatin and applied to leaves were determined by measuring the amount of gelatin that remained on the leaf disk after handling just prior to being fed to larvae. A 0.1% solution of rose bengal (Sigma) was mixed with gelatin and applied to 10 leaf disks from each plant species as described above. After drying, leaf disks were placed in 0.1 M K phosphate buffer and stirred with gentle heating to wash off all the gelatin. Absorbances were read at 496 nm, which is the peak of the visible spectrum for rose bengal. The mean amount of gelatin that stuck to the leaves was calculated based on a standard curve. On cotton, tomato, and lettuce, 5.88 $\pm$ 0.36, 4.58 $\pm$ 0.36, and 4.08 $\pm$ 0.13 μl of the 10 μl of the gelatin mixture applied to leaf disks still remained at the time they were fed to larvae.

Survival Analysis

Survival data were analyzed by logistic regression to determine if the probability of an insect dying could be predicted from plant phenolase levels (POD and/or PPO) as affected by the manipulation of plant chemistry (Kalbfleisch and Prentice, 1980; Collett, 1994). Parameter coefficients β with a positive sign indicate a variable that increases the probability of an insect's dying; negative coefficients decrease the probability of dying. In addition, we compared the frequency of dead and live insects between selected treatments by using data pooled from the two replicates by chi-square analysis with Bonferroni's correction for the number of paired comparisons (Steel and Torrie, 1980). We pooled all data for analyses because the mortality levels characterized by artificial diet showed considerable variation between replicates.

To evaluate whether free radical generation may be the mechanism whereby

baculoviral disease is inhibited, we regressed total percent mortality for each treatment on each plant species as a function of mean free radical generation (as micromoles of free radical/AOS equivalents) (Steel and Torrie, 1980). This is because free radical generation was not performed on the same foliage samples used for bioassay. However, free radicals were assayed with the same plant cultivars under the same conditions as the bioassay on a different day.

To evaluate whether free radical generation may be mediated, at least in part, by POD activity, we regressed mean free radical generation for each foliar treatment as a function of mean foliar POD activity for each treatment. This analysis was performed on pooled data.

RESULTS

We addressed three important questions that may explain, at least in part, inhibition of viral disease on plants (Figure 1): (1) Is quinone formation catalyzed by PPO sufficient to explain this effect? (2) Is POD activity, with the production of free radical intermediates, capable of causing this effect? (3) Is redox cycling among phenolic species, involving clastogenesis with subsequent generation of AOS, capable of causing this effect?

POD activity and free radical generation were linked to each other and to biological effects on viral disease. In all three plant species, application of each test chemical to foliage (Table 1) affected POD and/or PPO activities and free radical generation, the degree and nature of which depended upon the chemical and/or plant species (Tables 2–5). When larvae received viral inoculum on treated foliage, mortality varied with POD activity for both insect species and both viruses (AcMNPV and HzSNPV) (Tables 6–8). The higher the activity of POD, the lower the mortality by virus. Furthermore, free radical generation appeared to be mediated, at least in part, by POD activity. Free radicals increased linearily as a function of increasing POD activity in foliage [equation: free radicals = 161 + 1.1(POD); $R^2 = 0.55$, $F = 36$, $df = 1,30$, $P < 0.0001$]. As a consequence, as free radicals increased, larval mortality decreased linearly for all plants combined (Figure 3) and for each plant species analyzed separately [cotton: % mortality = 76 − (11) $\log_e$ (free radicals), $R^2 = 0.62$, $F = 17$, $df = 1,13$, $P = 0.0011$; tomato: % mortality = 55 − (7.5) $\log_e$ (free radicals), $R^2 = 0.63$, $F = 15$, $df = 1,11$, $P = 0.0027$]. For lettuce, mortality decreased asymptotically (as a quadratic function) with increasing free radical generation [% mortality = 21 − 0.11(free radicals) + 0.00006(free radicals)^2; $R^2_{adj} = 0.62$; $F = 6.7$, $df = 2, 5$, $P = 0.0384$].

In untreated control foliage, cotton produced 2.7× more free radicals than tomato (one-way ANOVA $F = 7.6$, $df = 2,12$, $P = 0.0075$; PLSD, $P = 0.0022$) and 1.2× more than romaine lettuce (PLSD: $P = 0.1403$). Crushed lettuce produced 2.2× more free radicals than did tomato (PLSD: $P = 0.0412$).

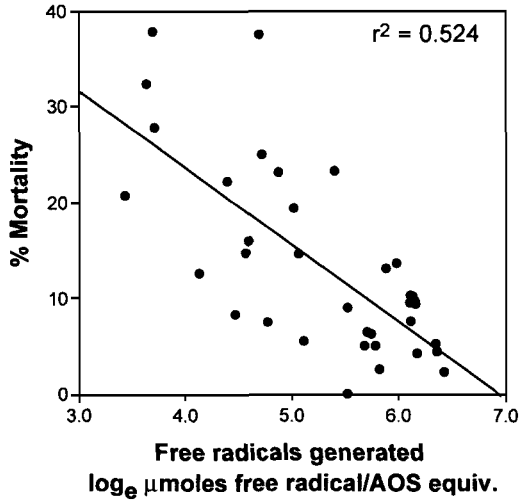

FIG. 3. Influence of free radical generation in foliage on viral disease. A variety of prooxidants, antioxidants, phenolic substrates, and/or semi-purified phenolases were applied to folliage of cotton, tomato, or lettuce. Larvae of *H. virescens* received viral inoculum (30 polyhedral occlusion bodies/larva) on treated foliage. Free radical generation (in H_2O_2 equivalents from the standard curve in Figure 2) was measured for most of the treatments (but not on the same samples). As free radicals increased, larval mortality by virus decreased {equation: % mortality = $55 - 7.9[\log_e(\text{free radicals})]$; $F = 36$; $df = 1,33$; $P < 0.0001$}.

In contrast to POD, PPO was correlated with enhanced viral disease. The higher the PPO activity of treated foliage, the greater the probability of an insect dying from viral disease on tomato (*H. virescens* only, Table 7) or lettuce (Table 8).

As the influence of a particular test chemical on viral disease is discussed below, be aware that classification of a given phenolic species as an antioxidant or prooxidant in Tables 2–5 was based on how we tested our hypothesis. A given phenolic species may behave as a prooxidant or an antioxidant, depending upon a multitude of factors (Ahmad, 1995; Duffey and Stout, 1996).

Effect of Phenolases on Viral Disease

In Vitro Bioassay. To determine whether quinone formation by itself may account for inhibition of viral disease, we treated occlusion bodies of AcMNPV during oxidation of a single phenolic species at several different concentrations (CHA, caffeic acid, or catechin) catalyzed by PPO or POD in vitro. No effects on viral disease were seen for either enzyme regardless of the substrate used

(two-way ANOVA: treatment $F = 0.37$, $df = 1,24$, $P = 0.55$; enzyme $F = 0.18$, $df = 2,24$, $P = 0.91$).

In Vivo Bioassay. We then examined the effects of phenolic oxidation by phenolases on viral disease in vivo by applying semipurified PPO to foliage, which produced apparently conflicting results. On the one hand, application of PPO enhanced viral disease (Table 2), which is consistent with the regressional results described above for tomato and lettuce showing that larval mortality increased with increasing PPO activity (Tables 7 and 8). Furthermore, boiling PPO before applying to tomato attenuated viral disease compared to the control, supporting the notion that PPO activity may enhance viral disease (Table 2). On the other hand, contradictory results were obtained on lettuce. PPO applied to lettuce increased free radical generation by 53% and inhibited viral disease (Table 2). Mortality of insects receiving the highest concentration of PPO (250 μg/ml) decreased 90% (Table 2, $\chi^2 = 6.56$, $P = 0.0105$, $N = 172$). Percent mortality of insects treated with PPO at four different concentrations (from high to low) were 1.0, 4.1, 4.1, and 4.17%, respectively. However, in these treatments POD activity also increased from 40 units in the control to 68 $\pm$ 1.5, 57 $\pm$ 2.8, 56 $\pm$ 14, and 55 $\pm$ 19 from highest to lowest PPO concentration, respectively.

In contrast to quinone formation catalyzed by PPO, POD activity and free radical generation influenced viral disease in a consistent manner in most treatments. Application of POD to cotton and tomato increased POD activity and free radical generation (Table 2). As a consequence of these factors, mortality of *H. virescens* by virus was diminished. For example, application of POD lowered mortality on cotton relative to control foliage by 66% ($\chi^2 = 4.13$, $P = 0.0424$, $N = 174$, although this was not significant with Bonferroni correction). In tomato, application of POD significantly decreased mortality by 73% ($\chi^2 = 8.44$, $P = 0.0037$, $N = 172$).

It was difficult to assess the impact of the dose–response test we performed by application of four concentrations of semipurified cotton POD to lettuce foliage because cotton POD appeared to inhibit foliar POD activity and free radical generation in lettuce (Table 2). In a separate assay, we tested POD activities by using the concentrations of semipurified POD applied to lettuce. We then added these values to the activities for the untreated lettuce foliage in the bioassay. The observed lettuce POD activities were markedly lower than the sum of these two values. Based on these calculations, supplementation with 100, 62.5, 31.25, and 15.6 μg/ml of semipurified POD should have produced POD activities of 180, 110, 60, and 35 units, respectively. However, the lettuce POD activities obtained were 68 $\pm$ 3.8, 57 $\pm$ 4.0, 38 $\pm$ 1.9, and 29 $\pm$ 3.4 units, respectively. Furthermore, mortality by virus relative to controls was enhanced in these treatments by 7.9, 9.9, 12, and 16%, respectively.

In contrast to cotton POD, HRP did not inhibit the observed lettuce POD

activities (Table 2). Consequently, HRP inhibited viral disease on cotton (42%), tomato (73%, $\chi^2 = 4.36$, $P = 0.00370$, $N = 175$), and lettuce (76%). Boiling semipurified cotton POD or HRP for 15 min only partially inhibited their activities (Table 2). Thus, reversal of the effect of viral inhibition by these enzymes was only partial.

Effects of Redox Cycling Between Catecholic Phenolics on Viral Disease. We tested whether cooxidation of phenolic species generates free radicals using CHA and rutin as the major model. We first examined the redox potential of a variety of catecholic phenolics and established that oxidation of CHA catalyzed by PPO or POD co-oxidized rutin.

Redox Potentials of Catecholic Phenolics. CHA had a higher E_h at all pHs tested (except pH 10) than did rutin (Table 9). This result is consistent with the observed cooxidation of these two phenolic species in mixture at pH 8 in the absence of phenolases, although the reaction rate is slow (≈ 7 units/min). Moreover, CHA had the highest redox potential at pH 7, 8, and 9 among all phenolics tested. Quercetin had the lowest redox potential at pH 8.

In order to evaluate whether cooxidation between phenolic species is an important process with the potential to inhibit viral disease, we first measured the rate of oxidation, catalyzed by phenolases, of a single vs. a mixture of phenolic species. We also obtained physical evidence showing that both phenolic species are oxidized during redox cycling. Finally, we demonstrated that cooxidation generates H_2O_2 and perhaps other radical species as well.

Spectrophotometric Evidence for Enzymatically Mediated Cooxidation of Rutin by Chlorogenoquinone Involving Clastogenesis. Quinone formation (measured as the ΔOD_{470} per minute) catalyzed by semipurified tomato PPO was 13 times higher in a mixture of 2 mM CHA and 2 mM rutin than in 2 mM

TABLE 9. REDOX POTENTIALS OF SELECTED TOMATO FOLIAR PHENOLICS

Phenolic	Redox potential (mV)[a]			
	pH 7	pH 8	pH 9	pH 10
Caffeic acid	389 ± 1.5b	352 ± 2.2c	230 ± 2.6e	339 ± 2.4ab
Catechin	355 ± 0.5c	300 ± 0.8d	256 ± 0.4c	244 ± 0.7c
CHA	419 ± 2.0a	376 ± 1.4a	352 ± 1.2a	331 ± 1.7b
Quercetin	NT[b]	258 ± 1.1e	351 ± 0.6a	350 ± 0.8a
Rutin	337 ± 2.5d	366 ± 1.2b	345 ± 0.9b	337 ± 2.8b

[a] Redox potentials are the mean ±SE of six replicates. Means not followed by the same letter within a column are significantly different at the 5% level by one-way ANOVA followed by Fisher's PLSD.

[b] The low solubility of quercetin did not permit preparation of a 0.4 mM solution at pH 7.

TABLE 10. SPECTROPHOTOMETRIC EVIDENCE OF CLASTOGENESIS AND FREE RADICAL GENERATION DURING COOXIDATION OF CHA AND RUTIN CATALYZED BY TOMATO PPO[a]

Inhibitor	Substrate		
	CHA	CHA/H_2O_2	CHA/rutin
None	149 ± 3.5a	256 ± 22a	1984 ± 28a
Catalase	188 ± 17a	241 ± 12a	1688 ± 40b
BHT plus mannitol	228 ± 5.5b	227 ± 7.9a	1592 ± 13c

[a]Units are 0.001 ΔOD/mg semipurified tomato PPO/min. Values are the mean ±SE of three replicates. Means followed by different letters within a column are significantly different at the 5% level. Means were compared by repeated measures ANOVA followed by Fisher's PLSD. Tomato PPO had no activity on rutin or guaiacol-H_2O_2. In the absence of inhibitors, PPO activity was significantly different among each of the above substrates (F = 1108, df = 9, 3, P = 0.0001).

CHA by itself (Table 10). Semipurified tomato PPO had no activity on rutin alone, with or without the addition of H_2O_2. Oxidation catalyzed by PPO of CHA, CHA plus H_2O_2, and CHA plus rutin were significantly different from each other (Table 10).

Likewise, semipurified cotton POD activity was 6.7× higher on the CHA–rutin mixture than on CHA alone (Table 11; unless otherwise noted all substrates tested with POD contained H_2O_2). POD activities increased in magnitude in the following order: rutin, CHA–rutin (no H_2O_2) (1.9× higher than rutin alone), CHA alone (3.7× greater than rutin alone), guaiacol, and CHA–rutin with H_2O_2. POD activities in these substrates were each significantly

TABLE 11. SPECTROPHOTOMETRIC EVIDENCE OF CLASTOGENESIS AND FREE RADICAL GENERATION DURING COOXIDATION BETWEEN CHA AND RUTIN CATALYZED BY COTTON POD[a]

Inhibitor	Substrate				
	CHA/H_2O_2	Rutin/H_2O_2	CHA/rutin	CHA/rutin/H_2O_2	Guaiacol/H_2O_2
None	792 ± 5.8a	215 ± 14a	414 ± 12a	5287 ± 168a	4720 ± 15.3a
Catalase	0 ± 0b	0 ± 0b	0 ± 0c	243 ± 56.3c	0 ± 0c
BHT plus mannitol	757 ± 29a	214 ± 20a	287 ± 3.2b	4833 ± 358b	3980 ± 73.7b

[a]Units are 0.001 ΔOD/mg semipurified cotton POD/min. Values are the mean ±SE of three replicates. Means followed by different letters within a column are significantly different at the 5% level. Means were compared by repeated measures ANOVA followed by Fisher's PLSD.

different from each other, except for guaiacol and CHA–rutin–H_2O_2 (repeated measures ANOVA, $F = 1092$, $df = 12,4$, $P = 0.0001$). POD activity in guaiacol–H_2O_2 and CHA–rutin–H_2O_2 were statistically equivalent. Thus, CHA can cooxidate rutin in the presence of either PPO or POD (Figure 1, equation 4).

In order to explain these results, we invoke the concept of clastogenesis (Figure 1, equation 4). Clastogenesis can be inferred during cooxidation between CHA and rutin catalyzed by either PPO or POD. Addition of catalase to the CHA–rutin mixture significantly decreased rate of oxidation by 15% (Table 10). In contrast, addition of catalase to CHA or CHA–H_2O_2 had no effect on the oxidation of these substrates by PPO. Furthermore, POD was able to oxidize the CHA–rutin mixture in the absence of added H_2O_2 (Table 11); this activity was completely quenched by the addition of catalase. Quinone formation was not attributable to cooxidation in the absence of POD because the same substrate mixture was used in the blank. Addition of catalase fully inhibited POD activities in all substrate combinations except CHA–rutin–H_2O_2; inhibition occurred but was not complete. Thus, we suggest that clastogenesis occurs as a consequence of cooxidation between catecholic phenolic substrates catalyzed by phenolases.

Free Radical Generation During Enzymatically Mediated Cooxidation Between CHA and Rutin. Cooxidation between CHA and rutin catalyzed by PPO or POD appears to produce free radicals in vitro. Addition of BHT plus mannitol to the CHA–rutin mixture significantly reduced PPO-mediated oxidation by 20% (Table 10). These free radical scavengers did not affect oxidation of CHA alone or CHA–H_2O_2 catalyzed by PPO. The BHT–mannitol combination also significantly inhibited oxidation of CHA–rutin, CHA–rutin–H_2O_2, and guaiacol–H_2O_2 catalyzed by POD by 44, 10, and 15%, respectively (Table 11). Furthermore, BHT and mannitol were tested separately as antioxidants in CHA–rutin–H_2O_2 with crude cotton enzyme extracts (Table 12). BHT and mannitol decreased POD activity by 55 and 33%, respectively, suggesting that free radicals are generated, including the highly reactive OH· as a result of redox cycling.

Quantitation of Cooxidation Between Rutin and CHA. To obtain physical evidence of the extent of oxidation of CHA and rutin during redox cycling catalyzed by phenolases, we chromatographed these substrates, singly or in mixture, at 30, 60, or 120 min following onset of the reaction. Both CHA and rutin in mixture disappeared rapidly in the presence of PPO (Figure 4). By the end of 2 hr, only 5% of either substrate was still detectable by TLC. In the presence of catalase or BHT plus mannitol, both phenolics in mixture disappeared more slowly; oxidation of rutin was more inhibited than that of CHA. Only 80% of the rutin in mixture disappeared in the presence of inhibitors compared to 95% in their absence. However, at the end of the 2-hr incubation

TABLE 12. EFFECTS OF PROOXIDANTS, ANTIOXIDANTS, OR COMBINATIONS OF BOTH ON COTTON POD ACTIVITIES IN THREE DIFFERENT SUBSTRATES IN VITRO[a]

Treatment	Substrate					
	Guaiacol/ H_2O_2	%Δ[1] in activity[b]	CHA/H_2O_2	%Δ in activity[b]	CHA/rutin/ H_2O_2	%Δ in activity[b]
Control	368 ± 5.2gi[b]		53 ± 0.58b		354 ± 22b	
Prooxidants						
Quercetin (1 μM)	420 ± 15fg	↑14	2.4 ± 0.62d	↓96*	NT	
Quebracho tannin (0.01%)	573 ± 33bcf	↑56*	671 ± 15a	↑1156*	558 ± 16a	↑58*
Ferulic acid (30 μM)	620 ± 18b	↑68*	0.31 ± 0.06d	↓99*	269 ± 17bd	↓24*
p-Coumaric (30 μM)	459 ± 14cf	↑25*	37 ± 4.2c	↓32*	270 ± 61bc	↓24
Quercetin + tannin	1843 ± 164a	↑400*	NT		NT	
Antioxidants						
BHT (20 μM)	275 ± 12j	↓25*	NT		159 ± 18e	↓55*
Mannitol (20 μM)	332 ± 20hij	↓10	NT		237 ± 15cd	↓33*
Prooxidant + Antioxidant						
Tannin + BHT	527 ± 15be	↓8[c]	NT		NT	
Tannin + mannitol	465 ± 34cdeg	↓19	NT		NT	
Ferulic acid + BHT	380 ± 20fgh	↓39*	NT		NT	
Ferulic acid + mannitol	530 ± 17bd	↓15	NT		NT	

[a]CHA = chlorogenic acid, BHT = butylated hydroxytoluene, NT = not tested. Concentrations are final concentrations. Values are the mean ±SE of three replicates. Units are ΔOD/g leaf tissue/min. Enzymes are crude extracts from cotton foliage. Letters within columns indicate significantly different means using repeated measures ANOVA followed by Fisher's PLSD. Asterisks next to change in activity indicate treatments within columns that are significantly different from the control.
[b]Percent change in activity is in comparison to the control.
[c]Change in activity compared to prooxidant treatment in the absence of an antioxidant.

period, the disappearance of CHA in mixture with rutin did not differ from the control (CHA alone).

CHA and rutin were also both oxidized in mixture in the presence of POD (Figure 5). However, POD did not oxidize as much of either substrate as did

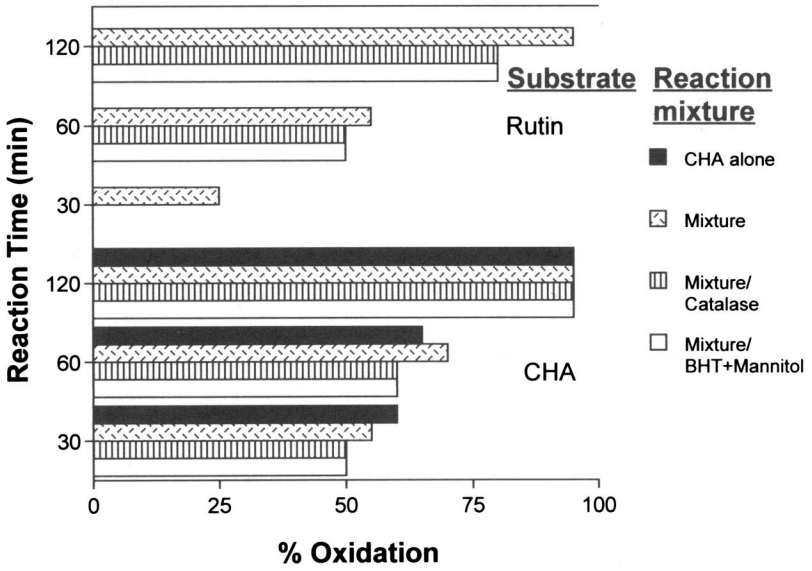

FIG. 4. Time course of cooxidation between chlorogenic acid (CHA) and rutin, catalyzed by tomato PPO, assayed separately or in mixture. The percent of the substrate that was oxidized at each time point was calculated by subtracting the initial amount of substrate (20 nmol) minus the amount that remained at the end of the reaction time. Aliquots of 1-ml of each reaction mixture were stopped and processed at each time point. A constant volume from each treatment was chromatographed on TLC plates at each time point and developed in 2% formic acid. The amount of unoxidized substrate remaining was semi-quantified by comparison to known standards chromatographed under the same conditions. Values shown are the mean of three replicates. R_f: CHA, 0.46; rutin, 0.24.

PPO by the end of the 2-hr incubation period. Addition of catalase to this mixture initially prevented oxidation of CHA, but after 1 hr, CHA began to disappear (autooxidation). Rutin in mixture was not oxidized at all in the presence of catalase. Addition of BHT plus mannitol slowed the rate of oxidation of both compounds. Less of either compound was oxidized by the end of 2 hr.

Effects of Cooxidation on Viral Disease In Vitro. The effects of redox cycling between phenolic species on viral disease was first examined in vitro. Cooxidation between CHA and rutin did not inactivate the virus in vitro whether the reaction was catalyzed by PPO or POD (treatment $F = 0.04$, $df = 1,6$, $P = 0.84$). Thus, we added lysolecithin to a set of treatments to determine the impact of a detergent on this reaction. Despite a nonsignificant result, POD (horseradish) appeared to negatively affect viral disease more in the presence of lysolecithin than in its absence. Horseradish peroxidase (HRP) inhibited mor-

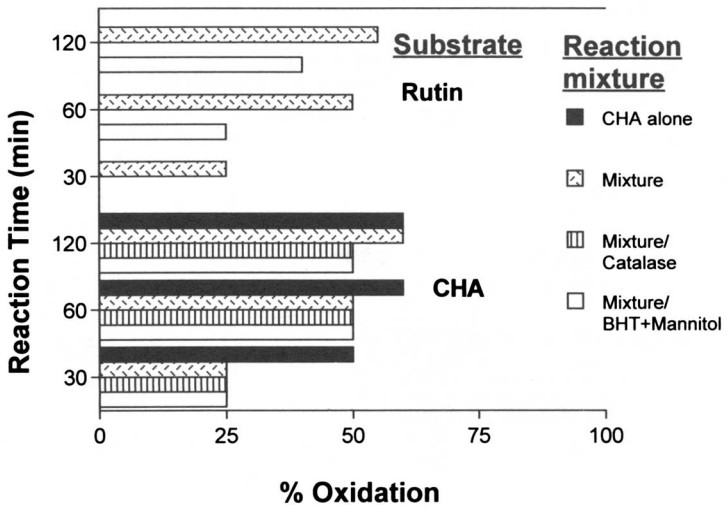

FIG. 5. Time course of cooxidation between chlorogenic acid (CHA) and rutin, catalyzed by cotton POD, assayed separately or in mixture. The percent of the substrate that was oxidized at each time point was calculated by subtracting the initial amount of substrate (20 nmol) minus the amount that remained at the end of the reaction time. Aliquots of 1-ml of each reaction mixture were stopped and processed at each time point. A constant volume from each treatment was chromatographed on TLC plates in 2% formic acid at each time point. The amount of unoxidized substrate remaining was semiquantified by comparison to known standards chromatographed under the same conditions. Values shown are the mean of three replicates. R_f: CHA, 0.46; rutin, 0.24.

tality 32 ± 1.2% and 25 ± 3.5% with and without lysolecithin, respectively. PPO did not inhibit AcMNPV, but did inhibit HzSNPV by 57% in the presence of lysolecithin only, but these treatments were not replicated.

In contrast to CHA and rutin, when occlusion bodies were incubated in a CHA plus catechin mixture with POD, significant viral inactivation occurred (Table 13). Viral inhibition by POD activity cannot be attributed simply to H_2O_2 because this treatment only inhibited mortality by 20% in the absence of enzyme (38 ± 6.5%, not significant). Furthermore, incubation of the virus with CHA plus catechin in the absence of H_2O_2 (quinone formation not detected) did not inhibit mortality (48 ± 2.3% and 47 ± 4.3% for the control and treatment, respectively). PPO had no effect on viral disease regardless of the substrate or inhibitor tested ($F = 0.88$, $df = 1,16$, $P = 0.47$). On the other hand, catalase was marginally better (54% inhibition) at protecting the virus from inactivation than any other inhibitor tested in the presence of POD (Table 13, inhibitor treatment $P = 0.06$, LSD $P = 0.02$).

TABLE 13. EFFECTS OF COOXIDATION BETWEEN 3 mM CHA AND 3 mM CATECHIN CATALYZED BY POD ON INFECTIVITY OF AcMNPV TO *H. virescens* INCUBATED AT pH 8 FOR 15 MIN AT A CONCENTRATION OF 30 OBs/μl[a]

Inhibitor	Control	Treatment	Disease inhibition (%)
Ascorbate	49 ± 13	29 ± 3.2	41
Galvinoxyl	38 ± 5.0	29 ± 5.5	25
Borate	62 ± 2.2	35 ± 14	40
Catalase	61 ± 17	26 ± 1.4	54

[a]Values are the mean ±SE percent mortality of three replicates. POD (horseradish peroxidase) activity was assessed in CHA/rutin in mixture in the absence of inhibitor. Activity = 300 units (ΔOD_{470}/min, 1 unit = 0.001). All treatments contained H_2O_2. The reaction was stopped with various inhibitors. Controls received inhibitor at the beginning of the incubation period. Control mortality was significantly greater than for treatments, but there were no significant differences among different inhibitors (treatment $F = 13$, $df = 1, 16$, $P = 0.0022$; inhibitor $F = 3.0$, $df = 3,16$, $P = 0.0645$).

Effects of Cooxidation on Viral Disease In Vivo. The impact of the application of CHA plus rutin to foliage on viral disease depended upon whether it was applied with (Table 2) or without (Table 4) a semipurified phenolase. When CHA plus rutin was applied to foliage in the absence of additional phenolase, this phenolic mixture appeared to act as an antioxidant by decreasing free radical generation (Table 4). However, viral disease was not significantly affected. On cotton, mortality did not increase significantly ($\chi^2 = 0.80$, $P = 0.3700$, $N = 168$) despite a marked decrease in POD activity and a 58% decrease in free radical generation. In tomato and lettuce, CHA plus rutin acted as an antioxidant by decreasing free radical generation 34 and 43%, respectively. As a result, viral disease was not enhanced on tomato and did not decrease on lettuce. CHA plus rutin had only a minor effect on viral disease in the absence of additional phenolase activity.

In contrast, application of CHA plus rutin in combination with semipurified POD markedly affected viral disease (Table 2). Application of a combination of POD and CHA–rutin to cotton increased POD activity by 200% and free radical generation by 72%. As a consequence, larval mortality by virus decreased significantly relative to the control (82%, $\chi^2 = 7.1$, $P = 0.0078$, $N = 171$) (Table 2). Furthermore, a significant decrease in larval mortality occurred when this combination was applied to tomato. In this treatment mortality decreased to zero ($\chi^2 = 17$, $P < 0.0001$, $N = 180$), POD activity increased by 529%, and free radical generation increased by 69% (Table 2). No mortality was seen in larval *H. zea* that received this treatment on tomato compared to 22% mor-

tality of control larvae (data not shown). Because lettuce POD activity was inhibited by the addition of semi-purified cotton POD, we could not accurately assess the impact of this treatment on lettuce.

The impact of application of semipurified POD in combination with CHA–rutin was inconsistent. On tomato, application of PPO increased larval mortality by 39% in *H. virescens* and inhibited free radicals by 71% (Table 2). For *H. zea* this combination on tomato did not affect mortality (data not shown). In contrast, this combination applied to lettuce enhanced free radical generation by 36%; as a consequence, larval mortality decreased by 87% (Table 2, $\chi^2 = 8.2$, $P = 0.0041$, $N = 176$). However, it is noteworthy that POD activity also increased in this treatment relative to the control.

Application of a single catecholic phenolic substrate in the absence of additional phenolase showed marked antioxidant activity. CHA and rutin applied separately to cotton dramatically inhibited POD activity on guaiacol by 91 and 69%, respectively. Application of CHA alone to cotton foliage enhanced viral disease by 135% ($\chi^2 = 5.1$, $P = 0.0241$, $N = 172$). However, rutin applied alone did not significantly affect disease ($\chi^2 = 3.5$, $P = 0.0599$, $N = 172$). The influence of catechin on viral disease suggests that it also functioned as an antioxidant. When catechin was applied to lettuce in the absence of additional phenolase, it inhibited foliar POD and PPO activities by 65 and 44%, respectively (Table 4; note enzymes were assayed using a CHA–mixture). It is noteworthy, however, that in a separate experiment, lettuce PPO activity was enhanced 22 times by the addition of an equal concentration of catechin to CHA alone, whereas this combination had no effect on POD activity on this substrate combination (data not shown). Application of catechin to lettuce also reduced free radical generation by 60% relative to the control (Table 4). As a consequence, virally induced mortality was increased 63%.

The preponderance of the evidence suggests that redox cycling among phenolic species involving clastogenesis has the ability to inhibit viral disease in the presence of POD but not PPO activity. On the other hand, if PPO activity predominates, catecholic phenolics appear to function as effective antioxidants, sparing the baculovirus from inhibition. Furthermore, application of high levels of catecholic phenolics protected the virus from inhibition if POD activities were inhibited by their application.

Effects of Prooxidants on Viral Disease

Effects of In Vitro Treatment of Virus with OH· on Disease. Incubation of viral inoculum with Fenton reagent in the absence of enzymes or phenolics significantly decreased viral disease by 48 ± 4.3% (27 ± 2.9%) compared to the inhibited (by mannitol) control (14 ± 2.2%) (Student's t test $t = 3.8$, $df = 4$, $P = 0.0198$). Mortality by virus treated with a buffer control was 26 ± 3.1% (not significant).

In Vivo Effects of Phenolic Prooxidants on Viral Disease Using a Diet Incorporation Method. We tested the effects of redox cycling catalyzed by PPO or POD of a CHA–rutin mixture in combination with catalytic amounts of two prooxidants, a monophenol (ferulic acid) and a catecholic phenolic (quercetin), on viral disease by incorporating these phenolics into artificial diet. We then applied a formulation of enzyme plus virus to small diet cubes that were subsequently fed to larvae of *H. virescens.* Viral disease was inhibited when semipurified cotton POD was applied to the phenolic diet. Ingestion of this phenolic mixture with 300 units of POD inhibited mortality 55 $\pm$ 2.4% compared to a boiled enyzme control and 55 $\pm$ 3.5% compared to the control diet (POD plus virus applied to diet without phenolics). POD at 100 units of activity reduced mortality by 37 $\pm$ 1.2% compared to the boiled enzyme control and 40 $\pm$ 0.61% compared to the control diet. PPO did not produce significant inhibition in any treatment. (Two-way ANOVA: treatment $F = 11$, $df = 1,6$, $P = 0.0161$; enzyme $F = 0.18$, $df = 2,6$, $P = 0.84$; PLSDs: POD 100 units $P = 0.0405$, POD 300 units $P = 0.0347$, PPO $P = 0.69$.)

Effects of Applying Prooxidants to Foliage on Viral Disease. To evaluate whether free radical generation is an important process in the attenuation of viral disease in vivo, we tested foliar application of a variety of prooxidants to cotton, tomato, and lettuce (only one prooxidant, quebracho tannin, was tested on lettuce). Quercetin applied to foliage increased both POD activity and free radical generation in cotton and lettuce (Table 3). In response, mortality by virus decreased on cotton by 67%. Quercetin applied to tomato, however, inhibited phenolase activities and free radical generation. However, this treatment did not significantly affect viral disease on tomato.

Monophenolic prooxidants applied to cotton inhibited viral disease on cotton, but enhanced disease on tomato (Table 3). Ferulic or *p*-coumaric acids applied separately to cotton increased free radical generation and inhibited viral disease by 37 and 27%, respectively (Table 3). In contrast to cotton, application of ferulic or *p*-coumaric acids to tomato inhibited free radical generation and POD activities. As a result, application of *p*-coumaric acid increased mortality on tomato by 65%. Thus, ferulic acid, *p*-coumaric acid, and quercetin functioned as prooxidants on cotton only.

Quebracho tannin was tested for its effects on free radical generation in cotton, lettuce, and tomato. In these plants, free radical generation increased, especially in tomato (247%, Table 3). In cotton, free radical generation increased by 30%, POD activity increased by 55%, and mortality dropped to zero. In lettuce, free radical generation increased by 42%, POD activity increased by 652%, and viral disease was reduced by 50% (Table 3). We also added lysolecithin in combination with quebracho tannin to determine if this detergent could reverse inhibition of viral disease by preventing possible tannin–protein aggregation (if this was the mechanism of inhibition). Application of lysolecithin

in combination with quebracho tannin had no effect on viral inhibition caused by the application of quebracho tannin.

The effects obtained by applying a variety of prooxidants to foliage strongly link both free radical generation and POD activity with each other and with viral inhibition, especially on cotton.

Effects of Prooxidants on Free Radical Generation Mediated by POD In Vitro. The above results obtained by applying prooxidants to foliage concur with in vitro testing of the effects of prooxidants on free radical generation in vitro. The ΔOD_{470} per minute during phenolic redox cycling catalyzed by POD was used as an index of free radical generation in mono- and/or diphenolic substrates in the presence of prooxidants. When a variety of prooxidants was tested for effects on oxidation of guaiacol, cotton POD activity was markedly enhanced (Table 12). Quebracho tannin (Figure 1, equations 4 and/or 6), ferulic acid alone (Figure 1, equation 7), *p*-coumaric acid alone (Figure 1, equation 7), or a combination of quercetin plus tannin (Figure 1, equations 4 and/or 6) significantly increased oxidation of guaiacol by 14, 56, 68, 25, and 400%, respectively (Table 12). Quercetin by itself was marginally effective at increasing POD activity ($P = 0.0605$). Furthermore, these prooxidant effects were significantly inhibited by adding free radical scavengers to these reaction mixtures. The prooxidant activity obtained by the addition of ferulic acid was decreased by BHT and mannitol, added separately, by 39 and 15%, respectively.

On lettuce or tomato, however, we reported that ferulic and *p*-coumaric acids did not enhance free radical generation or attenuate viral disease. This is consistent with in vitro testing of the addition of ferulic or *p*-coumaric acids to catecholic phenolic substrates oxidized by POD in vitro. Addition of these monophenols inhibited oxidation of CHA by POD (Table 12). Ferulic acid was particularly inhibitory to oxidation of CHA alone; activity dropped by 99% with this substrate (Table 12). However, inhibition of cooxidation between CHA and rutin by ferulic and *p*-coumaric acids was not statistically significant. Thus, ferulic and *p*-coumaric acids enhanced oxidation of monophenols and inhibited viral disease on cotton. In contrast, these monophenols did not significantly affect oxidation of catecholic phenolics or the extent of viral disease on tomato.

When quebracho tannin was added to a catecholic phenolic substrate, CHA, POD activity increased in vitro by 1156%, whereas oxidation of a CHA–rutin mixture was only moderately affected by addition of tannins (increase in activity of 58%) (Table 12). Quebracho tannin by itself was not a substrate for cotton POD at any concentration tested (1.00, 0.10, and 0.01%). In addition, the monomer, catechin, had no effect on cotton POD activity in CHA–rutin (data not shown). This finding is consistent with the above results whereby application of quebracho tannins to cotton or lettuce foliage increased free radical generation and inhibited viral disease.

Effects of Antioxidants on Viral Disease

We tested our ability to reverse the effects of POD activity and/or free radical generation as inhibitors of viral disease by applying a variety of antioxidants to foliage (Table 4). Note that on tomato, all antioxidants were applied in combination with POD because tomato by itself is not inhibitory to the virus. All antioxidants tested enhanced viral disease on at least one plant species. For example, ascorbate completely inhibited all phenolase activity when applied to cotton or tomato foliage. On cotton and tomato, mortality increased 63% ($\chi^2 = 2.0$, $P = 0.1499$, $N = 169$) and 432% ($\chi^2 = 11$, $P = 0.0079$, $N = 180$), respectively. We could not accurately assess the impact of ascorbate on free radical generation, however, because free radicals increase markedly in the presence of Fe^{2+}, giving ambiguous results (Koppenol and Butler, 1985).

Application of BHT enhanced viral disease on all three plants, bringing the percent mortality to the level of that obtained on artificial diet or higher (Table 4). For example, mortality on cotton increased 186% following application of BHT ($\chi^2 = 13.0$, $P = 0.0003$, $N = 179$). On tomato, BHT increased mortality by 263% ($\chi^2 = 7.0$, $P = 0.0081$, $N = 168$). BHT also inhibited POD and free radical activities in all three plant species.

Application of mannitol, an OH· radical scavenger, provided the best protection of all antioxidants tested on cotton. Mannitol did not affect cotton POD activity but did inhibit free radical generation and enhanced larval mortality compared to untreated cotton foliage by 238% (Table 4; $\chi^2 = 13$, $P = 0.0003$, $N = 172$).

Lutein, a water-soluble analog of β-carotene, did not scavenge free radicals as effectively as BHT based on the heme assay but was fairly effective at reversing free radical inhibition of viral disease (Table 4). Lutein moderately inhibited free radical generation in cotton (49%), but was only mildly inhibitory in tomato (15%) and lettuce (32%). As a consequence of lutein application, viral disease was enhanced on cotton by 91% relative to the control, such that mortality approached that obtained on artificial diet (25% compared with 30% on diet). Addition of lutein plus cotton POD to tomato allowed mortality to reach nearly the level on untreated control foliage (mortality for POD added to tomato without lutein was 6.25%).

Borate, which forms a chelate with catecholic phenolics (Pitzer and Babock, 1977), was tested on cotton and lettuce foliage. As expected, on cotton, borate did not affect POD activity using guaiacol as a substrate (Table 4). Application of borate, however, markedly enhanced viral disease on cotton by 215% ($\chi^2 = 11$, $P = 0.0008$, $N = 178$); free radical generation was not affected based on the heme assay. On lettuce borate inhibited POD and PPO activities, yet free radical generation increased according to the heme assay (Table 4). Application

of borate to lettuce did not enhance viral disease compared to the control. None of the insects died that were treated with the same concentration of borate applied to lettuce in the absence of virus.

Enzymatic Antioxidants. Catalase and SOD decreased free radical generation by 77 and 56%, respectively, on cotton. However, SOD was not as effective as catalase at sparing the virus. Catalase enhanced viral disease by 68% compared to the cotton control (Table 5). On tomato, catalase completely inhibited POD activity, had only a minor effect on PPO activity, decreased free radicals 76%, and increased larval mortality 231% (catalase was added to tomato in combination with POD). In contrast, SOD did not have a significant effect on viral disease on cotton (11% increase in larval mortality), perhaps because application of SOD did not inhibit POD activity (Table 5).

Effects of Antioxidants on Free Radical Generation Mediated by POD In Vitro. POD activity in a monophenolic substrate was inhibited by the addition of a free radical scavenger in vitro, supporting the above-observed biological effects on the virus. Addition of BHT significantly decreased POD activity in guaiacol by 25% in vitro (Table 12). Mannitol, however, had no effect on POD activity in vitro, suggesting that oxidation of guaiacol by POD does not generate $OH\cdot$. The ability of tannins to enhance POD activity in guaiacol was unaffected by adding BHT or mannitol (8 and 19% decrease in activity, respectively; Table 12).

DISCUSSION

In this study, we examined a series of chemical mechanisms to understand how phytochemicals influence disease in noctuid larvae caused by baculoviruses. To this end, we tested a series of chemical applications (phenolases, prooxidants, and/or antioxidants) to cotton, lettuce, and tomato foliage for effects on foliar phenolase activities and free radical generation. We then linked these chemical processes to subsequent effects on the extent of viral disease in larvae that ingested baculoviruses on treated foliage. The chemical interactions in foliage produced by these manipulations permitted us to address three critical questions outlined in the introduction regarding the processes whereby viral disease is attenuated on certain plant species. We will address each of these questions in turn.

Quinone Formation Catalyzed by PPO Was Not Sufficient to Explain Inhibition of Viral Disease. By designing a series of experiments with increasing complexity and scaling up from an in vitro to an in vivo approach, we were able to tease apart the influences of quinone formation from free radical generation. We demonstrated that quinone formation by itself was not an important process in the inhibition of viral disease. For example, viral disease was not

affected when polyhedral occlusion bodies were exposed to phenolic oxidation catalyzed by PPO in vitro, regardless of the phenolic species (singly or in mixture) tested. Furthermore, addition of a free radical scavenger (galvinoxyl) had no effect on quinone formation during redox cycling between CHA and catechin catalyzed by POD in vitro, yet the baculovirus was protected from inactivation. In fact, quinone formation as a consequence of PPO activity may enhance viral disease in some cases. For example, application of semipurified PPO to tomato foliage enhanced viral disease in vivo. Furthermore, logistic regression models incorporating the results of all chemical treatments applied to tomato and lettuce showed that as PPO activity increased, mortality by virus increased.

In contrast to the regression models, the application of semipurified PPO to lettuce appeared to inhibit viral disease. However, given that the observed POD activities in lettuce foliage were inhibited by application of cotton POD and enhanced by the application of tomato PPO, it is likely that inhibition of viral disease by application of PPO was in fact caused by lettuce POD activities. In point of fact, application of horseradish POD enhanced lettuce POD activity and inhibited viral disease. Why a combination of cotton and lettuce PODs was incompatible is unknown. It is possible that inhibition of lettuce POD by cotton POD occurred in a manner similar to inhibition of combinations of β-glucosidases from different plant species in the genus *Passiflora* (Spencer, 1988). Combinations of plant β-glucosidases from various related species in this genus frequently inhibited the ability of these enzymes to catalyze the hydrolysis of cyanogenic glycosides. It is also possible that our cotton POD preparations contained a protease that affects lettuce PODs but not tomato PODs.

POD Activity, Involving Production of Free Radical Intermediates, Is a Critical Process Profoundly Affecting Viral Disease in Noctuid Larvae. The preponderance of evidence suggests that inhibition of viral disease mediated by POD was caused by the generation of free radicals. The incidence of baculoviral disease varied as a function of POD activity in all three plant species. This result was seen both in vivo and in vitro. For example, application of semipurified POD to cotton and tomato foliage markedly inhibited disease, especially when applied with additional phenolic substrates (CHA plus rutin). Furthermore, incubation of polyhedra with CHA plus catechin in the presence of POD, or by applying POD to the surface of diet containing a mixture of phenolics, diminished the incidence of viral disease.

Although quinone formation by itself did not attenuate disease, we were able to link POD activity and free radical generation to each other and to biological effects on baculoviral disease in vivo by the application of a variety of antioxidants and prooxidants to foliage. For example, application of BHT or mannitol had minimal or no effect on foliar POD activities, yet these free radical scavengers prevented inhibition of viral disease. The use of ascorbate was less

straightforward because it serves both as a reducing agent and a free radical scavenger (Frei et al., 1989; Halliwell and Gutteridge, 1990). However, in this study, ascorbate probably functioned more as a reducing agent. Application of ascorbate completely inhibited POD activities in tomato and cotton, which subsequently enhanced viral disease on tomato, but did not reverse viral inhibition on cotton as effectively as did mannitol or BHT. Because ascorbate functions as a reductant even at low concentrations, but is only an effective free radical scavenger at high concentrations (Frei et al., 1989; Halliwell and Gutteridge, 1990; Buettner and Jurkiewics, 1993), we suspect that free radical generation was the primary process responsible for attenuating disease. At the concentration of ascorbate used in this experiment, ascorbate was probably more important as a reductant because of the prodigious oxidative activity of cotton. Oxidative stress from plant prooxidants can deplete ascorbate, thereby preventing it from functioning as a free radical scavenger (Englard and Seifter, 1986; Frei et al., 1989; Felton, 1995; Felton and Summers, 1995).

The generation of aryloxy free radical intermediates catalyzed by POD (in addition to semiquinones) may be an important process capable of attenuating viral disease in vivo and in vitro. Monophenolic prooxidants (ferulic or *p*-coumaric acids) applied to cotton foliage inhibited viral disease. Furthermore, addition of ferulic or *p*-coumaric acids to guaiacol in the presence of POD enhanced POD activity, whereas BHT inhibited this activity. These indirect measurements of free radical generation suggest that aryloxy radical intermediates (or AOS and/or other radical species) are generated during the oxidation of monophenols catalyzed by POD. Furthermore, generation of aryloxy radical intermediates may be a cause of subsequent inhibition of baculoviral disease, especially on cotton foliage which contains a number of monophenols (Bi et al., 1997).

Redox Cycling Among Phenolic Species Involving Clastogenesis and Generation of AOS and Other Free Radical Species Appear to Be Important Processes Contributing to Inhibition of Baculoviral Disease. During cooxidation between CHA and rutin, both phenolic species were oxidized during catalysis by either PPO or POD. Furthermore, we showed by the addition of catalase or mannitol and/or BHT that redox cycling by these phenolic species produced and was enhanced by generation of AOS (e.g., H_2O_2) and perhaps organic radicals as well. Application of CHA plus rutin in combination with semipurified POD to cotton or tomato foliage increased free radical generation and markedly inhibited viral disease. Furthermore, application of borate to cotton, which forms a chelate with *o*-dihydroxyphenolics (Pitzer and Babock, 1977), markedly reversed inhibition of viral disease on cotton. Viral inactivation also occurred when polyhedra were incubated with a catecholic phenolic redox couple plus POD in vitro (CHA plus catechin). Finally, the baculovirus was protected from inhibition on cotton and tomato by the application of catalase, suggesting that H_2O_2 is gen-

erated in crushed foliage, which subsequently leads to inhibition of viral disease. In fact, catalase provided the best protection from viral inactivation in vitro during redox cycling between CHA and catechin. These results support overwhelmingly our hypothesis that redox cycling among phenolics involving clastogenesis is a critical process leading to the inhibition of viral disease.

It is unclear why CHA plus rutin did not inhibit the virus in vitro, whereas CHA plus catechin did. These findings are indicative of a common problem encountered in the study of tritrophic interactions whereby in vitro and in vivo results do not always match. A possible explanation for this inconsistency may relate to the relative redox potentials of CHA, rutin, and catechin. Although we did not measure the redox potential of chlorogenoquinone, this quinone should have an even larger redox potential than the reduced form, CHA, given the prodigious increase in activity of POD on CHA and rutin in mixture. Furthermore, at pH 9, catechin had an even lower redox potential than rutin. As a consequence, cooxidation catalyzed by POD between CHA and catechin was even more rapid than between CHA and rutin. On CHA plus catechin, POD activity is ≈ 15 times higher than the activity of this enzyme on CHA alone (Hoover et al., unpublished), whereas POD activity on CHA plus rutin is seven times higher compared to the activity on CHA alone. Thus, perhaps cooxidation between CHA and catechin generates free radicals at a more rapid rate or to a greater extent during redox cycling than does cooxidation between CHA and rutin. Alternatively, the semiquinone of catechin may be more unstable (and thus, more reactive) than that of rutin.

In addition to clastogenesis, other AOS such as $OH\cdot$ and $O_2^{\cdot-}$ appear to be generated during phenolic oxidation and/or redox cycling mediated by POD. Of these radical species, $OH\cdot$ appeared to be particularly detrimental to the virus, whereas effects of $O_2^{\cdot-}$ on disease were difficult to discern. Hydroxyl radicals may directly (and/or indirectly) inactivate baculoviruses in vitro and in vivo. Mannitol, an effective scavenger of $OH\cdot$ (Elstner, 1987), quenched free radical generation and markedly enhanced viral disease when applied to cotton foliage. In fact, mannitol provided the best protection of the baculovirus among all antioxidants tested. Mannitol also inhibited redox cycling of CHA plus rutin catalyzed by POD in vitro, suggesting that $OH\cdot$ is formed during cooxidation of these phenolic species. It also appears that $OH\cdot$ can directly damage baculoviruses because polyhedra incubated with Fenton reagent in vitro were inactivated compared to the control that received mannitol at the beginning of the incubation period. However, oxidation of a monophenol (guaiacol) catalyzed by POD in the presence of mannitol was not inhibited, suggesting that $OH\cdot$ is not generated during oxidation of a single monophenolic substrate. In contrast to $OH\cdot$, $OH_2^{\cdot-}$, which may be produced during the formation of semiquinones (Singleton, 1987), did not affect viral disease. Although $O_2^{\cdot-}$ was apparently scavenged by the activity of SOD applied to cotton foliage, the reaction product,

H_2O_2, increased POD activity. Thus, the sum of these two competing influences on the biological activity of the virus was nil.

Inhibition of baculoviral disease by the application of a condensed tannin (quebracho tannin) appeared to be the result of free radical generation, not the result of precipitation of the virus by tannins. Previous studies with larval gypsy moth have suggested that tannins (at least hydrolyzable tannins) inhibit baculoviral disease by forming aggregates with the proteinaceous polyhedral occlusion body (Keating et al., 1990). We suggest, however, that some types of tannins may inhibit viral disease by prooxidant activity on phenolic redox cycling, which subsequently enhances free radical generation. In our study, if protein aggregation caused viral inhibition by quebracho tannin, application of a detergent (lysolecithin) in combination with tannins should have interferred with the ability of tannin/viral protein aggregates to form. Thus, we should have seen mitigation of the negative effects on viral disease caused by tannins. However, attempts to reverse viral inhibition by applying quebracho tannin in combination with lysolecithin did not mitigate the negative influence of tannins on mortality by virus. In contrast to detergent effects, quebracho tannins had marked prooxidant effects on POD activity. When a catalytic amount of quebracho tannin was added to a CHA plus rutin substrate mixture in the presence of POD, POD activity increased markedly. This activity was not attributable to contamination by catechin monomers because catechin inhibited this reaction (data not shown). Neither was the increased absorbance at OD_{470} the result of turbidity due to protein aggregation, because we did not observe turbidity in these reaction mixtures. Furthermore, other studies have strongly suggested that tannins can generate AOS in lepidopteran midguts (Bernays et al., 1980; Berenbaum, 1983; Steinly and Berenbaum, 1985; Barbehenn and Martin, 1994; Crawford et al., 1994). Thus, we suggest that redox cycling among a variety of phenolic species, including polyphenols, generates free radicals that subsequently inhibit viral disease.

Antioxidant or Prooxidant Activity of Different Classes of Phenolics. In general, whether a given phenolic used in our experiments acted as a prooxidant or antiooxidant depended upon the total chemical mixture. On cotton, monophenols enhanced free radical generation and inhibited viral disease; whereas catecholic phenolics had the opposite influence on these variables on all plant species tested. Many phenolics have the potential to act as powerful antioxidants (Torel et al., 1986; Singleton, 1987; Larson, 1988, 1995; Sato et al., 1996). In particular, hydroxycinnamic acids may scavenge AOS and electrophiles (Mayer, 1987; Huang et al., 1992) and can chelate metals (Huang and Ferraro, 1992). In our study, catechin, which is reported to be a radical scavenger (Torel et al., 1986; Puppo, 1992), inhibited redox cycling between CHA and rutin on lettuce catalyzed by POD. Thus, application of catechin to lettuce enhanced viral disease. Likewise, application of CHA plus rutin to cotton or tomato foliage

in the absence of added POD scavenged free radicals. Consequently, viral disease was enhanced on these plant species. CHA alone was particularly effective at preventing inhibition of viral disease.

In plants that contain predominantly catecholic phenolics such as tomato (Waiss et al., 1981; Isman and Duffey, 1982a,b), catecholic phenolics may function as effective scavengers of aryloxy radicals (and other radicals) formed by the addition of catalytic amounts of a monophenol. In this way, catecholic phenolics may protect the baculovirus from free radical inactivation. The aryloxy radical intermediate may redox cycle with a catecholic phenolic, producing a semiquinone radical and regenerating the reduced form of the monophenol. The semiquinone radical is probably better resonance-stabilized than the aryloxy radical, and thus, less reactive. Semiquinones in plant foliage have been reported to be remarkably stable (Crawford et al., 1994). For example, in contrast to cotton, ferulic or *p*-coumaric acids applied separately to tomato foliage did not inhibit viral disease. Phenolics in tomato foliage contain primarily CHA and rutin with small amounts of ferulic acid (Waiss et al., 1981; Isman and Duffey, 1982a,b) and catalytic amounts of quercetin (Duffey et al., unpublished). We demonstrated in vitro that addition of a small amount of ferulic or *p*-coumaric acid to CHA or CHA plus rutin inhibited oxidation of these catecholic phenolic substrates catalyzed by POD. Furthermore, application of CHA plus rutin to foliage inhibited POD activity using guaiacol as a substrate. Thus, catecholic phenolics may assume antioxidant activity in the presence of monophenols or vice versa.

On the other hand, cotton contains a rich mixture of mono-, di-, and polyphenols (Bi et al., 1997). Monophenols (ferulic or *p*-coumaric acids) applied to cotton foliage enhanced free radical generation and increased inhibition of viral disease on cotton. Application of a condensed tannin to cotton foliage (quebracho tannin) markedly attenuated viral disease and increased free radical generation. Quebracho tannin also dramatically increased oxidation of phenolic substrates catalyzed by POD. Furthermore, these prooxidant activities were inhibited to various degrees by free radical scavengers such as BHT or mannitol, suggesting that free radicals and/or AOS were generated during redox cycling among these phenolic compounds. These results suggest that, at least in cotton, monophenols (and polyphenols) function as prooxidants. Further, this prooxidant activity is likely the result of free radicals generated during redox cycling among mono, di-, and/or polyphenols. As mentioned above, aryloxy or aryl radicals produced from monophenols such as ferulic or *p*-coumaric acids are perhaps less resonance-stabilized and, thus, more reactive than catecholic phenolics. As a consequence of their greater reactivity, aryloxy, aryl radicals, and/or the AOS generated during this redox cycling are particularly detrimental to the baculovirus.

Our study suggests that the antioxidant or prooxidant behavior of ingested

phenols depends upon the chemical properties of the gut, the specific chemical structures involved, and the relative amounts and redox potentials of the chemicals present (see also Appel and Maines, 1995; Duffey and Stout, 1996; Johnson and Felton, 1996). Because some phenols may act as effective antioxidants (radical scavengers), some types of phenol-rich foliage may generate little oxidative stress in herbivores insects. This may explain why tomato does not inhibit baculoviral disease (Forschler et al., 1992; Hoover et al., 1998b) in noctuids.

Biological Bases for Free Radical Inhibition of Baculoviral Disease. The chemical and/or physiological mechanism(s) whereby free radicals inhibit baculoviral disease is beyond the scope of this paper, but clearly warrants further investigation. We suggest that there are multiple causes and effects for the inhibition of viral disease mediated by POD—direct effects of free radicals on the virus and indirect effects mediated through gut physiology. Viral inhibition may be a matter of the different abilities of the reactive end products formed by the oxidation of phenolics catalyzed by POD to react directly with the virus. Alternatively (and/or additionally), viral disease may be inhibited as the result of indirect effects of free radical generation mediated through midgut physiology. For example, reactive end products generated by phenolic redox cycling catalyzed by POD may cause co- and heteropolymer formation of proteins [see insect cuticle in Hasson and Sugumaran (1987)]. Given that the polyhedral occlusion body is primarily composed of protein (Vlak and Rohrmann, 1985; Whitt and Manning, 1987, 1988), the occlusion body is vulnerable to cross-linking (Felton and Duffey, 1990). It is also possible that virions released from the occlusion bodies form adducts with each other or with semiquinones, impairing their ability to fuse with receptors on the midgut epithelial cells to initiate an infection. In vitro treatment of polyhedra with POD and phenolic substrates (in mixture only) diminished the incidence of viral disease, suggesting that direct effects on viral integrity can occur during phenolic redox cycling. What is not clear is whether binding of phenolics to polyhedra lessens their ability to release infectious virions, or whether the virions themselves are affected by these processes.

In contrast to free radical generation, we obtained substantial evidence that quinone formation does not interfere with viral disease. On the contrary, quinones may protect the virus from inactivation. It is possible that quinones function as free radical/AOS sinks. Quinones may cross-link with the occlusion bodies and/or virions, scavenging radical species and protecting the virions from attack by these radicals.

An additional mechanism of attenuation of viral disease may be mediated through midgut physiology of the host insect. There is substantial evidence supporting this hypothesis. First, there is mounting evidence that inhibition of viral disease is functionally a three-way interaction. It has been demonstrated by others that treating the baculovirus in isolation from the insect has not resulted

in inhibition of viral disease (Keating et al., 1990; Forschler et al., 1992; Young et al., 1995). In those instances in which we obtained viral inhibition in vitro, the degree of inhibition was not as marked or as consistent as that obtained on plants. A more important explanation for the limited impact of in vitro treatments of polyhedra on viral disease, of course, is that it is nearly impossible with an in vitro approach to mimic realistically all of the chemical and physical interactions that occur in vivo.

Second, the production of reaction products that produce free radicals that attack proteins and lipids (Summers and Felton, 1994) create oxidative stress in the insect midgut and damage to midgut cells. Damaged gut cells may be subsequently sloughed, thereby eliminating the infection from the insect before the virus has an opportunity to spread systemically. H_2O_2 generated in the gut lumen poses a particularly serious threat to midgut cells. Because of its relative stability and ability to cross cell membranes, H_2O_2 generated in the gut can diffuse into midgut cells where it can serve as a source of highly toxic AOS, $OH\cdot$, peroxyl radicals, and hydroperoxides (Summers and Felton, 1994, 1996; Bi and Felton, 1995). Evidence that the ingestion of phenols results in AOS generation in midgut cells includes observations of increased levels of peroxidized lipids and oxidized proteins in the midgut of *H. zea* feeding on previously wounded soybean foliage (Summers and Felton, 1994; Bi and Felton, 1995) and possibly in the fatal midgut lesions in several species of herbivorous insects (Bernays et al., 1980; Steinly and Berenbaum, 1985). In fact, horseradish peroxidase can form lipid hydroperoxides directly by catalyzing the oxidation of linoleic and arachadonic acids in the presence of H_2O_2 (Garner, 1984). Insects are very susceptible to damage by lipid peroxidation because lipids, including cholesterol, are essential components of cell membranes (Ahmad et al., 1991). Sloughing of infected midgut cells prior to the establishment of a systemic infection may be an important mechanism of resistance to baculoviral infection. This process has been hypthesized as a critical mechanism causing developmental resistance to baculovirus infection, which is observed within and among instars (Engelhard and Volkman, 1995; Washburn et al., 1998). It is possible that induction of lipid peroxidation by H_2O_2 and other AOS generated by redox cycling results in damage to the receptor sites on the gut wall. This process may prevent infection by inhibiting the ability of the virions to fuse with receptors. Although the insect midgut is a rich source of enzymatic antioxidants and ascorbate (Ahmad, 1995), it is possible that gut enzymes in *H. virescens* and *H. zea* cannot adequently protect the baculovirus from inhibition on cotton. Alternatively, the virus may be irreversibly damaged during maceration of cotton foliage in the foregut before the virus reaches the midgut.

None of the above biological mechanisms need be considered as mutually exclusive; all of these processes may occur to some extent depending upon the plant and insect species involved.

Formulation. Our results suggest that formulation of baculoviruses with catecholic phenolics, catalase, and/or free radical scavengers (e.g., mannitol, BHT, or lutein) may provide improved efficacy on plants such as cotton that currently inhibit baculoviral disease in noctuids. In our study, mannitol provided the best protection from inactivation on cotton among all of the antioxidants tested. Antioxidants enhanced viral disease on treated relative to untreated cotton foliage with decreasing effectiveness as follows: mannitol, borate, BHT, CHA, lutein, catalase, ascorbate, and SOD. In another study, a group of antioxidants were tested for their ability to protect the baculovirus from inactivation by UV irradiation (Ignoffo and Garcia, 1994), which is known to generate free radicals. Catalase provided the best protection, which supported the authors' hypothesis that free radicals caused inactivation of field-applied viral insecticides. In fact, many years ago it was proposed that the inactivation of *Bacillus thuringiensis* in the field by UV may arise from photoactivation of amino acids such as tryptophan, which provides free radicals and H_2O_2 (Anasnthaswamy and Eisenstark, 1976; Ignoffo and Garcia, 1978).

CONCLUSIONS

Redox cycling among phenolic species involving clastogenesis appears to be a critical driver of the generation of free radicals, which subsequently attenuates viral disease. Our results suggest that free radical generation in crushed foliage may be a useful predictor of the performance of baculoviruses as pest control agents on plants. In general, monophenols applied to cotton foliage produced considerable prooxidant activity, whereas application of catecholic phenolics in the absence of high POD activity produced antioxidant activity. We suspect that catecholic phenolics are able to step down the electronic energy of free radicals generated during redox cycling catalyzed by POD, particularly in plants that contain a predominance of catecholic phenolics such as tomato (Isman and Duffey, 1982a,b) and lettuce (Sharples, 1964). On the other hand, radicals formed from monophenols are perhaps less resonance-stabilized and, thus, more reactive. In contrast to tomato, unstable monophenolic radicals (aryloxy and/or aryl radicals) may predominate in plants like cotton. An additional possibility is that cotton foliage is particularly inhibitory to baculoviral disease as a consequence of redox cycling among monophenolics, catecholic phenolics, and/or condensed tannins. Redox cycling among these diverse phenolic substrates may also involve clastogenesis, with the production of reactive oxygen species that are responsible for inhibiting baculoviral disease in noctuid larvae.

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INFLUENCE OF INCREASING HERBIVORE PRESSURE ON MODIFICATION OF GLUCOSINOLATE CONTENT OF SWEDES (*Brassica napus* spp. *rapifera*)

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Abstract—The effect of increasing herbivore pressure, in the form of larval feeding damage by the turnip root fly, *Delia floralis*, on the glucosinolate content of swede roots (*Brassica napus* ssp. *rapifera*) was investigated. Only one of the 14 root glucosinolates detected, 3-indolyl methyl glucosinolate, rose significantly with increasing levels of insect attack. Although other root glucosinolate concentrations altered following damage, the induced changes were no greater from inoculation with 20 eggs/root than with 5 eggs/root. Swedes roots that had been damaged by *D. floralis* contained approximately three times the concentration of total indolyl glucosinolates of control roots. This change was strongly influenced by a fourfold increase in the concentration of 1-methoxy-3-indolyl methyl glucosinolate. The total glucosinolate concentration found in swede roots remained unchanged overall as a result of a fall in the concentration of five of the aliphatic glucosinolates, which balanced the rise in aromatic glucosinolates. The relevance of these results to studies of crucifer–insect interactions are discussed.

Key Words—Herbivore pressure, glucosinolate, induced response, turnip root fly, *Delia floralis*, *Brassica napus*, root damage.

INTRODUCTION

Glucosinolates are a group of secondary plant compounds found in the *Brassicaceae* and related families, with approximately 100 different side chains bonded

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to a core molecule containing sulfur and nitrogen. Glucosinolates mediate insect behavior, acting as either stimulants or deterrents for feeding and oviposition, depending on the insect species, the glucosinolate involved, and the concentration present. Several are known to act as stimulants to crucifer-feeding insects and to influence oviposition or feeding behavior of over 20 crucifer specialist insects (see reviews by Städler, 1992 and references therein).

Plant injury, from a range of sources, can be a major factor in modifying plant glucosinolate content. Wounding *Brassica* plants affects both aliphatic and aromatic glucosinolates, but the specific effect is dependent on the plant species and age, the tissue studied, and the extent and timing of damage (Bodnaryk, 1992; Birch et al., 1992; Griffiths et al., 1994). Koritsas et al. (1989) found that damage to the leaf lamina, petiole, and stem of oilseed rape (*B. napus* ssp. *napus*) by the cabbage stem flea beetle (*Psylliodes chrysocephala* L.) led to a reduction of aliphatic and an accumulation of indolyl glucosinolates in damaged tissues. Damage to the roots of oilseed rape plants by larvae of the turnip root fly (*Delia floralis* Fall.) resulted in individual indolyl glucosinolates increasing by up to 88%, while aliphatic glucosinolates were largely reduced or unaffected (Birch et al., 1990).

Fifteen glucosinolates have been found in swede (*Brassica napus* ssp. *rapifera*) (Mullin et al., 1980; Carlson et al., 1981; Adams et al., 1989; Birch et al., 1992). However, the natural variation in the number and concentration is made more complicated by the selective induction of individual glucosinolates following damage to the plant. A linear increase in the extent of physical damage (needle punctures) to oilseed rape plants resulted in progressively smaller incremental increases in 3-indolyl methyl glucosinolate (Bodnaryk, 1992). Topical application of methyl jasmonate and jasmonic acid also induced altered glucosinolate metabolism in some plants. Logarithmic increases in the applied concentrations of these two jasmonates, up to 10 nmol/seedling, led to linear increases in 3-indolyl methyl glucosinolate, which then appeared to reach a plateau at approximately eight times the concentration found in control plants (Bodnaryk, 1994). Application of methyl jasmonate and jasmonic acid does not have the same effect on all glucosinolates. Doughty et al. (1995) found progressive increases in 3-indolyl methyl and 1-methoxy-3-indolyl methyl glucosinolate with jasmonate dose, while the effect on six other glucosinolates was less consistent.

The role of glucosinolates in insect–plant relationships remains the subject of considerable research, and it is important to the understanding of the dynamic nature of this system to consider the effect of increasing herbivore pressure on their levels. Several are known to stimulate the oviposition of *D. radicum* L. (Städler, 1978; Roessingh et al., 1992; Braven et al., 1996) and *D. floralis* (Simmonds et al., 1994). In addition, glucosinolate volatile breakdown products (e.g., isothiocyanates) were found to be attractive to larvae at low concentration

but became repellent at increased concentrations (Koštál, 1992). Consequently, it is important to understand how concentrations of individual glucosinolates vary with increasing insect feeding damage to the root. The aim of this study was to investigate how increasing herbivore pressure caused by *D. floralis* larval feeding damage to swede roots affected the induced qualitative and quantitative changes in specific glucosinolate concentrations.

METHODS AND MATERIALS

Biological Material. Eight genotypes of swede (*Brassica napus* spp. *rapifera*) were selected to represent a range of susceptibility, tolerance, and partial resistance to *Delia floralis*. Angus and, to a lesser extent, Melfort have exhibited partial resistance to turnip and cabbage root flies in a number of field experiments (Birch, 1988; Ruuth, 1988; Wilson et al., 1990). Doon Major is used as a standard susceptible genotype in experiments against both turnip root fly and cabbage root fly (Birch, 1988, 1989; Ruuth, 1988; Wilson et al., 1990). Marian and derived lines (M4M2 a-c and Nmm3 a-d) are tolerant to cabbage root fly damage at the seedling stage (Wilson et al., 1990), and GRL aga has been shown to be resistant to turnip root fly (Birch, 1989). Plants were grown in 10-cm pots containing a 3:1 mixture of Levington Universal compost and sand in a glasshouse with a temperature range of 16–21°C.

At the 8–10 true leaf stage, plants were inoculated with *D. floralis* eggs, less than 24 h old, from a laboratory culture, at a rate of 5, 10, or 20 eggs per plant and arranged with control plants in a series of randomized blocks with eight replicates per treatment. The percentage pupation for different treatments was between 51% and 59%. There was no significant difference in the percentage of eggs that produced pupae either on different plant genotypes or for different inoculation levels (Hopkins et al., 1993). Consequently, the number of eggs applied to each plant equates to the approximate feeding pressure exerted by the larvae. Six weeks after the inoculation, plants were harvested and the roots separated from the aerial parts at the hypocotyl. The roots were then weighed individually and frozen prior to freeze drying and milling.

Chemical Analysis. Glucosinolates were from freeze-dried and milled roots extracted by boiling a 1-g subsample in 20 ml of 70% v/v aqueous methanol at 80°C for 15 min. The contents of the flask were filtered and the residue reextracted with a further 20 ml of 70% v/v aqueous methanol for 5 min. The combined filtrates (containing the glucosinolates) were concentrated to 10 ml. Extracts were desulfated on A-25 DEAE Sephadex minicolumns by a method based on that of Heaney and Fenwick (1980). Each column was left overnight with 0.02 ml of aryl sulfatase to convert glucosinolates to desulfoglucosinolates, which were eluted the following morning with 2 ml of distilled water. Individual

desulfoglucosinolates were separated and quantified by HPLC, utilizing a method based on that of Spinks et al. (1984). Identification of desulfoglucosinolate peaks was by coelution with standards supplied by the Food Research Institute, Norwich, UK, unless otherwise stated (Table 1).

Statistical Analysis. Data were analyzed with the assistance of staff from Biomathematics and Statistics Scotland by using Genstat (Genstat, Sun/Unix version 5.0, release 2.2, 1990) to perform analysis of variance on untransformed data.

RESULTS

Total Glucosinolates. The mean total glucosinolate content of undamaged swede roots was 30.6 mmol/kg, did not vary ($P > 0.05$) between genotypes, and was unaffected ($P > 0.05$) by *Delia floralis* root damage (Figure 1). The roots contained 14 individual glucosinolates, one benzyl, four indolyl, and nine aliphatic compounds (Table 1), and there was considerable variation in the glucosinolate composition. Undamaged roots contained 7.1–27.0 mmol/kg of aromatic glucosinolates (combined benzyl and indolyl), which was, on average, 38% of the total glucosinolate content. When contrasted with control plants, *D. floralis* damage at 5 eggs/plant resulted in significant ($P < 0.001$) increases in both the concentration (Figure 1) and the proportion (Figure 2) of aromatic glucosinolates. Increasing herbivore pressure, by increasing the number of *D. floralis* eggs applied to each plant, did not ($P > 0.05$) further increase the change in the concentration or the proportion of aromatic glucosinolates.

Aromatic Glucosinolates. The only benzyl glucosinolate, 2-phenylethyl, varied ($P < 0.001$) between plant genotypes (2.4–11.2 mmol/kg), but it did not contribute to the rise in the proportion of aromatic glucosinolates, remaining unaltered ($P > 0.05$) by *D. floralis* damage (Figure 3). In contrast, the four indolyl glucosinolates were all affected. There was a fivefold variation ($P = 0.010$) in the indolyl glucosinolate content of the control roots tested, from 3.0 to 15.8 mmol/kg. While control swedes contained a mean of 6.4 mmol/kg of indolyl glucosinolates, those inoculated with 5, 10, and 20 *D. floralis* eggs contained 15.3, 18.0, and 17.8 mmol/kg of indolyl glucosinolates, respectively. This increase ($P < 0.001$) in concentration following *D. floralis* damage related to a change from an average of 21% in control roots to approximately 50% in damaged roots (Figure 2). Although the initial increase was significant, relative to the control plant, there was no difference ($P > 0.05$) between the indolyl glucosinolate content of swedes under the three herbivore pressure regimens.

The change in the indolyl glucosinolate content was dominated by two compounds. The dominant compound was 1-methoxy-3-indolyl methyl glucosinolate, which overall comprised, on average, 27% of the total glucosinolate

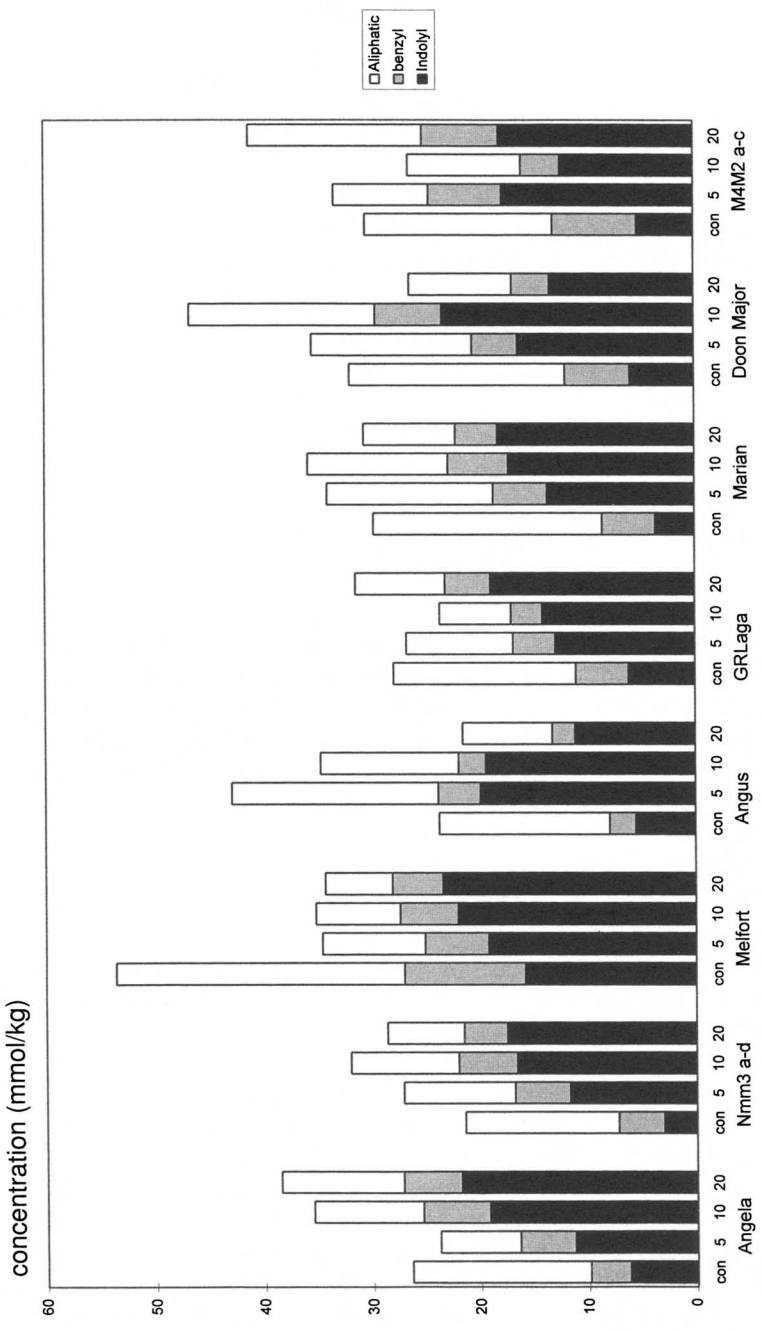

FIG. 1. The concentration of aliphatic, benzyl, and indolyl compounds in the roots of undamaged swede and swede inoculated with increasing numbers of *D. floralis* eggs.

TABLE 1. INDIVIDUAL GLUCOSINOLATES DETECTED IN THE ROOTS OF SWEDE

Class	Name	Trivial name
Indolyl	3-indolyl methyl	glucobrassicin
	1-methoxy-3-indolyl methyl	neoglucobrassicin
	4-methoxy-3-indolyl methyl	4-methoxyglucobrassicin
	4-hydroxy-3-indolyl methyl	4-hydroxyglucobrassicin
Benzyl	2-phenylethyl	gluconasturtiin
Aliphatic	2-hydroxy-3-butenyl	progoitrin
	prop-2-enyl	sinigrin
	4-methyl sulfinylbutyl[a]	glucoraphanin
	2-hydroxypent-4-enyl	napoleiferin
	5-methylsulfinyl pentyl	glucoalyssin
	but-3-enyl	gluconapin
	3-methylthiopropyl[a]	glucoiberverin
	pent-4-enyl	glucobrassicanapin
	4-methyl thiobutyl	glucoerucin

[a] Identification based on retention time under similar conditions.

content. The mean 1-methoxy-3-indolyl methyl glucosinolate content of control roots was 2.8 mmol/kg (genotypes varied significantly from 1.5 to 7.0 mmol/kg). The glucosinolate content of herbivore-damaged roots was elevated ($P < 0.001$) compared to controls. Swede roots inoculated with 5, 10, or 20 *D. floralis* eggs contained 9.5, 11.1, and 10.8 mmol/kg of 1-methoxy-3-indolyl methyl glucosinolate, respectively (Figure 3). The same pattern of response was found for 4-methoxy-3-indolyl methyl glucosinolate, for which overall concentrations were somewhat lower. The mean 4-methoxy-3-indolyl methyl glucosinolate content of control swedes was 1.85 mmol/kg, and there was a significant interaction ($P < 0.05$) between herbivore pressure and plant genotype. As can be seen (Figure 4), there was a universal trend towards an increase in the concentration of 4-methoxy-3-indolyl methyl glucosinolate with damage, but individual genotypes responded differently. The variation in response ranged from that found in cv. Melfort, in which the 4-methoxy-3-indolyl methyl glucosinolate concentrations of inoculated plants were not significantly higher ($P > 0.05$) than the concentrations in control plants, to Angela, which showed a clear stepwise response to increasing herbivore pressure.

The only glucosinolate detected that showed a consistent clear stepwise response to increasing herbivore pressure was 3-indolyl methyl glucosinolate (Figure 3). The 3-indolyl methyl glucosinolate content of controls varied ($P < 0.001$) between 0.25 and 2.78 mmol/kg, with a mean concentration of 1.11 mmol/kg. The concentrations of 3-indolyl methyl glucosinolate in *D. floralis*-

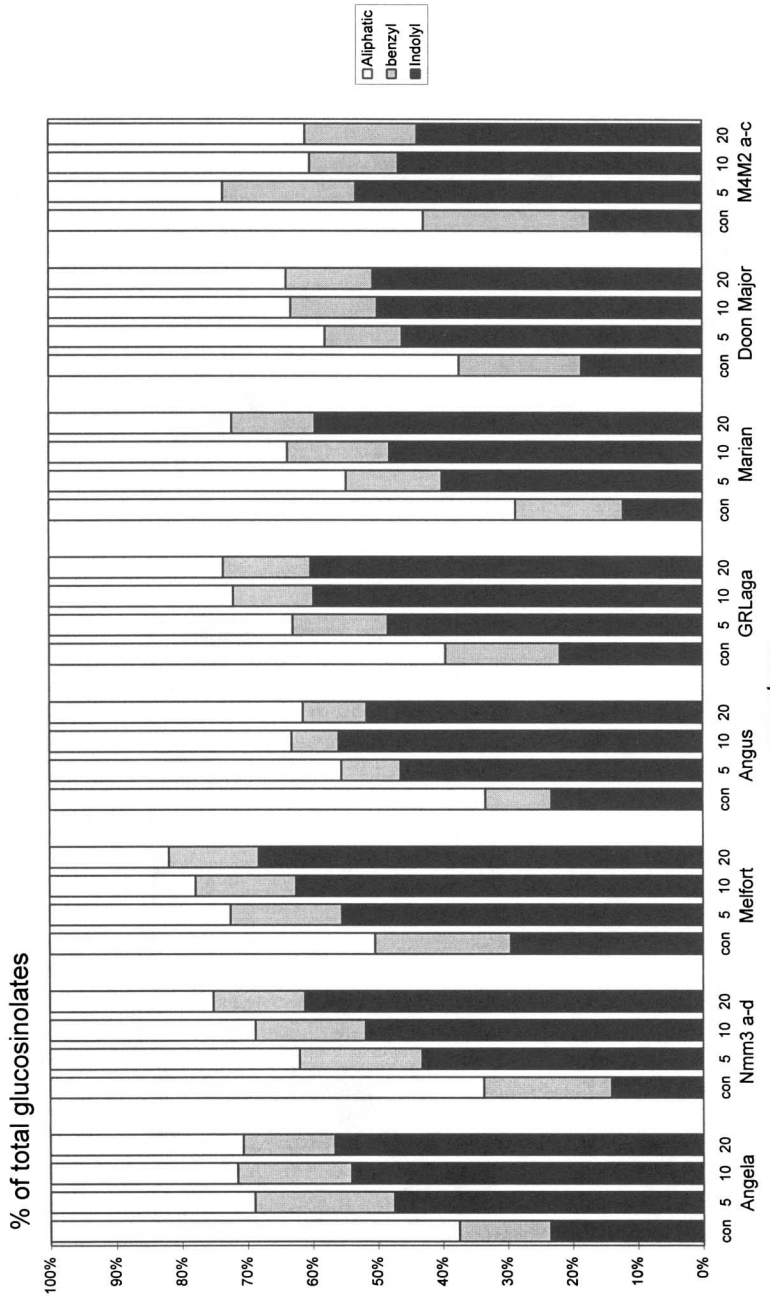

FIG. 2. Variation in the proportion of aliphatic, benzyl, and indolyl glucosinolates (percentage of total glucosinolates) in swede roots with genotypes and increasing *D. floralis* egg numbers.

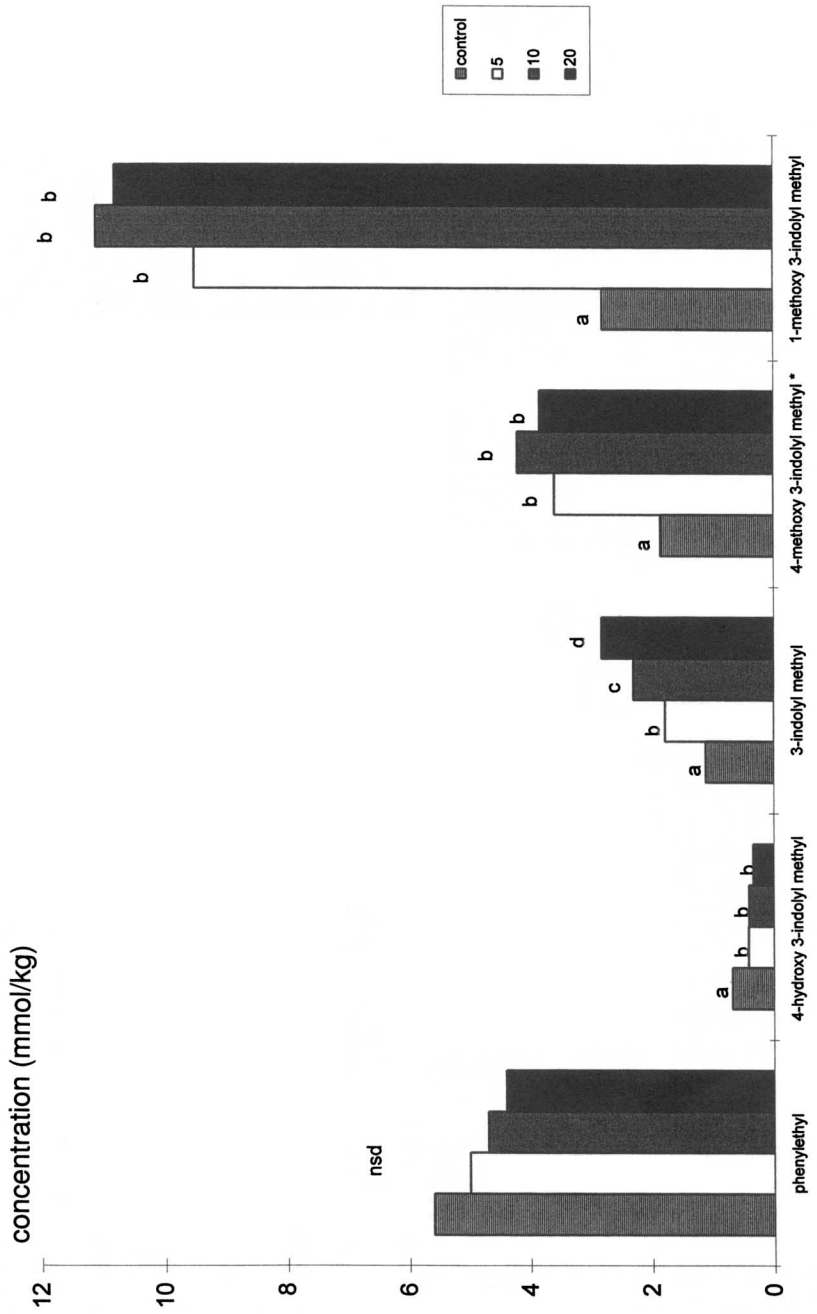

FIG. 3. Variation in the concentration of five aromatic glucosinolates with increasing *D. floralis* egg numbers (columns with different letters within a compound group are statistically different, $P < 0.05$). Bars represent the mean of the eight genotypes tested. There was no interaction between genotype and herbivore pressure except in the concentration of 4-methoxy-3-indolyl methyl glucosinolate, which is shown in more detail in Figure 4.

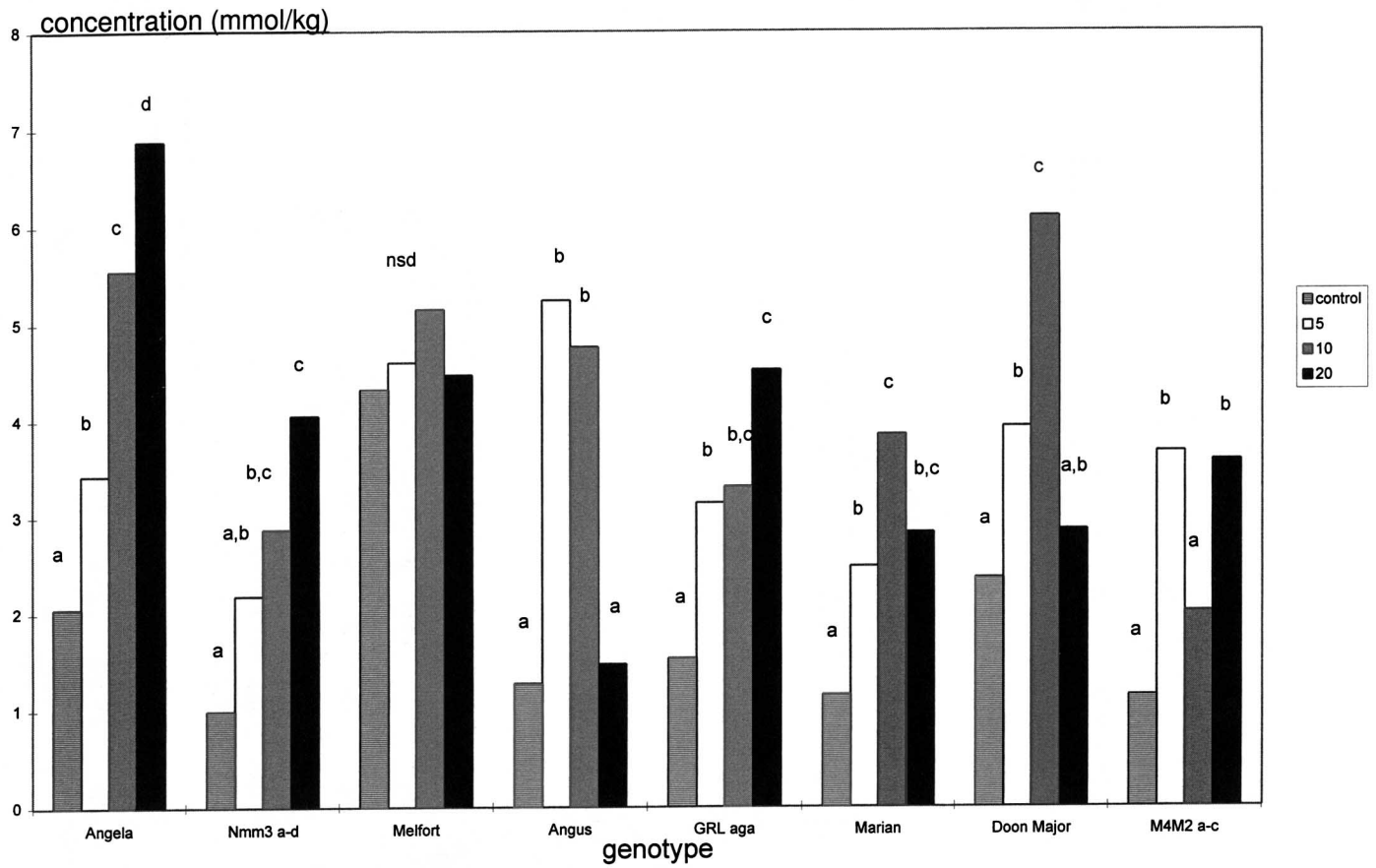

FIG. 4. Variation in the concentration of 4-methoxy-3-indolyl methyl glucosinolate between different genotypes of swede when damaged by increasing numbers of *D. floralis* larvae (columns with different letters within a genotype group are statistically different, $P < 0.05$).

damaged roots showed a ($P < 0.001$) progressive increase with increasing herbivore pressure. 3-Indolyl methyl glucosinolate concentrations in roots inoculated with 5, 10, and 20 turnip root fly eggs were 1.77, 2.30, and 2.82 mmol/kg, respectively.

The effect of damage on the concentration of 4-hydroxy-3-indolyl methyl glucosinolate differed from the effect on the other three indolyl compounds. The 4-hydroxy-3-indolyl methyl glucosinolate content of control swedes ranged ($P < 0.001$) from 0.23 to 1.65 mmol/kg and had a mean concentration of 0.68 mmol/kg. This was the only aromatic glucosinolate for which the control plants contained a higher ($P = 0.005$) concentration than *D. floralis*-damaged roots. The mean 4-hydroxy-3-indolyl methyl glucosinolate concentrations of plants inoculated with 5,10, and 20 eggs of the turnip root fly were 0.42, 0.41, and 0.34 mmol/kg, respectively. There was no ($P > 0.05$) difference between the 4-hydroxy-3-indolyl methyl glucosinolate concentrations of the swedes inoculated at the three levels.

Aliphatic Glucosinolates. Aliphatic glucosinolates comprised 50–71% of the total glucosinolate content of roots of control swedes. There was no difference ($P > 0.05$) in the aliphatic glucosinolate content of the different genotypes. However, *D. floralis* damage resulted in a ($P < 0.001$) decrease in the concentration of aliphatic glucosinolates in the roots both in real terms (Figure 1) and as a proportion of total glucosinolates (Figure 2). The proportion of aliphatic glucosinolates found was not further reduced by increasing herbivore pressure.

The individual aliphatic glucosinolates showed remarkable consistency in the changes found following plant responses to larval feeding damage. The dominant aliphatic glucosinolate was 2-hydroxy-3-butenyl glucosinolate (Figure 5), which did not vary ($P > 0.05$) between genotypes and represented over 60% of the aliphatic glucosinolates found in control roots. The control plants contained a mean concentration of 11.4 mmol/kg of 2-hydroxy-3-butenyl glucosinolate, while *D. floralis*-damaged roots contained significantly ($P < 0.001$) less of this compound. Plants inoculated with 5, 10, and 20 *D. floralis* eggs contained 7.4, 6.8, and 5.6 mmol/kg of 2-hydroxy-3-butenyl glucosinolate, respectively. However, there was no ($P > 0.05$) difference between the 2-hydroxy-3-butenyl glucosinolate concentrations of the swedes inoculated with different numbers of eggs.

The concentrations of the other aliphatic glucosinolates were all far lower than that of 2-hydroxy-3-butenyl glucosinolate. The pattern detected for 2-hydroxy-3-butenyl glucosinolate was reflected, however, in all the aliphatic glucosinolates that showed a response to damage. The mean concentrations of compounds found in control plants were 2.4 mmol/kg of 5-methylsulfinyl pentyl glucosinolate, 0.15 mmol/kg of prop-2-enyl glucosinolate, 1.37 mmol/kg of 4-methyl sulfinylbutyl glucosinolate, and 0.35 mmol/kg of but-3-enyl glucosi-

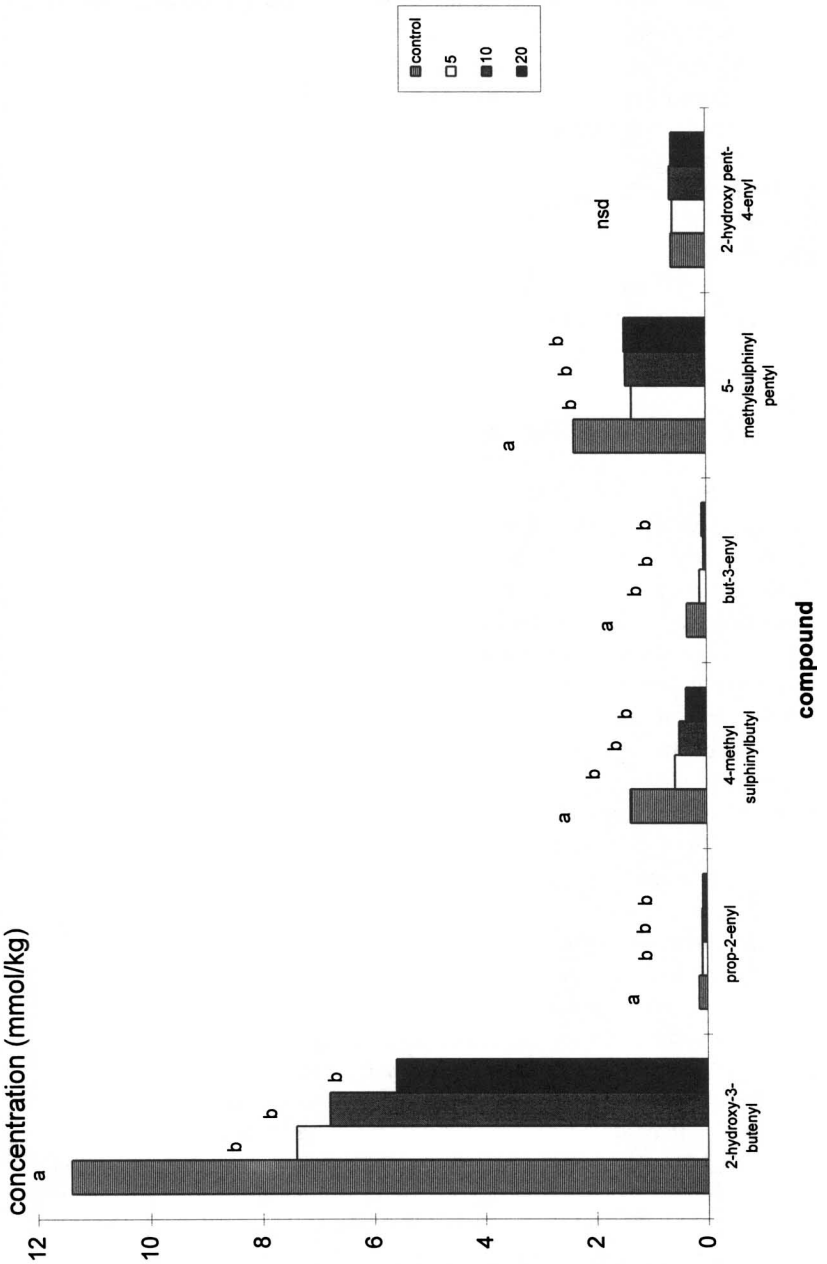

FIG. 5. Variation in the concentration of six aliphatic glucosinolates with increasing *D. floralis* egg numbers (columns with different letters within a compound group are statistically different, $P < 0.05$). Bars represent the mean of the eight genotypes tested, there was no interaction between genotype and herbivore pressure except in the concentration of 2-hydroxypent-4-enyl glucosinolate, which is shown in more detail in Figure 6.

nolate. For these four compounds, an initial decrease in concentration following root damage was followed by no further decrease with increasing herbivore pressure. 2-Hydroxypent-4-enyl glucosinolate showed no overall change ($P > 0.05$) in concentration with damage (Figure 5), but there was an interaction ($P = 0.019$) between treatment and genotype. The concentration of 2-hydroxypent-4-enyl glucosinolate was largely unaffected by *D. floralis* damage, but in Doon Major it rose and in the genotypes Marian and M4M2 a-c it fell (Figure 6). Three aliphatic compounds, 3-methylthiopropyl, pent-4-enyl, and 4-methyl thiobutyl glucosinolate, did not vary ($P > 0.05$) with genotype or damage. They were minor components comprising, in total, approximately 5% of the total glucosinolate content with average concentrations of 0.28, 0.10, and 1.41 mmol/kg.

DISCUSSION

Within this study, 3-indolyl methyl glucosinolate responded to increasing herbivore pressure with a series of incremental increases in concentration. In doing so, it was comparable with the response elicited by increasing physical damage, in the form of multiple pinholes in the leaf (Bodnaryk, 1992) and by chemical induction, in the form of topical application of jasmonic acid or methyl jasmonate (Bodnaryk, 1994; Doughty et al., 1995). For all three cited studies, damage to the aerial part of the plant led to progressively smaller incremental increases in the concentration of 3-indolyl methyl glucosinolate in the leaves. 1-Methoxy-3-indolyl methyl glucosinolate, which increased considerably with the application of jasmonates (Doughty et al., 1995) also underwent a large rise in concentration in this study (Figure 3). In this study, 2-hydroxy-3-butenyl glucosinolate exhibited a trend towards further reduction at higher herbivore pressures, although there was no difference between the three herbivore pressures applied. The constraints of *Delia floralis* as an organism prevented testing herbivore pressure over the logarithmic gradients that can be applied with physical damage or chemical induction studies. The responses to *D. floralis* larval feeding damage found were generally consistent between swede genotypes. Only two glucosinolates showed an interaction between herbivore pressure and plant genotype, and the differences were not generally pronounced.

Clossais-Besnard and Larher (1991) concluded from studies on oilseed rape (*Brassica napus* L.) that glucosinolates act as storage molecules for nitrogen, carbon, and sulfur and are accumulated at the growing points of plants when young seedlings photosynthesize and increase their biomass rapidly. The accumulation in the shoots of growing plants was especially marked for 2-hydroxy-3-butenyl glucosinolate (Clossais-Besnard and Larher, 1991). The rapid fall in the root concentration of 2-hydroxy-3-butenyl glucosinolate in this study may

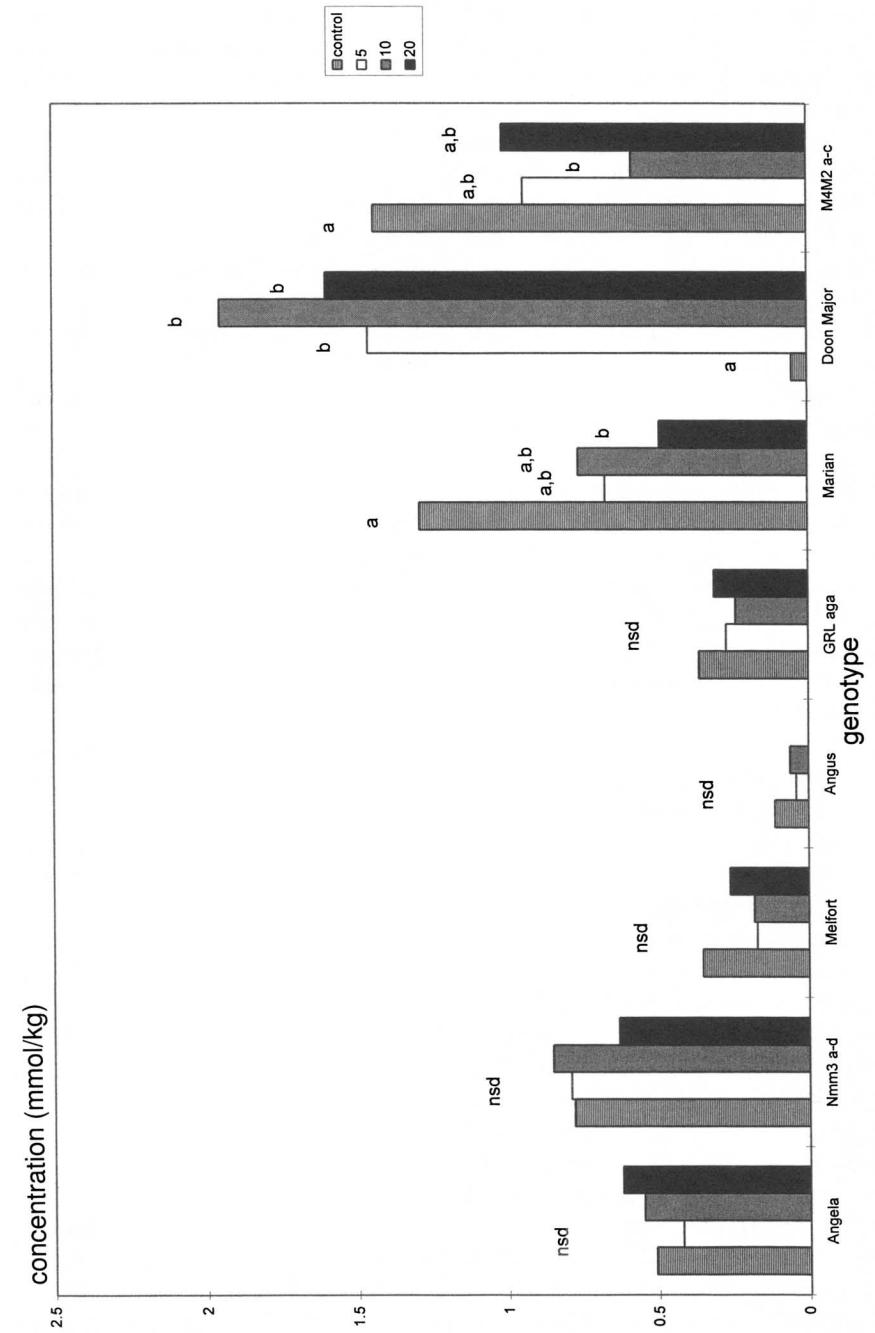

FIG. 6. Variation in the concentration of 2-hydroxypent-4-enyl glucosinolate between different genotypes of swede when damaged by different numbers of *D. floralis* larvae (columns with different letters within a genotype group are statistically different, $P < 0.05$).

be associated with a change in root function from growth to repair following damage. Swede roots that have been damaged by *D. floralis* also contain less sugars than undamaged roots (Hopkins et al., 1993, 1995), possibly indicative of a simultaneous suspension of their storage function.

In this study, the majority of root glucosinolates influenced by increasing severity of *D. floralis* attack responded in a nonincremental pattern. Induced increases were typified by a large initial change in concentration at 5 eggs/root, but by smaller (not statistically significant) variations in glucosinolate concentration over the range of 5–20 eggs inoculated per root. This was true for both 2-hydroxy-3-butenyl and 1-methoxy-3-indolyl methyl glucosinolates, which dominated the switch in the composition of damaged swede roots from aliphatic to indolyl glucosinolate compounds. 1-Methoxy-3-indole methyl and 2-hydroxy-3-butenyl glucosinolate represented means of nearly 60% of the indolyl and aliphatic glucosinolates, respectively. In consequence, both compounds dominated the response of the plants to root damage.

Interestingly, 3-indolyl methyl glucosinolate, which was induced in a stepwise response to increasing *D. floralis* larval damage, is a compound that strongly stimulates oviposition by adult *D. floralis* (Simmonds et al., 1994) and *D. radicum* (Roessingh et al., 1992; Braven et al., 1996). The impact of conspecific and physical damage to the host selection of *D. floralis* is currently under investigation. In the related species, *D. radicum*, conspecific larval damage to roots can initially make plants more attractive as an oviposition site, partially due to changes in leaf surface chemistry (Baur et al., 1996). A time-course study on several *Brassica* attacked by *D. floralis* larvae also demonstrated that root damage can systematically induce changes in specific leaf glucosinolates known to be behaviorally active as oviposition stimuli (Birch et al., 1996). While conspecific damage can make a plant more attractive at low to moderate levels of root damage, ovipositing *D. radicum* adults avoid plants with heterospecific damage to aerial parts. *D. radicum* adults also are deterred from oviposition by chemicals in the frass of garden pebble moth caterpillars, *Evergestis forficalis* L. (Jones and Finch, 1987; Jones et al., 1988) and by the presence of aphids on the plant (Finch and Jones, 1989). Changes in plant chemistry resulting from conspecific and heterospecific damage may significantly influence the probability of conspecific competition via altered oviposition preference.

Several specific glucosinolates appear to stimulate monophagous and oligophagous crucifer specialists while deterring many polyphagous insects. It is probable that changes in the glucosinolate composition of plants can alter the host status of a plant (Birch et al., 1992, 1996; Siemens and Mitchell-Olds, 1996). Indolyl glucosinolates act as oviposition or feeding stimulants to a wide range of *Brassica*-feeding insects (reviewed Städler, 1992), including the turnip root fly (Simmonds et al., 1994), cabbage root fly (Roessingh et al., 1992), diamondback moth (Reed, 1989), and cabbage butterfly (Traynier and Truscott,

1991). Feeding of the cabbage stem flea beetle, *Psyllioides chrysocephala* L., is influenced by the presence of glucosinolates, particularly 3-indolyl methyl glucosinolate, in the feeding substrate (Bartlet et al, 1994).

Variations in the concentrations of individual compounds following biotic or abiotic stress to a plant may provide signals to the insect concerning not only the status of the plant as a suitable host, but the current physiological condition of the plant. While concentration-related effects of single glucosinolates on insect behavior are becoming known, the consequences of interactions between plant compounds in complex mixtures remain largely unknown (e.g., Simmonds et al., 1994) and add to the complexity of glucosinolate–insect interactions. However, it is clear that glucosinolates are part of a dynamic interaction between phytophagous insects and their host plants, whatever their functional and evolutionary associations.

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TASK-RELATED DIFFERENCES IN THE CUTICULAR HYDROCARBON COMPOSITION OF HARVESTER ANTS, *Pogonomyrmex barbatus*

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Abstract—Colonies of the harvester ant, *Pogonomyrmex barbatus*, perform a variety of tasks. The behavior of an individual worker appears to depend on its recent history of brief contacts with ants of the same and other task groups. The purpose of this study was to determine whether task groups differ in cuticular hydrocarbon composition. We compared the cuticular hydrocarbon composition of ants collected under natural conditions as they performed one of three tasks: patrolling (locating food sources), foraging, or nest maintenance. Task groups differed significantly in the relative proportions of classes of hydrocarbon compounds, as well as in individual compounds. Relative to nest maintenance workers, foragers and patrollers had a higher proportion of straight-chain alkanes relative to monomethylalkanes, dimethylalkanes, and

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alkenes. There was no significant difference in the chain length of *n*-alkanes among the task groups. Foragers did not differ in hydrocarbon composition from patrollers. Colonies differed significantly from one another in hydrocarbon composition, but task groups differed in consistent ways from colony to colony, suggesting that the mechanism responsible for task-related hydrocarbon composition was the same in all colonies. *P. barbatus* workers switch tasks during their lifetimes, suggesting that cuticular hydrocarbon composition changes during adulthood as well. Nest maintenance workers are probably younger than foragers and patrollers and perform very little of their work outside of the nest. Task-related hydrocarbon differences detected here may be associated with worker age, and/or the abiotic characteristics (temperature, humidity, and ultraviolet light) of the interior and exterior work environments.

Key Words—Cuticular hydrocarbons; Formicidae; *Pogonomyrmex barbatus*; task allocation; *n*-alkanes.

INTRODUCTION

Ant colonies perform a variety of tasks outside the nest. In the harvester ant *Pogonomyrmex barbatus* (F. Smith), exterior tasks include foraging, traveling up to 20 m from the nest in search of seeds and bringing them back for storage inside the nest; patrolling, the early-morning search for the day's seed sources; and nest maintenance work, carrying excavated soil out of the nest and depositing it a few centimeters from the entrance (Gordon, 1986). Nest maintenance workers inside the nest repair and maintain the chambers, plaster the walls with moist soil that dries to a hard, adobe-like surface, and pile up dry, discarded soil near the nest entrance. Unlike foragers and patrollers, most of a nest maintenance worker's task is performed inside the nest, with only brief trips outside.

Colonies adjust the number of workers engaged in each task according to colony needs and changing conditions (Gordon, 1986, 1987). When more food is available, more ants forage; when the summer rains flood the nests, more ants do nest maintenance work. Changes in the number of ants performing a task can occur because ants move between active and inactive states; for example, at times when extra nest maintenance work is being done, the numbers foraging decrease because foragers tend to remain inactive inside the nest (Gordon, 1989). Changes in the number of ants performing a task can also occur because ants switch from one task to another. When abundant food appears, nest maintenance workers and patrollers will switch tasks to forage, and once they become foragers, they will not switch back (Gordon, 1989). When new nest maintenance workers are needed they are recruited from other, probably younger, workers inside the nest (Gordon, 1989).

Task allocation, the process that results in the appropriate numbers of ants performing each task, operates without any central control or hierarchical organization (reviewed by Gordon, 1996). Individuals must use simple cues to decide which task to perform and when to perform it actively. Recent work suggests

that interaction patterns influence task decisions in harvester ants; the task a harvester ant performs depends in part on its recent history of brief antennal contacts with ants of the same and other task groups (Gordon and Mehdiabadi, unpublished data). Can one ant discern the task of another through antennal contact?

In many insect species, the identity and relative abundance of lipids on the cuticle appear to be important in social signaling. Bioassays in which insects are presented with extracts of cuticular lipids have demonstrated that at least some social insects perceive and respond to differences in lipid composition (reviewed by Howard, 1993). For example, *Reticulitermes* termites reacted more aggressively to heterospecific cuticular extracts (containing only hydrocarbons) than to conspecific extracts (Bagnères et al., 1991). Similarly, workers of the ant *Camponotus vagus* responded more aggressively toward nestmates coated with cuticular extracts from another colony than toward nestmates coated with cuticular lipids from their own colony. Although such extracts may contain a variety of compounds, hydrocarbons are the most abundant component of the cuticular lipids of many insect species (Jackson and Blomquist, 1976). Several studies have demonstrated that cuticular hydrocarbon composition differs among species and among social insect colonies, suggesting that cuticular hydrocarbons might play an important role in species and nestmate recognition (Bonavita-Cougourdan et al., 1987; Nowbahari et al., 1990; Gamboa et al., 1996; Dahbi et al., 1996).

Cuticular hydrocarbon composition can vary among workers within a social insect colony as well. Studies of *Camponotus* ants (Bonavita-Courgourdan et al., 1993; Lavine et al., 1990) and of termites (Howard et al., 1982; Haverty et al., 1996) showed that workers in different task groups also differed in cuticular hydrocarbon composition. Task-related differences in the cuticular hydrocarbon profiles of *P. barbatus* workers might allow ants to distinguish, in the course of brief antennal contacts, the task of the ants they meet. Here we investigate whether *P. barbatus* workers in distinct task groups differ in cuticular hydrocarbon composition.

METHODS AND MATERIALS

Ant Collections. Ants were collected from three *P. barbatus* colonies in October 1996 near Rodeo, New Mexico, USA. Colonies were estimated to be at least five years old, based on the width of the pebble-covered mound surrounding the nest entrance. Ants were collected while they performed one of three tasks: foraging, patrolling, or nest maintenance work (Gordon, 1986). Foragers were collected as they returned to the nest carrying seeds. Patrollers were identified by their characteristic zig-zag path of movement with the abdomen tucked under the thorax. Nest maintenance workers were collected after they exited the nest carrying dirt. Ants were placed directly onto Dry Ice and

shipped overnight to Stanford University, where they were stored at −70°C until chemical analyses were performed.

Chemical Analysis. Cuticular components were extracted from each ant separately by immersing the ant in 1 ml of pentane (Sigma, St. Louis, Missouri) for 10 min. Suspensions were gently shaken during the first and last minute of the soak period. Ants were then removed from vials and the extracts were dried under a stream of N_2. The residue was redissolved in 50 μl of chloroform. Chloroform is less volatile than pentane, and we used it as the final solvent in order to reduce variability in sample concentration prior to analysis. Samples were analyzed by using combined gas chromatography–mass spectrometry (HP5990 series II/5971). Aliquots of 1 μl were introduced by spitless injection onto a capillary column (SPB-1 fused silica, 30 m, 0.25 mm ID, 0.25-μm film thickness; Supelco, Bellefonte, Pennsylvania); samples were purged after 1 min. Helium was the carrier gas, flowing at 1 ml/min. The injector temperature was kept at 300°C. The oven was kept at 100°C during injection, and then the temperature was increased rapidly from 100 to 220°C at 25°C/min, then more slowly from 220 to 310°C at 3°C/min (Provost et al., 1993). To confirm consistency of retention times among runs, samples of a standardized mixture of alkanes were interspersed among ant samples in each run.

To assist in identifying the compounds found on individual ants, a concentrated extraction was prepared by soaking a sample of 42 ants from five colonies in 3 ml of hexane for 1 hr at 50°C with occasional mixing. One milliliter of extract was dried under a stream of N_2 and the residue resuspended in 50 μl chloroform. Samples were analyzed with an HP 5890/5971A gas chromatograph–mass selective detector. Aliquots of 1–5 μl were introduced by 2-min splitless injection onto a DB-1 fused silica capillary column (30 m × 0.32 mm, 0.25-mm film thickness, J&W Scientific, Folsom, California), with helium as the carrier gas. The injector temperature was 350°C. The temperature was 170°C during injection, and was then increased to 260°C at 30°C/min, 260–295°C at 2.5°C/min, 295–350°C at 15°C/min, followed by a constant 350°C for 3 min. A standard mixture containing various straight-chain hydrocarbons (boiling point calibration sample #1, Hewlett Packard, Palo Alto, California) was also analyzed as above, with the exception that 1 μl of sample (~10 mg/ml in chloroform) was injected in split mode with a split ratio of 50:1.

Mass spectra were analyzed and compounds identified according to Nelson et al. (1980). Peaks that appeared in individual ants were matched to peaks in the more concentrated sample on the basis of relative elution time and mass spectra. Identification of compounds from the concentrated sample was used to confirm the identity of compounds found in individual ants.

Data Analysis. The relative abundance of each compound was estimated as the proportional peak area from total ion chromatograms. All compounds that

constituted at least 1% of the total hydrocarbon abundance on at least one ant were included in the analyses. We used linear discriminant analysis to determine whether ants of different task groups or different colonies had different relative abundances of cuticular hydrocarbons. Data were transformed by taking the arcsine of the square root of the proportion under each peak. We included only the five most abundant hydrocarbon compounds in the discriminant analysis of single compounds, for two reasons: (1) Large numbers of independent variables relative to the sample size can lead to significant discrimination where no actual grouping exists (Panel on Discriminant Analysis and Clustering, 1989). (2) Scarce compounds had poorer signal-to-noise ratios than abundant compounds and therefore were likely to bring little information to the discriminant analysis. We tested for differences in the relative abundance of classes of hydrocarbon compounds (alkanes, monomethylalkanes, dimethylalkanes and alkenes), as well as single compounds.

Linear discriminant analyses were performed by using SPSS Professional Statistics (Norusis/SPSS, Inc. 1994). Discriminant scores were calculated for each ant as the position of that data point along a new axis, the linear combination of variables that provided the best discrimination among groups. When there were only two groups in the analysis, discriminant scores of ants in different groups were compared with analysis of variance with k and $n_1 + n_2 - (k + 1)$ degrees of freedom, where k is the number of variables and n_i is the number of ants per group (Selvin, 1995). When discriminant analysis was applied to more than two groups, analysis of variance could no longer be used. In this case we applied an approximate test of the overall effectiveness of the discriminant function by using Wilks' lambda (λ), transformed to approximate a chi-square distribution as follows: $\chi^2 = -m \log(\lambda)$; where $m = [N - 0.5(k + g) - 1]$, where N is the number of ants, and g is the number of groups (Selvin, 1995). Discriminant scores were then used to predict group membership (Norusis/SPSS, Inc. 1994), by using a technique based on Bayes' rule (Hand, 1981; Norusis/SPPS, Inc., 1994).

We also compared the relative abundance of long- versus short-chain n-alkanes among the task groups. We calculated the proportion of the total n-alkane ion abundance contributed by n-alkanes greater than, or equal to, 28 carbons, the median chain length, and analyzed the data with one-way ANOVA.

RESULTS

Thirty-four cuticular hydrocarbons were detected on *P. barbatus* workers (Table 1). Hydrocarbon compounds were of four classes, n-alkanes, monomethylalkanes, dimethylalkanes, and alkenes. Several long-chain fatty acids (hexadecanoic acid, 9,12-octadecadienoic acid, and undecyesterdodecanoic acid)

TABLE 1. AVERAGE PERCENT COMPOSITION OF CUTICULAR HYDROCARBONS RECOVERED FROM *P. barbatus* ANTS FROM 3 COLONIES[a]

Component	TIC peak no.[b]	Colony B F (9)	Colony B P (10)	Colony B N (5)	Colony C F (10)	Colony C P (9)	Colony C N (10)	Colony E F (9)	Colony E P (10)	Colony E N (8)
Alkanes										
n-Tricosane	1	3.6	3.5	3.3	2.8	2.6	2.2	2.0	2.1	2.5
n-Tetracaosane	2	1.0	1.3	1.6	0.5	0.4	0.3	0.0	0.0	0.6
n-Pentacosane[c]	3	19.8	22.9	13.5	25.2	23.0	17.2	20.6	20.4	15.5
n-Hexacosane	6	2.0	2.3	2.0	2.2	1.8	2.1	2.1	2.1	2.3
n-Heptacosane[c]	8	5.6	7.6	4.4	7.8	6.4	5.9	8.0	9.5	6.6
n-Octacosane	13	0.0	0.1	0.6	0.7	0.3	0.4	0.5	0.4	0.2
n-Nonacosane[c]	15	4.2	4.5	3.8	4.5	4.6	5.1	4.5	4.9	4.0
n-Triacontane	20	1.1	0.9	0.7	0.5	0.6	0.7	0.5	0.6	1.0
n-Hentriacontane	23	4.0	3.5	3.0	3.9	4.1	4.4	4.5	4.8	4.4
n-Tritriacontane	29	0.7	0.3	0.3	0.7	0.3	0.7	0.4	0.4	0.3
Total *n*-alkanes		42.1	46.8	33.3	48.7	44.1	39.1	43.2	45.1	36.6
Monomethylalkanes										
13-Methylpentacosane	4	2.2	2.3	3.1	2.3	2.6	3.0	2.4	2.6	3.0
7-Methylpentacosane	5	0.8	0.2	0.7	0.3	0.0	0.1	0.0	0.0	0.0
13-Methylheptacosane[c]	9	5.2	5.5	5.4	6.3	6.3	7.3	6.6	6.8	7.4
7-Methylheptacosane[c]	10	4.1	4.1	3.6	4.0	4.1	4.4	4.2	4.4	4.7
5-Methylheptacosane	11	0.6	0.1	1.4	0.3	0.5	1.2	0.4	0.4	1.1
15-Methylnonacosane	16	7.6	6.8	7.7	6.4	7.4	8.0	7.4	7.5	8.1
9-Methylnonacosane	17	2.7	2.5	2.6	2.8	2.6	2.7	2.6	3.3	2.8
7-Methylnonacosane	18	3.9	3.8	3.8	2.9	3.1	2.9	2.8	3.0	2.8

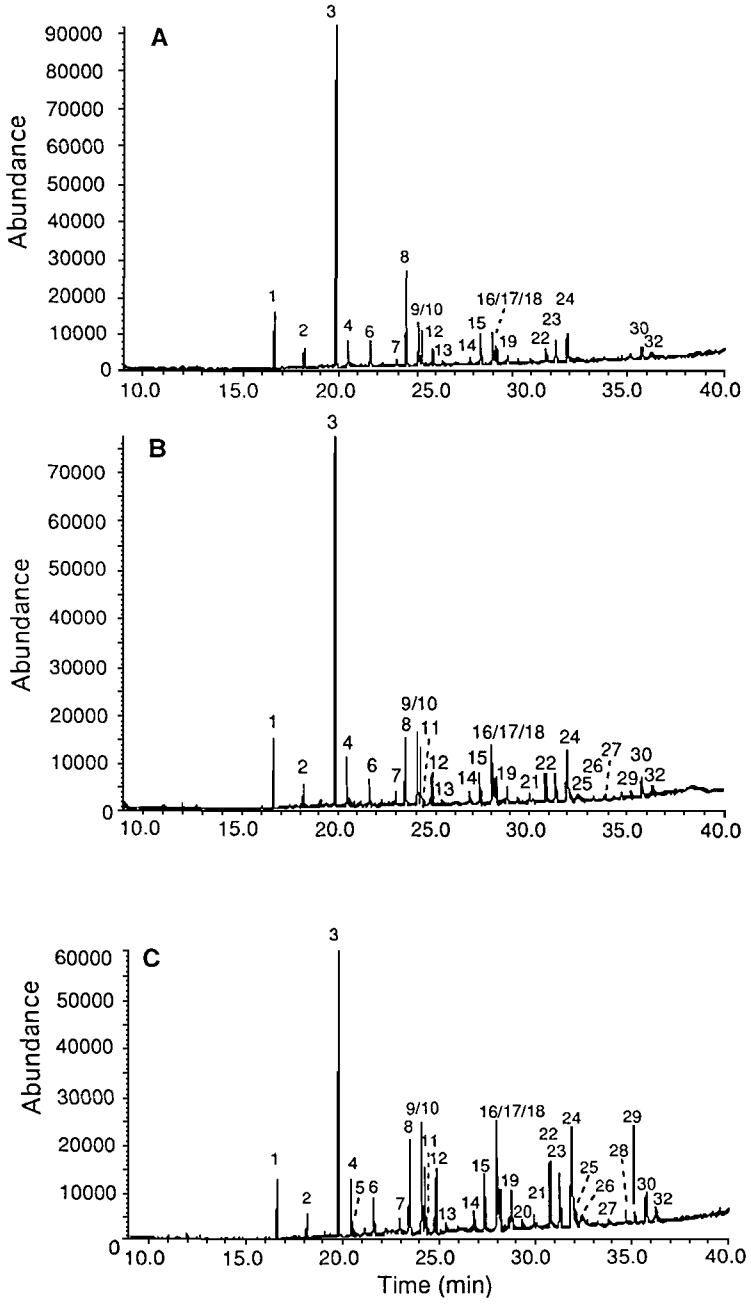
9-Methylhentriacontane	25	0.7	0.3	0.2	0.5	0.6	0.7	0.3	0.1	0.0
7-Methylhentriacontane	26	0.2	0.8	2.0	0.9	1.1	1.1	1.2	0.7	1.0
Total monomethylalkanes		27.4	26.3	29.1	26.6	27.8	0.1	27.5	28.5	29.9
Dimethylalkanes										
7,13-Dimethylheptacosane	12	3.3	3.0	4.1	3.2	3.2	4.1	2.5	3.4	4.2
7,13-Dimethylnonacosane	19	2.3	1.7	2.9	2.2	2.5	2.7	2.3	2.2	2.3
13,15-Dimethylnonacosane	21	1.1	1.3	1.0	0.3	0.9	0.6	0.8	0.6	1.3
x,y-Dimethyltriacontane[d]	24	8.1	6.9	7.4	7.4	8.3	7.8	9.0	8.4	8.1
13,15-Dimethylhentriacontane	27	0.5	0.5	0.3	0.1	0.0	0.7	0.4	0.0	0.2
x,y-Dimethyldotriacontane[d]	30	4.1	3.4	3.1	2.8	3.2	3.1	3.8	3.6	3.6
9,21-Dimethyltritriacontane	31	0.6	1.5	1.9	0.4	0.0	0.0	0.3	0.0	0.3
13,17-Dimethyltritriacontane	32	1.4	0.3	0.0	0.8	1.7	1.4	1.1	1.1	1.5
x,y-Dimethyltetratriacontane[d]	33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0
13,19-Dimethylpentatriacontane	34	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.6	0.0
Total dimethylalkanes		20.8	18.3	20.4	17.1	19.8	19.7	20.0	20.0	22.5
Alkenes[e]										
Heptacosene	7	1.1	1.3	1.6	1.0	1.3	1.2	1.2	0.7	1.6
Nonacosene	14	2.3	2.2	2.6	1.1	1.8	1.8	2	1.3	1.4
Hentriacontene	22	4.7	3.7	4.9	2.9	3.8	3.7	4.0	3.3	4.0
Tritriacontene	28	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Total alkenes		8.1	7.2	9.1	5.0	6.9	6.9	7.1	5.3	7.0

[a] Ants were collected while they were performing three tasks: foraging (F), patrolling (P), and nest maintenance (N). The number of ants analyzed per sample appears in parentheses.

[b] Total ion chromatograph (TIC) peak numbers correspond to those in Figure 1.

[c] One of the five most abundant compounds, used in statistical comparisons.

[d] x · y-methylation branch points undetermined.


A
B
C
Abundance
Time (min)
10.0
15.0
20.0
25.0
30.0
35.0
40.0

were also present in small quantities (< 1% on average). It is possible that other lipid components were present, but only in trace quantities. Figure 1 shows representative chromatograms for the three task groups.

Ants from different colonies and task groups expressed the same set of cuticular compounds (Table 1). However, there were differences in the relative abundance of those compounds. Colonies differed somewhat in the proportions of the four major classes of hydrocarbons on the cuticle ($\lambda = 0.79$, $\chi^2 = 17.4$, $df = 8$, $P = 0.03$); however, classification based on the discriminant analysis scores from these four classes of compounds was poor, misclassifying fully 48% of individuals overall (6/24 ants in colony 1, 18/29 in colony 2, and 14/27 in colony 3 were misclassified). Colonies differed more strongly in the relative proportions of the five most abundant cuticular hydrocarbon compounds ($\lambda = 0.33$, $\chi^2 = 83.0$, $df = 10$, $P < 0.001$). Prediction of colony membership on the basis of discriminant scores from the five single compounds led to an overall misclassification frequency of 21% (6/24 ants in colony 1, 4/29 in colony 2, and 7/27 in colony 3 were misclassified).

Despite the variation among colonies, the cuticular hydrocarbon profiles of ants in different task groups differed in a similar fashion both within and among colonies. For example, nest maintenance workers in all colonies tended to have relatively low levels of *n*-alkanes and relatively high levels of branched alkanes, in comparison to other task groups (Figure 2). All further analyses are based on individuals' deviations from their colony means, for each compound or class of compounds. This enables task group differences within colonies to be combined across colonies. We use the term "Standardization" to identify this part of the data analysis.

Initial inspection of the data using principal components analysis suggested that nest maintenance workers differed in hydrocarbon profile from foragers and patrollers, but foragers could not be distinguished from patrollers. On the basis of this observation, we compared task groups by using two discriminant analyses: nest maintenance workers versus foragers and patrollers, and foragers versus patrollers.

The relative abundance of *n*-alkanes, monomethylalkanes, dimethylalkanes, and alkenes on the cuticle differed significantly between nest maintenance workers and foragers/patrollers ($\lambda = 0.62$, $F_{4,75} = 11.25$, $P < 0.001$; Figure 3a). Predicted classification based on discriminant scores from this analysis misclassified 21% of workers (5/23 nest maintenance workers and 12/57 forager/

FIG. 1. Representative chromatograms of ants collected while they were performing one of three tasks: (A) foraging; (B) patrolling, or (C) nest maintenance. All three ants were from the same colony.

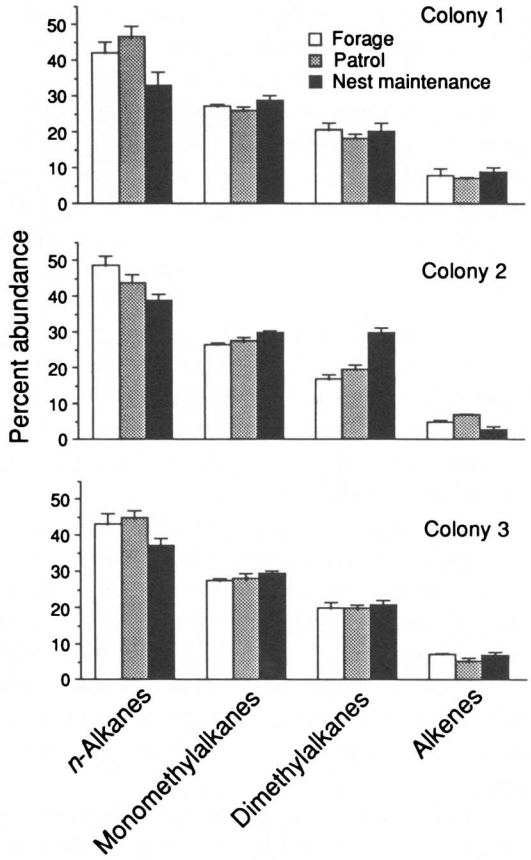


FIG. 2. Mean proportional abundance of major classes of cuticular hydrocarbons from *Pogonomyrmex barbatus* workers in three colonies. Sample sizes range from 5 to 10 per colony/task. Bars are standard errors.

patrollers). In general, workers performing nest maintenance expressed relatively lower concentrations of *n*-alkanes, and relatively higher concentrations of methyl-branched alkanes and alkanes, than foragers and patrollers (Figure 4). There was no significant difference among the three task groups in the proportion of long-chain *n*-alkanes (more than 28 carbons) to total *n*-alkane ion abundance ($F_{2,77} = 2.8$, $P = 0.08$; overall mean $= 0.25 \pm 0.1$ SE, $N = 80$).

Nest maintenance workers and foragers/patrollers differed in the relative abundance of the five most abundant compounds as well ($\lambda = 0.67$, $F_{5,74} =$

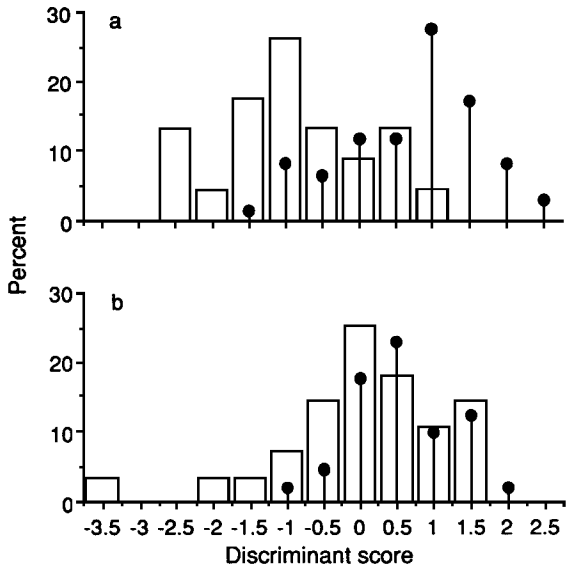


FIG. 3. Frequency distribution of linear discriminant analysis scores for ants of three task groups. Discriminant analysis yielded a new variable that was a linear function of the proportional abundance of the four major hydrocarbon classes (alkanes, monomethylalkanes, dimethylalkanes, and alkenes). The discriminant scores shown here are the position of each ant along this new axis. Similarity in discriminant scores reflects overall similarity in chemical composition. Graph (a) compares the scores of nest maintenance workers (bars) and the group composed of foragers and patrollers (spikes); graph (b) compares the scores of foragers (bars) and patrollers (spikes). See text for significance tests.

abundance of the five most abundant compounds as well ($\lambda = 0.67$, $F_{5,74} = 7.1$, $P < 0.001$). Not all compounds contributed equally to this result. The proportion of the most abundant compounds, *n*-pentacosane, *n*-heptacosane, and 13-methylheptacosane, varied considerably between nest maintenance workers and forager/patrollers, while 7-methylheptacosane and *n*-nonacosane varied little (Figure 5). Prediction of task group (nest maintenance versus forage or patrol) from discriminant scores based on the five most abundant compounds misclassified 25% of workers (4/23 nest maintenance workers and 16/57 foragers/patrollers).

Foragers and patrollers did not differ in the proportion of *n*-alkanes, monomethylalkanes, dimethylalkanes, and alkenes on the cuticle ($\lambda = 0.92$, $F_{4,52} = 1.1$, $P = 0.3$; Figures 3b and 4). Discriminant scores based on classes

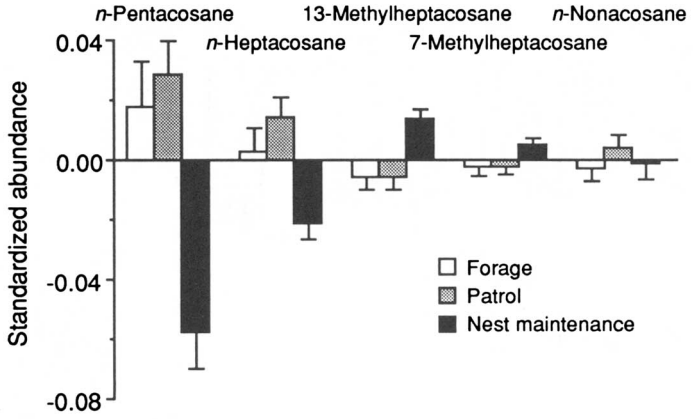


FIG. 4. Differences among task groups in the proportion of major cuticular hydrocarbon classes. Data were standardized for differences among colonies by subtracting the within-colony mean proportional abundance for each hydrocarbon class from the proportional abundance of that hydrocarbon class on each ant. A value of zero for *n*-alkanes, for example, would mean no difference between the proportional abundance of *n*-alkanes on an ant and the mean proportional abundance of *n*-alkanes for that ant's colony as a whole. Bars represent standard errors; $N = 80$ ants.

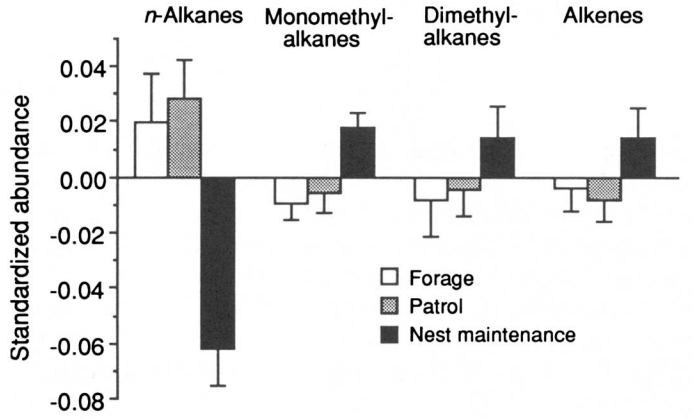


FIG. 5. Differences among task groups in the mean proportion of the five most abundant cuticular hydrocarbon compounds. Data were standardized among colonies (see Figure 4 legend for explanation of the standardization). Bars represent standard errors; $N = 80$ ants.

of compounds were poor (analysis misclassified 39% workers: 12/28 foragers and 10/29 patrollers). There was also no difference in the proportion of the five most abundant cuticular hydrocarbons on foragers and patrollers ($\lambda = 0.95$, $F_{5,51} = 0.5$, $P = 0.9$; Figure 5). Discriminant scores from this analysis misclassified 35% of workers overall, 11/28 of foragers and 9/29 of patrollers.

DISCUSSION

The results demonstrate that there are differences in cuticular hydrocarbon composition between some harvester ant task groups. Previous work showed that the extent to which one worker group actively performs a task affects the activity of other worker groups (Gordon, 1987). Brief antennal contacts with workers engaged in the same or different tasks may influence an ant's subsequent behavior. Recent laboratory work suggests such contacts do influence transitions in ant behavior from one task to another and between activity and inactivity (Gordon and Mehdiabadi, unpublished data). Task-related differences in cuticular hydrocarbon composition may permit one ant to determine the task of another during a brief antennal contact.

Contact among ants within a colony probably transfers some quantity of cuticular lipids from one worker to another, but contact among *P. barbatus* workers in different task groups was evidently not sufficient to homogenize their hydrocarbon profiles. Recent work by Soroker et al. (1995) demonstrated that hydrocarbons are regularly exchanged among workers of the ant *Cataglyphis niger*. Cuticular hydrocarbons were transferred to the postpharyngeal gland, probably through self-grooming, and then exchanged with other ants primarily through trophallaxis. Whether this exchange of hydrocarbon material was enough to eliminate differences among task groups in *C. niger* is not known. Observations of *P. barbatus* colonies dwelling in transparent nest boxes in the laboratory suggest that trophallaxis among adults is rare. Low rates of postpharyngeal hydrocarbon exchange may preserve task-related hydrocarbon differences in this species.

Despite colony differences in cuticular hydrocarbon composition, *P. barbatus* task groups tended to differ from one another in consistent ways from one colony to the next. In all three colonies surveyed, nest maintenance workers relative to foragers and patrollers were characterized by a lower ratio of straight-chain alkanes to branched and unsaturated hydrocarbons. Differences in the relative abundance of many single compounds of nest maintenance workers and foragers/patrollers also tended to be consistent among colonies. Similarity among colonies in task-related hydrocarbon patterns is noteworthy, because it suggests a common mechanism may operate to produce task group differences. Unlike foragers or patrollers, nest maintenance workers perform relatively little of their

work outside the nest. Differences in the hydrocarbon composition of exterior workers (foragers) and wholly interior workers (brood-tenders) were demonstrated for the ant species *Camponotus vagus* (Bonvita-Courgourdan et al., 1993). Taken together, these results suggest that changes in the chemical composition of the cuticle may be associated with the transition between interior and exterior work.

How do task-related differences in cuticular hydrocarbon composition arise? We consider two, not mutually exclusive, hypotheses to be likely. First, both the transition to exterior work and changes in the composition of the cuticle may correlate with age (as suggested by Bonavita-Cougourdan et al., 1989). In many ant species, including several species of *Pogonomyrmex*, younger ants work inside the nest, whereas older ants perform exterior tasks (MacKay, 1983; Wilson, 1971; Hölldobler and Wilson, 1990). In response to experimental perturbation, workers of *P. barbatus* switch between external tasks in a predictable manner, from nest maintenance to foraging or patrolling (Gordon, 1989). If the distribution of this short-term task switching mirrors the way an ant moves from one task to another over the months of its lifetime, then nest maintenance workers should be younger on average than foragers and patrollers. If age is a cause of differences in cuticular hydrocarbon composition among task groups, then our data suggest that older ants produce more *n*-alkanes relative to methyl-branched alkanes and alkenes than younger ants. Similar increases in the proportion of cuticular *n*-alkanes with increasing adult insect age have been reported for the hemipteran *Triatoma infestans* (Juarez and Brenner, 1985) and the moth *Trichoplusia ni* (de Renobales and Blomquist, 1983).

Second, environmental conditions experienced by ants in different task groups may induce changes in the cuticle. Workers that perform their tasks outside the nest are likely to experience markedly different abiotic conditions from those inside the nest, including higher temperature, lower humidity, and exposure to ultraviolet light. Cuticular lipids are the primary passive barrier to water loss, and the composition, as well as the quantity, of hydrocarbons appears to play a role in the desiccation resistance (Hadley, 1994). Waterproofing is thought to result from weak molecular interactions among long-chain, nonpolar molecules (Lockey, 1976; Toolson et al., 1979; Toolson, 1982). Compounds of long chain length are generally thought to enhance desiccation resistance (reviewed by Gibbs, 1998), although this is not always the case (Gibbs et al, 1998). Both within and between species, arthropods dwelling in warm, dry environments tend to have hydrocarbons of longer chain length than their counterparts in more mesic environments (Hadley, 1977, 1978; Hadley and Schultz, 1987). Methyl-branching and unsaturation probably also affect cuticle permeability. Increases in the relative abundance of methyl-branched alkanes and alkenes in simple mixtures with *n*-alkanes result in a decrease in the melting

temperature, suggesting that both disrupt lipid packing (Gibbs, 1995; Gibbs and Pomonis, 1995) and thus may reduce waterproofing. In *Melanoplus sanguinipes*, acclimation to warm temperatures results in a greater relative abundance of straight-chain relative to branched-chain alkanes (Gibbs and Mousseau, 1994). The proportional abundance of cuticular *n*-alkanes on *P. barbatus* foragers and patrollers was about 20% higher than on nest maintenance workers. This greater relative abundance of *n*-alkanes may enhance the desiccation resistance of foragers and patrollers and is consistent with their greater exposure to the desert environment. Foragers and patrollers in this study did not, however, have alkanes of longer average chain-length than nest maintenance workers. Further work will be required to determine whether abiotic conditions are responsible for a shift toward saturated alkanes in *P. barbatus* foragers and patrollers.

Differences among task groups in cuticular hydrocarbon composition may assist in our understanding of how work is organized in social insect colonies. If *P. barbatus* can perceive cuticular hydrocarbons, differences in the composition of the cuticle among task groups may provide workers with information about the task of ants they encounter. Future work should focus on whether ants perceive and respond to task-related differences in cuticular hydrocarbon composition.

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EFFECTS OF ALLELOCHEMICAL STRESS PRODUCED BY *Sicyos deppei* ON SEEDLING ROOT ULTRASTRUCTURE OF *Phaseolus vulgaris* AND *Cucurbita ficifolia*

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Abstract—The allelopathic potential of the cucurbit *Sicyos deppei* has been reported. Aqueous leachates (1% w/v) of *S. deppei* significantly inhibited the radicle growth of several test plants. Root tips of treated plants were thicker, with a brownish color as compared to control roots. Light microscope observations of treated root tips of *Phaseolus vulgaris* showed that cells around the quiescent center appeared compressed. Tissues were disorganized and lacked evident cell differentiation. *Cucurbita ficifolia* did not show these differences in root morphology. Scanning and electron microscope observations showed that treated roots of both *P. vulgaris* and *C. ficifolia* had ultrastructural alterations of certain organelles, the plasma membrane, and cell walls. Root cap cells of treated roots showed amorphous and inactivated (nondividing) nuclei, mitochondria, and endoplasmic reticulum. Vacuoles in treated cells showed invaginations that may correspond to engulfing of damaged cellular components.

Key Words—Allelopathy, allelochemicals, *Sicyos deppei*, *Phaseolus vulgaris*, *Cucurbita ficifolia*, cell ultrastructure, root morphology.

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INTRODUCTION

Allelopathic effects are one of many stresses with which plants must cope in their environment. Allelochemical interactions between plants have been implicated in the patterning of vegetation and weed growth in agricultural systems (Aldrich, 1987). In recent years the study of allelopathy has received considerable attention, as allelochemicals released to the environment may decrease crop yield (Chou, 1993) or they may offer potential as natural herbicides.

During the last decade, our laboratory has conducted several studies on different aspects of allelopathy in both natural and agronomic ecosystems (Anaya et al., 1987, 1988, 1990, 1992). *Sicyos deppei* G. Don (Cucurbitaceae), locally known as "atatana" or "chayotillo," is an endemic weed that grows in the temperate central part of Mexico. It grows extensively and aggressively climbs over other plants. This rapid growth allows *S. deppei* to cover the soil quickly and to eliminate other weeds almost completely. It is also considered a harmful weed in field crops, decreasing productivity. Previously, an aqueous leachate (1% w/v) of *S. deppei* was tested on seed germination and seedling growth of eight plants: corn (*Zea mays* L); bean (*Phaseolus vulgaris* L); squash (*Cucurbita pepo* L); bottle gourd (*Cucurbita ficifolia* L); lettuce (*Lactuca sativa* L.); tomato (*Lycopersicum esculentum* L); amaranth (*Amaranthus hypochondriacus* L); and barnyardgrass (*Echinochloa crussgalli* L). The leachate significantly inhibited the root growth of all test species (Hernández-Bautista et al., 1996). Currently, a biodirected fractionation study is examining the most active organic extracts to isolate the active compounds.

Allelochemicals may affect many physiological processes, with visible effects being secondary signs of primary ones occurring at the molecular level (Einhellig, 1989). Inhibition typically results from the combined action of multiple allelochemicals (Einhellig, 1996). Several studies provide evidence that allelochemicals have a primary effect on root growth and root tip ultrastructure (Lovett, 1982; Kutchan et al., 1986; Levitt et al., 1984; Lovett and Potts, 1987; Aliotta et al., 1994; Gasparikova et al., 1996). In order to understand better the physiological effects or modes of action of allelochemicals, one must have a greater knowledge of these processes, especially at the ultrastructural level. The purpose of the present study was to analyze the effects of an aqueous leachate of *Sicyos deppei* on the primary root tip ultrastructure of *Phaseolus vulgaris* and *Cucurbita ficifolia*.

METHODS AND MATERIALS

Seed Material. Seeds of bottle gourd (*C. ficifolia*) and beans (*P. vulgaris*, var. Flor de Mayo) were obtained from a local market in Tulyehualco, D.F.,

Mexico. *S. deppei* was collected from a crop field at Xochimilco, Mexico D.F. in July 1996.

Bioassays. Seeds were germinated in an aqueous leachate (1% w/v) of dried leaves of *S. deppei*, in Petri dishes with agar (1%) as substrate. Leachate was prepared by soaking dried leaves in distilled water for 3 hr. The leachate was filtered through a Millipore membrane (0.45 μm), and its osmotic pressure was measured with a freezing-point osmometer (Osmmete A, Precision Systems, Inc.). Osmotic pressure was 15–17 mosm/liter. Controls contained only distilled water. Ten seeds were placed on each Petri dish and kept in the dark at 27°C (four replicates of each treatment were made). After 48 hr, radicle lengths were measured and data analyzed by ANOVA. Root tips (3 mm) were harvested and fixed for microscopy.

Light and Electron Microscopy. Three-millimeter root tip segments were fixed for 3 hr in 3% glutaraldehyde in 10 mM sodium phosphate buffer, pH 7.2, and 60 mM sucrose. They were postfixed for 3 hr in 2% osmium tetroxide in sodium phosphate buffer (pH 7.2). Roots were embedded in fresh epon resin (Epon 812, Spi-Chem) and polymerized for 48 hr at 60°C. Sections (3–4 μm) were made on a Reichert-Jung Ultracut microtome and subsequently stained with fresh 0.05% (w/v) toluidine blue for light microscopy. For transmission electron microscopy (TEM), 600–800 Å sections were made and stained with 2% (w/v) uranyl acetate and 2% (w/v) lead citrate. After fixing, specimens were examined on a Jeol 1200 EXII transmission electron microscope. Specimens for scanning electron microscopy (SEM) were coated with gold and examined on a Jeol JSM-5410LV microscope. For the TEM, three regions of the root tips of the two plant species were used: (1) cap tissue (statocyts present); (2) the peripheral region where the secretory cell layers are located; and (3) the initial cell complex of the root cap, which is a portion of the quiescent center of the root tip.

RESULTS

The effect of the aqueous leachate on *P. vulgaris* and *C. ficifolia* is shown in Figure 1. Radicle growth of both species was significantly inhibited 80% by the leachate. Radicles of treated seedlings became brownish, thick, and brittle. Treated radicles lacked mucilage secretions compared with mucilage droplets observed on control roots. Previous studies have shown that the primary target of many allelochemicals is the root, especially during its development (Lorber and Muller, 1976; Levitt et al., 1984; Anaya and Pelayo-Benavides, 1997). Radicle growth is characterized by high metabolic rates, and at this time roots are highly susceptible to environmental stresses, such as phytotoxic compounds present in the soil. Other studies have shown that cells of the root cap suffer

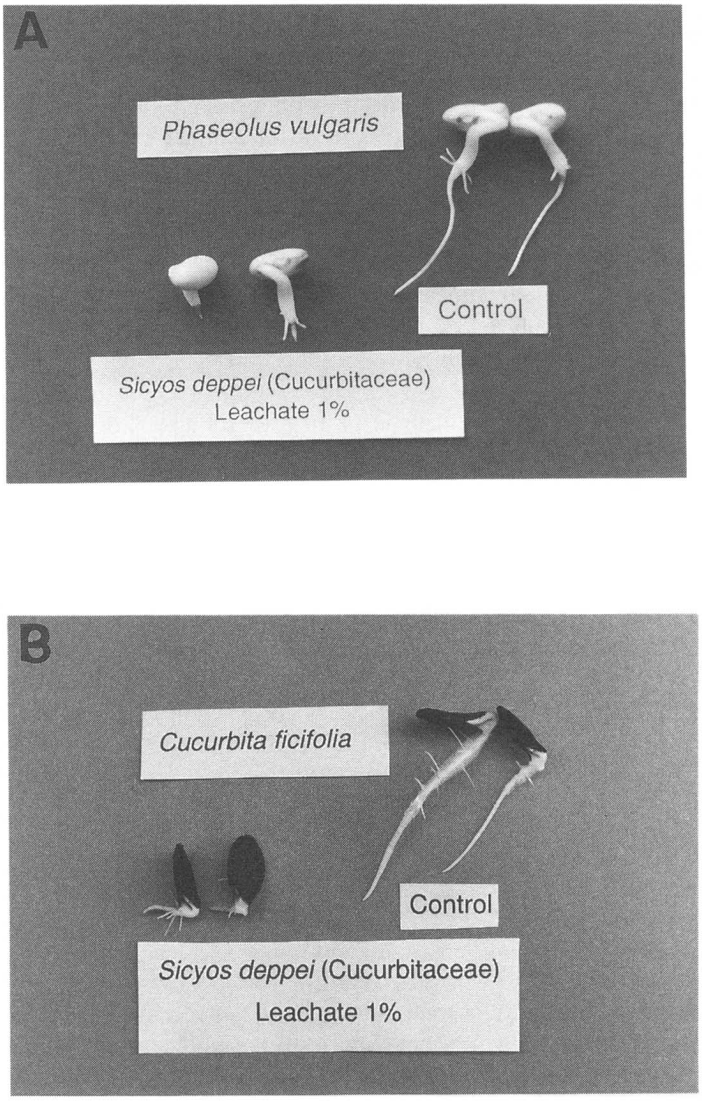

FIG. 1. Effects of an aqueous leachate (1% w/v) of *S. deppei* dried leaves on radicle growth of *P. vulgaris* (A) and *C. ficifolia* (B).

damage at the plasma membrane level with some cell organelles also disrupted (Lovett, 1982; Lovett and Potts, 1987; Anaya and Pelayo-Benavides, 1997).

Light Microscopy. Light photomicrographs of control bean roots (Figure 2A and B) show a normal pattern of growth from the root cap to the columella. Treated bean roots (Figure 2C and D) show a well-defined zone (between parentheses in Figure 2C) where cells appear compressed. This zone corresponds to the quiescent center and to the apical meristem whose cells are well defined in control roots, but reduced and compact in treated roots. Root cap cells in the treated bean seedlings look smaller and show similar numbers of statoliths to the controls, but they are in different positions (see arrows in Figure 2B and D). Moreover, the cells of the columella in treated roots showed a larger number of smaller vacuoles compared with control cells.

Control root tips of bottle gourd (Figure 3A and B) show a well-defined differentiation pattern of tissues that is not observed in treated roots (Figure 3C and D). The meristematic zone in treated bottle gourd roots does not look as compressed as in treated bean roots. Columella cells of treated bottle gourd roots show a large number of vacuoles, as observed with bean root cells (see arrows in Figure 3D). In this area, the cells are apparently still dividing, but there is no elongation growth.

Transmission Electron Microscopy. The electron microscope observations were divided into three sections: (1) cells of the root cap, (2) meristematic zone and quiescent center, and (3) peripheral cells that surround the tip. Treated bean root cap cells (Figure 4B and C) show irregular nuclei with unorganized chromatin. Nucleoli are inconspicuous and smaller and more dense than in control cells, where they are large, indicative of actively dividing growing cells. Control cell nucleoli also exhibit well-defined granular, fibrillar, and vacuolar zones. Vacuoles with storage material (protein and lipid bodies) are larger and more evident in treated root cells (Figure 4b). Arrows in Figure 4C indicate invaginations in the vacuoles, similar to those described by Lovett and Ryuntyu (1992) in sunflower root tip cells exposed to thornapple allelochemicals. These authors state that the invaginations are the remains of cellular components that have been damaged during allelopathic stress. Figure 5 shows meristematic cells of control (Figure 5A) and treated bean roots (Figure 5B). Meristematic cells of treated roots contain proplastids (P) with a large number of starch grains (S), an irregular nucleus with a less apparent nucleolus, as in the case of the root cap cells, and swollen mitochondria (M) (see arrows in Figure 5B). Treated cells appear to be dividing but are smaller than controls. Vacuoles in treated cells are abundant, irregular, and show invaginations, and also are smaller than control ones. Primary cell walls are marked with asterisks in Figure 5B.

Bean periphery cells are shown in Figure 6. Treated roots (Figure 6B) show cells where the plasma membrane (PM) is separated from the cell wall as in

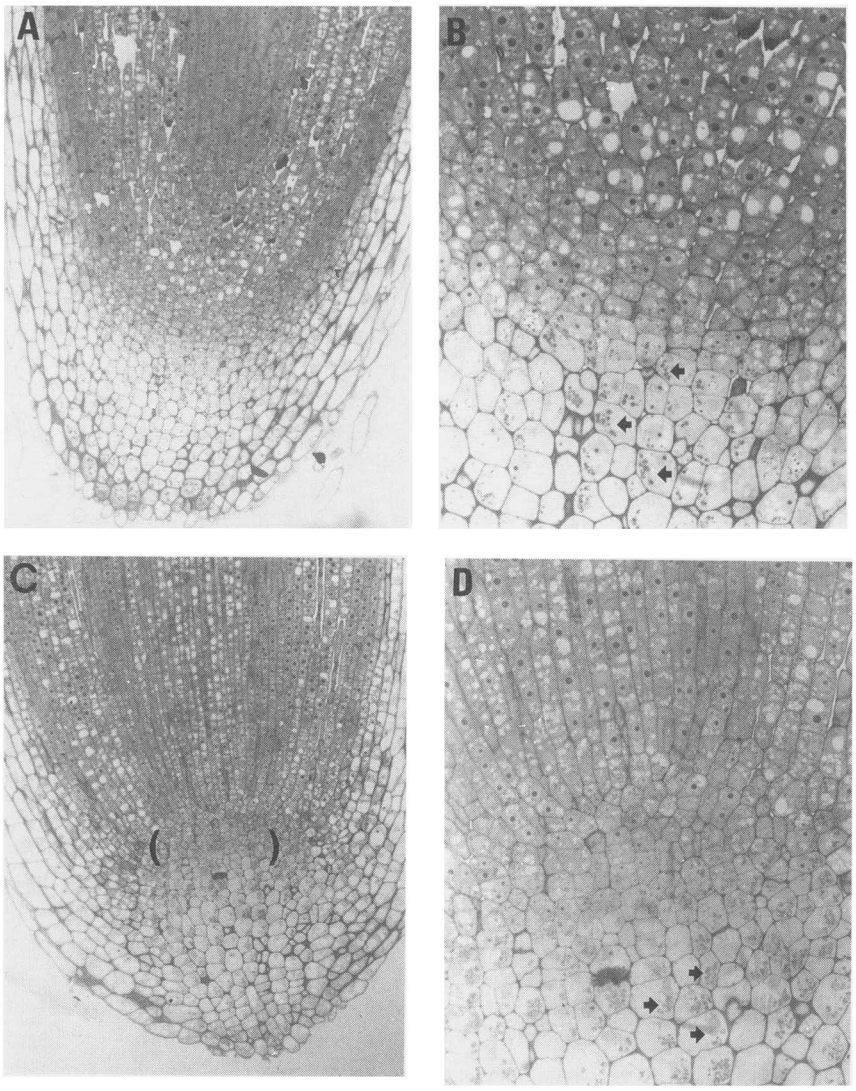

FIG. 2. Median longitudinal sections of *P. vulgaris*. Root tips after two days of germination: Control (A and B) and *Sicyos*-treated (C and D). Compressed cells between parentheses. Arrows point to statoliths.

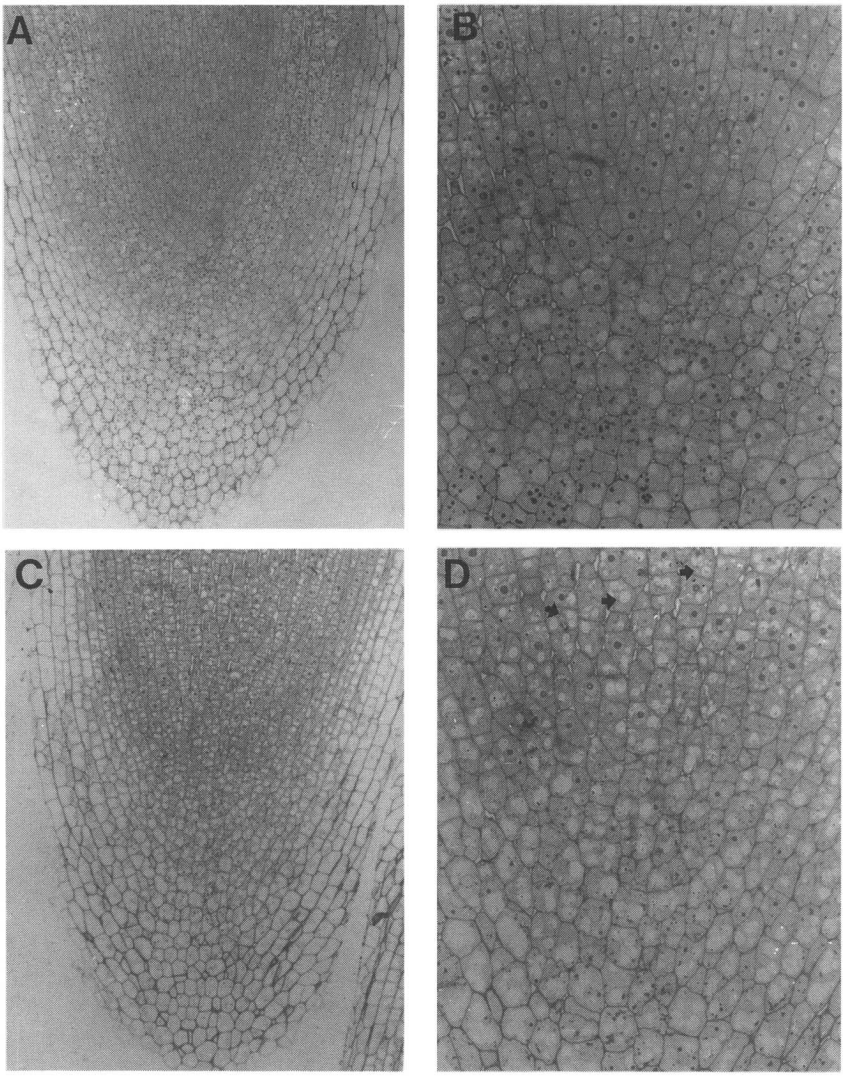

FIG. 3. Median longitudinal sections of *C. ficifolia*. Root tips after two days of germination: control (A and B) and *Sicyos*-treated (C and D). Arrows point to vacuoles.

plasmolized cells. Vacuoles (V) in treated cells are irregular and look fragmented; some have invaginations containing cellular components. In control cells (Figure 6A), the vacuoles show material probably for mucilage secretion, which is not apparent in treated root cells. Cell walls in treated root cells are

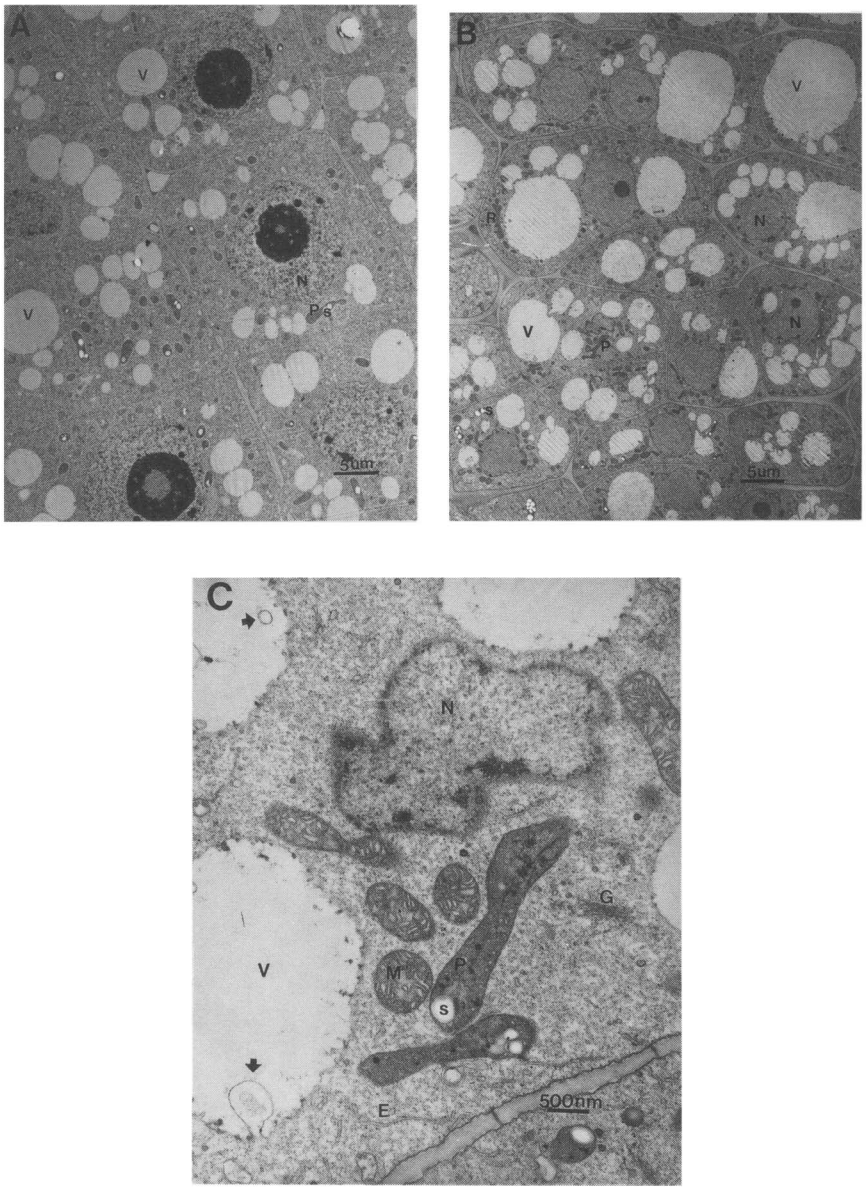

FIG. 4. Control (A) ×1500 and *Sicyos*-treated (B) ×1500 and (C) ×12K root bean cap cells. Nuclei (N) of treated cap cells are irregular and chromatin appear unorganized. Arrows indicate apparent engulfing of damaged cellular components inside of vacuoles (V) in treated root cells.

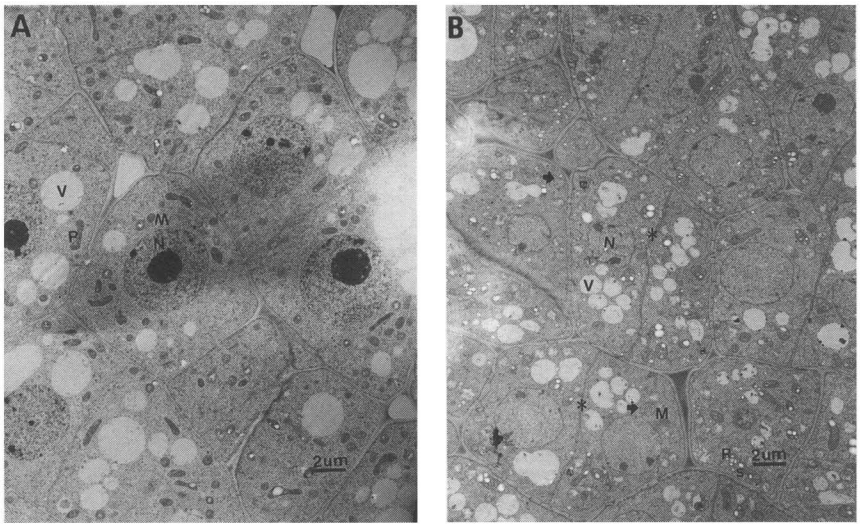

FIG. 5. Control (A) and *Sicyos*-treated (B) meristematic bean cells, ×2500. Asterisks indicate dividing cells. P = proplastid; N = nucleus; V = vacuole; M = mitochondrion.

also irregular with narrowing zones (see arrows in Figure 6B). Spherical bodies can be observed in treated roots, sometimes associated with plastids lacking starch granules, like proplastids (asterisks in Figure 6B).

Treated root cap cells of bottle gourd (Figure 7B) exhibit small and irregular nuclei, with large vacuoles containing irregular and fragmented vesicles and granules not found in cells of control root cap cells (Figure 7A). Plastids of bottle gourd root cap cells show immature development (Figure 7A) as in treated bean periphery cells (Figure 6B). The plasma membrane is separated from the cell wall (CW) in treated cells (see arrows in Figure 7B). Figure 8A shows control and Figure 8B treated bottle gourd meristematic root. Treated cells show amorphous and irregular nuclei, as well as larger storage vacuoles. Controls show dividing cells with well-defined nuclei and nucleoli.

Bottle gourd periphery cells are shown in Figure 9. There are no dramatic changes in treated roots compared with those of the controls (Figure 9A and B). However, in treated cells, there are more but smaller storage vacuoles than in the controls (arrows in Figure 9B).

Scanning Electron Microscopy. Observations of bean roots by SEM were divided into root tips and epidermal cells. Figure 10A and B shows root tip and epidermal cells from control roots. Figure 10C and D shows treated bean root tip and epidermal cells. Control tip cells look well defined with conspicuous

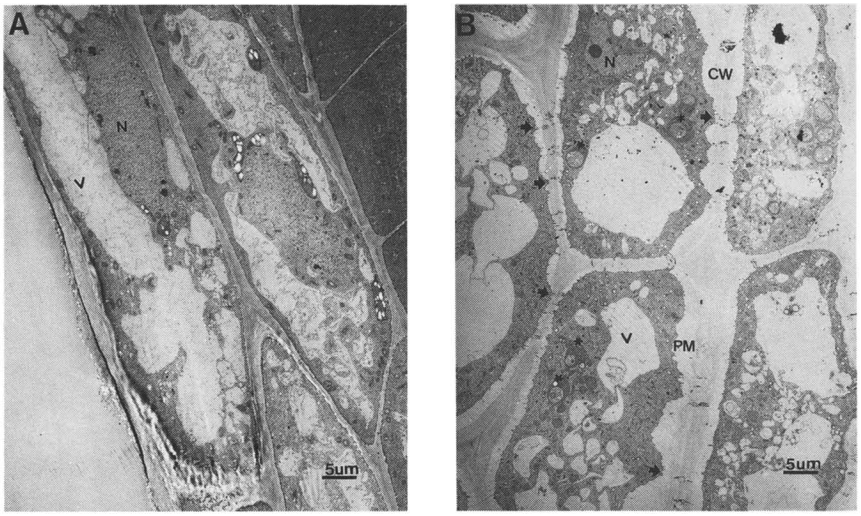

FIG. 6. Control (A) and *Sicyos*-treated (B) peripheral or epidermal bean cells, ×1500. Arrows indicate where plasma membrane (PM) is retracted from cell wall (CW). V = vacuole.

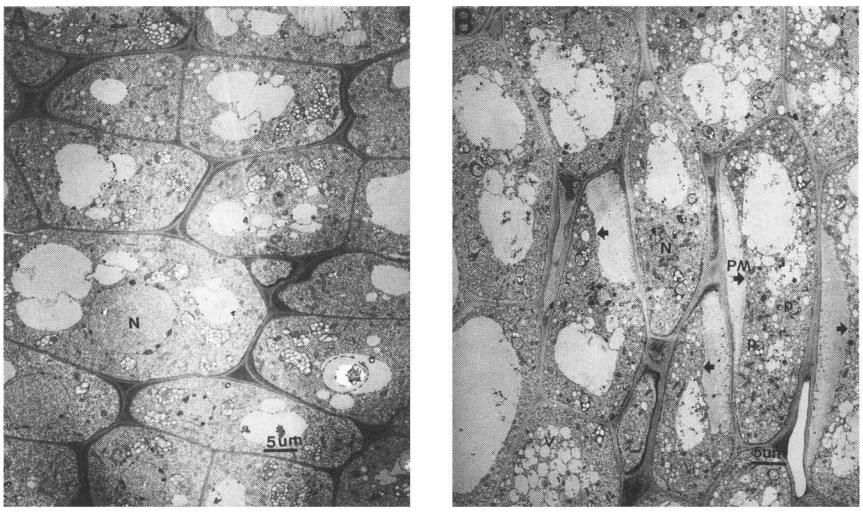

FIG. 7. Control root-cap cell (A) and *Sicyos*-treated cap bottle gourd cells (B), ×1500. Arrows indicate where plasma membrane (PM) is retracted from cell wall (CW). P = plastid.

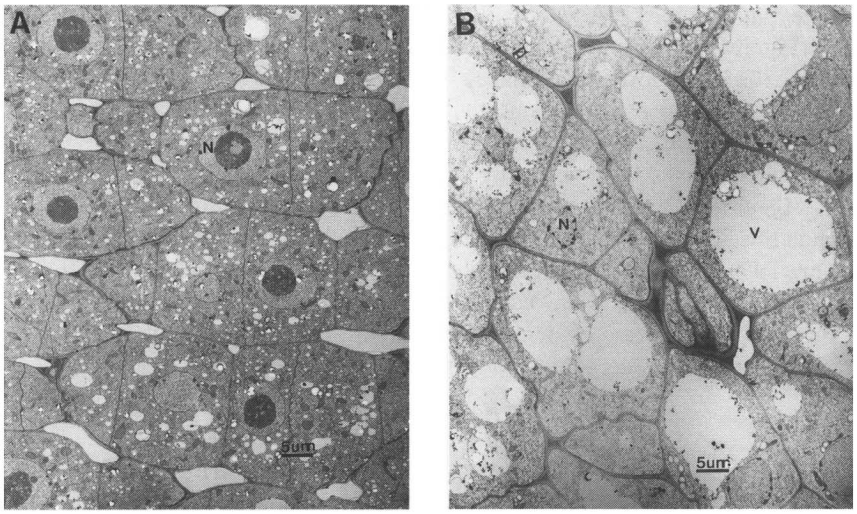

FIG. 8. Control (A) and *Sicyos*-treated (B) meristematic bottle gourd cells, ×1500. Note irregular nuclei (N) and large vacuoles (V).

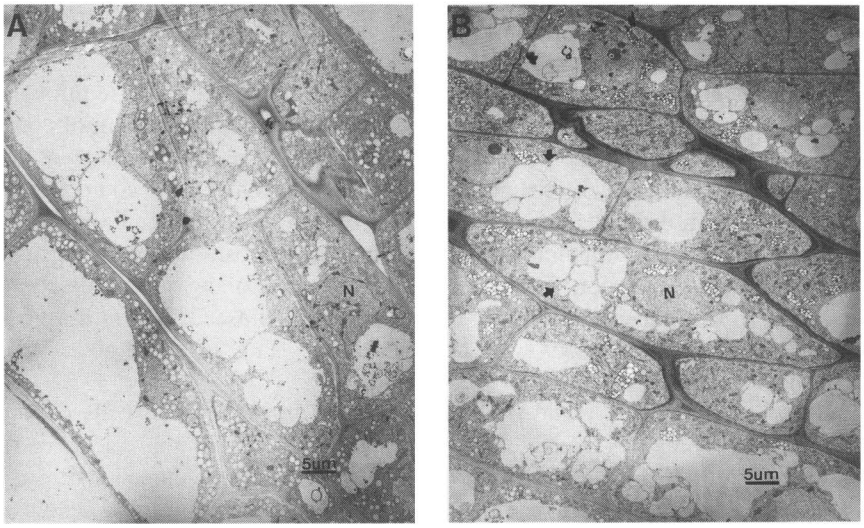

FIG. 9. Control (A) and *Sicyos*-treated (B) peripheral bottle gourd cells, ×1500. Arrows indicate smaller vacuoles in treated cells.

crevices and cavities (arrows in Figure 10A) compared with those of treated cells (Figure 10C). Control epidermal cells are normally elongated; however, treated cells have an irregular shape, with a greater number of sloughing cells, compared with those of the controls. This sloughing of cells leaves exposed inner layers in treated roots (asterisks in Figure 10D).

Figure 11A and B shows the tip and epidermal cells from *C. ficifolia* control roots. Figure 11C and D shows tip and epidermal cells from treated roots. Treated tips appear more spherical and wider than control tips. As in bean roots (Figure 10B), control bottle gourd root epidermal cells are elongated. In *Sicyos*-treated roots, more cells sloughed off, leaving inner cell layers exposed (asterisks in Figure 11D). Treated epidermal tissues look disintegrated as in beans. Cell borders in treated bottle gourd roots are also undefined.

DISCUSSION

Levitt et al. (1984) described the effects of two concentrations of alkaloids from jimson weed (*Datura stramonium* L.) and from sunflower (*Helianthus annuus* L.) seedlings at the subcellular level. Electron microscopy showed no obvious structural damage in cells from the alkaloid-treated seedlings. Treated cells showed distinct nuclei, other organelles, and intact membranes. However, they contained aggregations of electron-dense structures bounded by double membranes, identified as amyloplasts. Treated cells also contained greater quantities of lipid than control cells, and vacuolation was more pronounced. Lorber and Muller (1976) found damage to various organelles in cucumber (*Cucumis sativus* L.) root tip cells treated with volatile inhibitors (mostly terpenes) from *Salvia leucophylla*. They suggested that these lipid globules may arise through abnormal metabolism within terpene-treated seedlings, either as a result of poor utilization of nutrients or as a result of the blocking of a metabolic pathway by the allelochemicals. They also suggested that these lipids may be decomposition by-products from organelles and membranes. The presence of membrane fragments indicated that structural breakdown and decomposition was occurring within terpene-treated root tips. Lovett (1982) observed increased vacuolation and other apparent disruptions on the root tip cells of linseed (*Linum usitatissimum* L.). Similar alterations were observed in our study of bottle gourd and bean roots.

Vigil et al. (1989) found that as a result of drought stress, cells of the apical and basal regions of radicles of cotton embryos were highly vacuolated, particularly in the basal region. The vacuolar compartment occupied a considerable area and often consisted of small rather than (typically) large vacuoles. Cortical parenchymal cells appeared dramatically different from controls, and there was an absence of normal organelle development, especially protein bod-

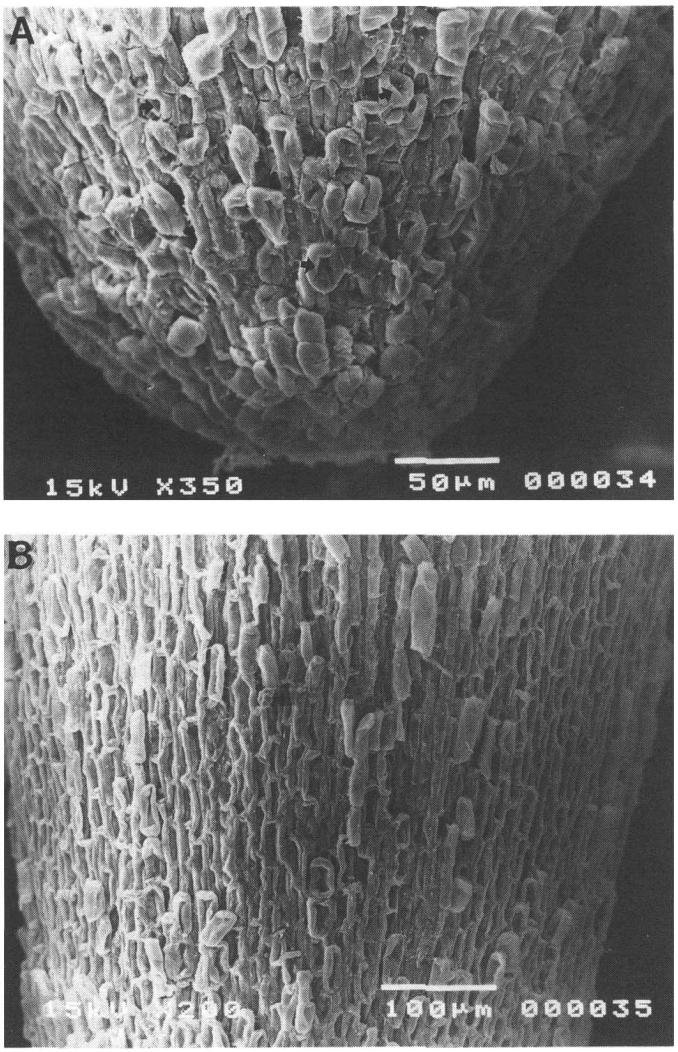

FIG. 10. Control (A) and *Sicyos*-treated bean root tips (C), ×350. Cells from the epidermis of control (B) and treated bean roots (D), ×200. Note sloughing cells in treated samples.

ies. These changes were similar to those observed in both bean and bottle gourd root cap cells treated with *S. deppei*.

The toxin amilovorin from the bacteria, *Erwinia amylovora*, caused plasmolysis of xylem parenchyma apple stem cells (Hanchey, 1981). An increase

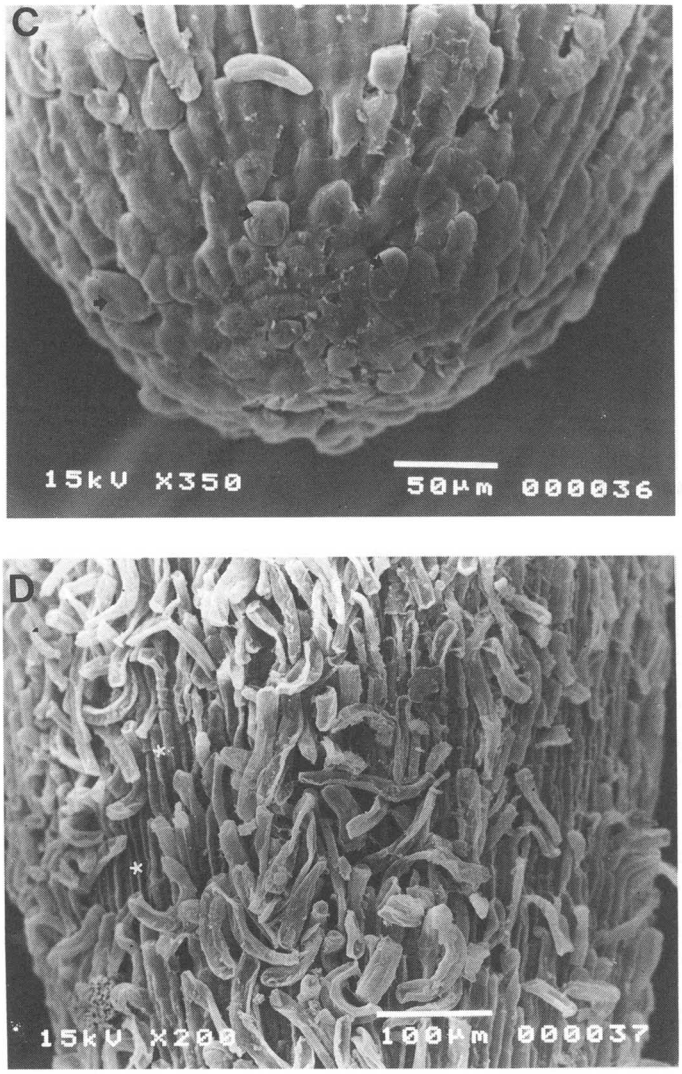

FIG. 10. Continued.

in the number of vacuoles has been observed in bean plants treated with toxins such as cercosporine from *Cercospora beticola*. The *S. deppei* leachate caused plasmolysis in peripheral cells of bean roots and an increase in the number of vacuoles. These results suggest that general responses, such as the plasmolyzed cells, occur when root tip cells are exposed to chemical toxins.

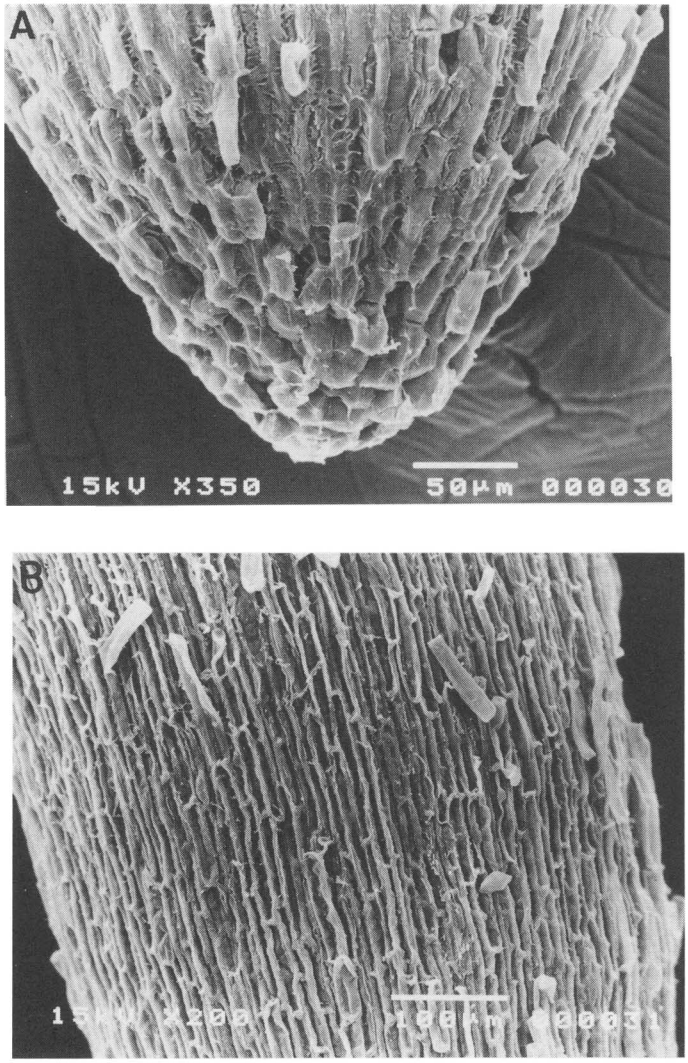

FIG. 11. Control (A) and *Sicyos*-treated bottle gourd root tips (C), ×350. Cells from the epidermis of control (B), and treated bottle gourd roots (D), ×200. *Points to exposed cell layers from sloughed off cells.

Root tip cell ultrastructure of *L. usitatissimum* radicles, treated with benzylamine (isolated from *Camelina*) at the highest (1 mg/ml) concentration, indicated that root development was arrested in an early stage (Lovett, 1982). Lipid levels were abundant in the cells, and nuclei and most organelles were indistin-

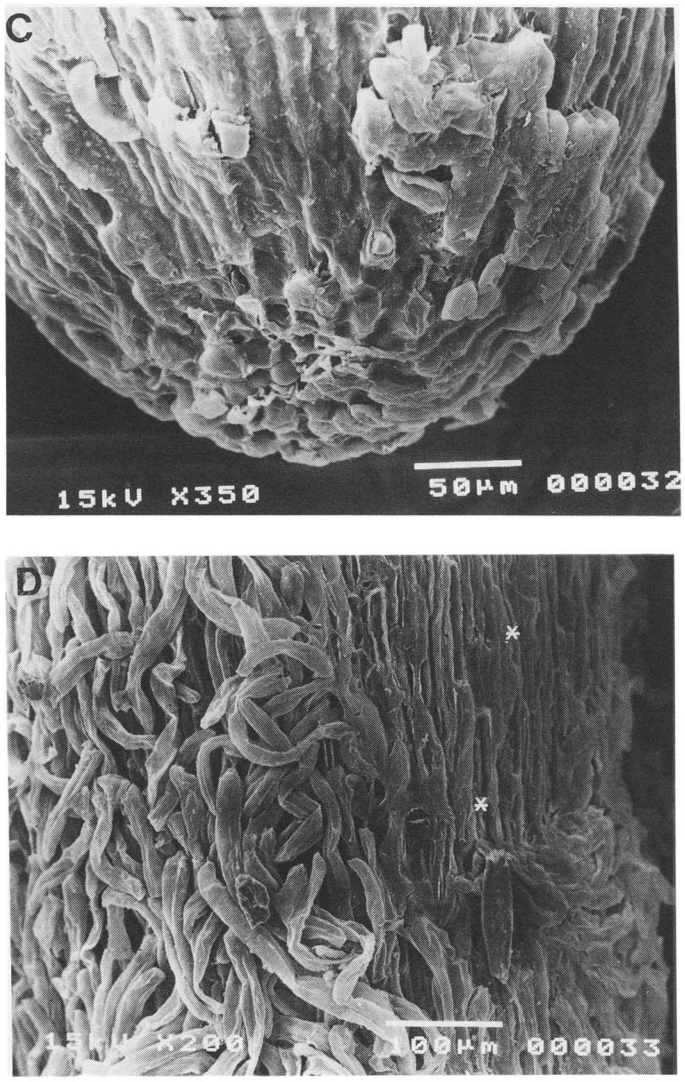

FIG. 11. Continued.

guishable. At a lower level of benzylamine (0.10 mg/ml), many vacuoles were present, and the nucleoli were poorly defined, suggesting some abnormality. In contrast, cells from control plants were well organized, with the organelles appearing normal with nuclei and nucleoli well defined. In our study, we found a similar nuclear dysfunction in treated bean and bottle gourd roots, and this nuclear dysfunction also was observed previously in cells of dehydrated seeds

of *Turbina corymbosa* after imbibing water for 4 hr (Laguna-Hernández et al., 1984). In another study, roots of seedlings of *Pisum sativum* treated with amitrole were swollen with a reduction in the frequency of mitotic figures in root meristems and mitotic aberrations (Arntzen et al., 1970). A brownish color and a somewhat shrunken area also was observed just above the root tip within seven days after treatment. In untreated flax root apices, the dividing cells had large nuclei and dense cytoplasm. The most obvious difference between the control and treated roots was the increase of vacuolation in almost all cells, as observed in this study.

Fayez and Kristen (1996) used electron microscopy to study the effects of three herbicides (chlorsulfuron, norflurazon, and triallate) on the root caps of young seedlings of *P. sativum*, *P. vulgaris*, and *Vicia faba*. All three herbicides caused changes in root cap ultrastructure, and the outermost cell layer of the secretory tissue was destroyed. Chlorsulfuron damaged amyloplasts in gravity-sensing cells (statocytes). Radicle growth was reduced by all three herbicides. This inhibition might be due partially to root cap damage, as the radicle directly contacts herbicides in the soil. This also has been shown with allelochemicals (Anaya and Pelayo-Benavides, 1997). The secretory function of the root cap at this early stage is not fully developed, and consequently there is no mucilage or slime released. As the root cap is vulnerable at this stage of development, an herbicide or allelochemical could easily penetrate the cell walls and enter the secretory layers of the root cap. Fayez and Kristen (1996), by using fluorescence microscopy found that chlorsulfuron caused thickening of the cell walls, but that there was no sloughing of cells from the tip of the root cap, as occurred in our study. Flabuariari and Kristen (1996) exposed seeds of *Zea mays* to low doses of the sulfonylurea herbicides, chlorsulfuron and metsulfuron-methyl. Scanning electron microscopy of root tip surfaces indicated that slime secretion was inhibited at a herbicide concentration of 1.5 mg/liter. Transmission electron microscopy revealed obvious changes to the nuclei and deformation of radial cell walls in the primary root cortex at 0.012 and 1.5 mg/liter for both herbicides.

The ultrastructural changes that we observed for both bean and bottle gourd roots may be an indication of alterations at the gene expression level or a consequence of an effect on protein synthesis. This assumption is based on the changes observed to nuclei and nucleoli, the lack of mucilage secretion in root cap cells, and the inhibition of root growth. Further research will be conducted on mitotic index differences between control and treated cells and also on the possible involvement of Ca^{2+} in the extension of cell walls (Virk and Cleland, 1990) during allelochemical stress, such as that produced by *S. deppei* leachate. Currently, we are studying the effects of *S. deppei* allelochemicals on protein synthesis and gene expression of some crop plants.

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SEX PHEROMONE OF APPLE BLOTCH LEAFMINER, *Phyllonorycter crataegella*, AND ITS EFFECT ON *P. mespilella* PHEROMONE COMMUNICATION

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Abstract—(*Z*)-10,(*Z*)-12-Tetradecadienyl acetate (*Z*10,*Z*12-14:OAc) and (*E*)-10,(*E*)-12-tetradecadienyl acetate (*E*10,*E*12-14:OAc) are sex pheromone components of the apple blotch leafminer (ABLM), *Phyllonorycter crataegella*. Compounds extracted from female pheromone glands were identified by coupled gas chromatographic-electroantennographic detection (GC-EAD) analyses, retention index calculations of EAD active compounds, and by comparative GC-EAD analyses of female ABLM-produced and authentic (synthetic) compounds. In field experiments in apple *Malus domestica* orchards in Connecticut, *Z*10,*Z*12-14:OAc alone attracted ABLM males. Addition of *E*10,*E*12-14:OAc to *Z*10,*Z*12-14:OAc at 0.1:10 or 1:10 ratios enhanced attractiveness of the lure. Geometrical isomers *Z*10,*E*12- or *E*10,*Z*12-14:OAc at equivalent ratios were behaviorally benign and slightly inhibitory, respectively. In field experiments in British Columbia, *Z*10,*Z*12-14:OAc plus *E*10,*E*12-14:OAc did not attract *Phyllonorycter* moths, supporting the contention that ABLM is not present in the fruit growing regions of British Columbia. *Z*10,*Z*12-14:OAc added to *P. mespilella* pheromone, (*E*)-4,(*E*)-10-dodecadienyl acetate, strongly inhibited response by *P. mespilella* males. Recognition of the ABLM pheromone blend by allopatric *P. mespilella* males suggests a phylogenetic relationship and previous sympatry of these two *Phyl-*

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lonorycter spp. If pheromonal attraction of ABLM males were reciprocally inhibited by *P. mespilella* pheromone, a generic *Phyllonorycter* pheromone blend could be tested for pheromone-based mating disruption of the apple leaf-mining *Phyllonorycter* guild in North America.

Key Words—Lepidoptera, Gracillariidae, *Phyllonorycter crataegella*, *Phyllonorycter mespilella*, sex pheromone, (*Z*)-10,(*Z*)-12-tetradecadienyl acetate, (*E*)-10,(*E*)-12-tetradecadienyl acetate, (*E*)-4,(*E*)-10-dodecadienyl acetate, interspecific effects.

INTRODUCTION

The apple blotch leafminer (ABLM), *Phyllonorycter crataegella* (Clemens), is a trivoltine major pest of apple, *Malus domestica* Borkh., in New England and eastern New York (Weires et al., 1980; Maier, 1981). High populations have been associated with early leaf abscission (Maier 1983), reduced fruit size, premature fruit ripening, and reduced fruit set the following year (Reissig et al., 1982). Resistance to insecticidal organophosphates and carbamates used in ABLM control (Green and Prokopy, 1991) can lead to outbreaks (Weires, 1977), rendering alternative means of control desirable.

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); it may also be a viable management alternative for ABLM populations.

Although highest efficacy in mating disruption may be achieved when the disruptant(s) closely resembles the pheromone blend emitted by female moths (Roelofs, 1978; Minks and Cardé, 1988), release of pheromone components from sympatric, coseasonal moths should also be considered for disorientation of mate-seeking males. For example, 2% of (*E*)-11-tetradecenyl acetate (*E*11–14:OAc), a synergistic pheromone component in the fruit-tree leaf roller, *Archips argyrospilus* (Walker) (Roelofs et al., 1974), when added to the pheromone blend of the European leaf roller, *Archips rosanus* (L.), disrupts the response of *A. rosanus* males (Vakenti et al., 1988). Such use of pheromone components from congeners may also be applicable for control of the ABLM and related species.

Our research objectives were to identify and field test the female ABLM sex pheromone blend, and determine its effects on pheromonal communication of congeneric *P. mespilella* (Hübner).

METHODS AND MATERIALS

Insects

Apple leaves infested with overwintering ABLM pupae were collected from apple orchards in Connecticut (April 1994) and sent to Simon Fraser University. Male and female pupae were removed from leaves and kept separately in filter-paper lined Petri dishes at 20°C and a photoperiod of 14L : 10D. Eclosed adults were collected and held at 18°C until used.

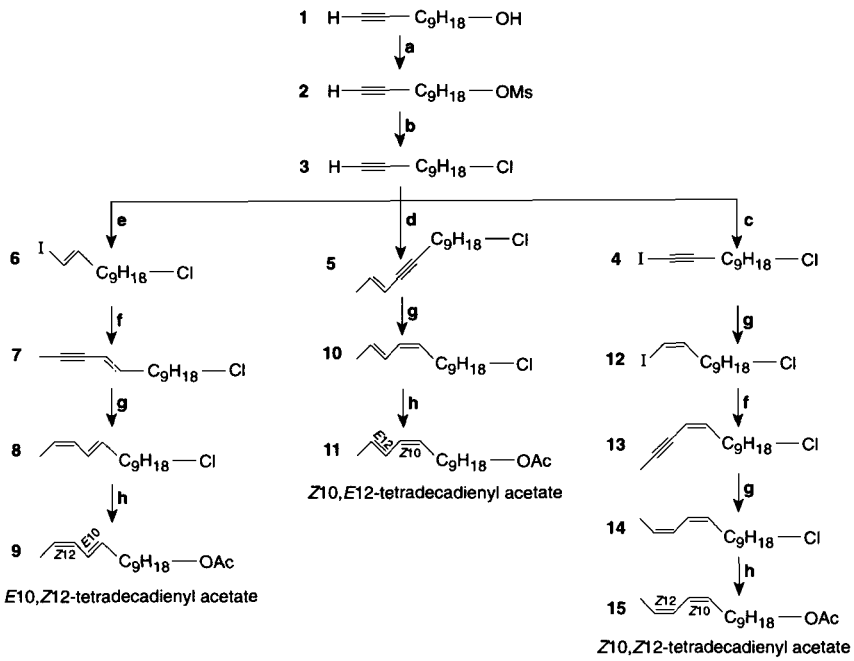
Pheromone Analysis

At the onset of the photophase and during peak calling activity (Green and Prokopy, 1991; Maier, unpublished data), abdominal tips (>90) with pheromone glands of 1- to 2-day-old virgin females were removed and extracted for about 5 min in HPLC-grade hexane. Fifteen aliquots of 1–2 female equivalents (FE) of pheromone gland extract were subjected to gas chromatographic–electroantennographic detection (GC-EAD) analyses (Arn et al., 1975) on three fused silica columns (30 m × 0.25 or 0.32 mm ID) coated with DB-5, DB-210, or DB-23 (J&W Scientific, Folsom, California). For retention index calculations (Van Den Dool and Kratz, 1963) of EAD-active compounds, standards of aliphatic hydrocarbons C_{14}–C_{23} were chromatographed under identical conditions. To determine double-bond positions in candidate pheromone components, four series of synthetic tetradecenyl acetates [(*E*)-3, -5, -7, -9, -11; (*E*)-4, -6, -8, -10, -12; (*Z*)-4, -6, -8, -10, -12; and (*Z*)-3, -5, -7, -9, -11 plus Δ13–14 : OAc] were analyzed by GC-EAD. An aliquot of 41 FE of pheromone extract was analyzed by GC-mass spectrometry, but quantities of candidate pheromone components were too low to obtain mass spectra.

Syntheses

(*E*)-10,(*E*)-12-Tetradecadienyl acetate (chemical and geometrical purity 99% and 98%, respectively) was purchased from the Institute for Plant Protection (Wageningen, The Netherlands). (*E*)-10-Dodecenyl acetate (chemical and geometrical purity 95% and 97%, respectively) was available from previous research (Gries et al., 1993). Instruments for characterization of compounds and general synthetic procedures are similar to those previously described (Gries et al., 1997).

10-Undecyne Methanesulfonate (2; Figure 1) (Crossland and Servis, 1970). To a solution of 10-undecyn-1-ol (**1**) (20.16 g, 0.12 mol) and Et_3N (25.47 mL, 0.18 mol) in CH_2Cl_1 (175 ml) was added dropwise MsCl (11.2 ml, 0.144 mol) at −10°C. After stirring for 45 min at −10°C, the mixture was poured into ice water (100 ml). The organic layer was separated and the aqueous layer



E10,Z12-tetradecadienyl acetate

Z10,E12-tetradecadienyl acetate

Z10,Z12-tetradecadienyl acetate

FIG. 1. Scheme for the syntheses of tetradecadienyl acetates: **a**: Et_3N, $MeSO_2Cl$; **b**: LiCl, DMF; **c**: 1. BuLi, THF, 2. I_2, THF; **d**: 1. THF, BuLi, $ZnBr_2$; 2. (*E*)-1-bromopropene, 5% $Pd(PPh_3)_4$; **e**: 1. DIBAL, hexane, 2. I_2: **f**: 1. Propyne, THF, BuLi, 2. $ZnBr_2$, 5% $Pd(PPh_3)_4$; **g**: 1. $(Sia)_2BH$, THF, 2. CH_3COOH; **h**: $CH3COO^-K^+$, DMF, 70°C.

extracted with ether (3 × 200 ml). The combined organic extracts were washed with 10% HCl (v/v; 100 ml), sat. $NaHCO_3$ (200 mL), H_2O (200 ml), sat. NaCl (200 ml), and dried over anhyd. Na_2SO_4. The syrup obtained after solvent evaporation was flash chromatographed with hexane–ether (50:50) as eluents to yield **2** (27.0 g, 91%); IR (film): 3288, 3025, 2930, 2856, 2114, 1465, 1353, 1174, 973, 948, 823, 721, 629 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.29–1.41 (10H, m), 1.49 (2H, H-8, m), 1.74 (2H, H-2, m), 1.94 (1H, H-11, t, J = 2.6 Hz), 2.18 (2H, H-9, td, J = 7.0, 2.6 Hz), 3.01 (3H, $-OSO_2C\underline{H}_3$, s), 4.22 (2H, H-1, t, J = 6.6 Hz); CI-MS (isobutane) [*m/z* (relative intensity)]: 303 (M^+ + 57, 7), 247 (M^+ + 1, 7), 233.(7), 207 (68), 199 (7), 193 (8), 192 (7), 177 (7), 169 (39), 151 (100), 137 (30), 123 (31), 111 (29), 109 (96); anal. calcd. for $C_{12}H_{22}O_3S$: C, 58.50; H, 9.00; found C, 58.72; H, 8.85.

11-Chloro-1-undecyne (3; Figure 1). Lithium chloride (7.6 g, 0.18 mol) was added to a solution of **2** (22 g, 0.09 mol) in dry DMF (250 ml) and stirred

for 24 hr at room temperature. The mixture was then diluted with water (200 ml) and extracted with hexane (3 × 200 ml). Combined hexane extracts were washed with 10% HCl (v/v; 200 ml), sat. $NaHCO_3$ (200 ml), H_2O (200 ml), sat. NaCl (200 ml), and dried over anhyd. Na_2SO_4. The organic layer was filtered through a bed of silica gel and evaporated to yield **3** (16.0 g, 97%); IR (film): 3303, 2930, 2856, 2116, 1462, 1305 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.26–1.43 (10H, m), 1.49 (2H, H-4, m), 1.76 (2H, H-10, m), 1.93 (1H, H-1, t, J = 2.6 Hz), 2.18 (2H, H-3, td, J = 7.0, 2.6 Hz), 3.53 (2H, H-11, t, J = 6.8 Hz); CI-MS [*m/z* (relative intensity)]: 243 (M^+ + 57, 14), 189 (10), 187 (M^+ + 1, 24), 173 (15), 159 (15), 151 (25), 145 (24), 137 (46), 133 (26), 131 (50) 125 (25), 123 (49), 119 (36), 117 (30), 113 (31), 111 (87), 173 (15), 159 (15), 151 (25), 145 (24), 137 (46), 133 (26), 131 (50), 125 (49), 119 (36), 117 (30), 113 (31), 111 (87), 109 (100), 107 (34), 105 (21); anal. calcd. for $C_{11}H_{19}Cl$: C, 70.76; H, 10.26; found C, 70.99; H, 10.16.

11-Chloro-1-iodo-1-undecyne (4; Figure 1) (Dieck and Heck, 1975). Butyllithium (17.74 ml, 0.44 mol) was added to **3** (8.25 g, 0.044 mol) in THF (100 ml) at −78°C. The mixture was stirred 1 hr while warming to −10°C. After recooling to −78°C, I_2 (11.12 g, 0.044 mol) in THF (100 ml) was added dropwise from a dropping funnel. The mixture was stirred for 15 min at −78°C, and allowed to warm to room temperature. Water (200 ml) was added and the mixture extracted with hexane (3 × 200 ml). Combined hexane extracts were washed with 10% (v/v) $Na_2S_2O_3$ (200 ml), 10% HCl (v/v; 200 ml), sat. $NaHCO_3$ (200 ml), H_2O (200 ml), sat. NaCl (200 ml), and dried over anhyd. Na_2SO_4. The organic layer was filtered through a bed of silica gel and evaporated to yield **4** (13.7 g, 99%); IR (film): 2928, 2855, 2148, 1463, 1309 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.26–1.43 (10H, m), 1.49 (2H, H-4, m), 1.76 (2H, H-10, m), 2.35 (2H, H-3, t, J = 7.1 Hz), 3.53 (2H, H-11, t, J = 6.8 Hz); EI-MS [*m/z* (relative intensity)]: 312 (M^+, 4), 206 (15), 207 (20), 193 (26), 185 (6), 180 (60), 165 (100); anal. calcd. for $C_{11}H_{18}ClI$: C, 42.26; H, 5.80; found C, 42.15; H, 5.68.

11-Chloro-1-iodo-(Z)-1-undecene (12; Figure 1). The disiamylborane reagent was prepared as described (Zweifel and Poiston, 1970). The reagent (0.042 mol) was cooled to 0°C and a solution of **4** (9.0 g, 0.028 mol) in THF (20 ml) was added. The reaction mixture was allowed to warm to room temperature, stirred 5 hr, and treated with 99% acetic acid (30 ml) and stirred an additional 48 hr at room temperature. Water (200 ml) was added, and the solution was extracted with hexane (3 × 200 ml). Combined hexane extracts were washed with 10% HCl (v/v; 200 ml), sat. $NaHCO_3$ (200 ml), H_2O (200 ml), sat. NaCl (200 ml), and dried over anhyd. Na_2SO_4. The crude product obtained after solvent evaporation was flash chromatographed with hexane as eluent to yield **12** (7.0 g, 78%); IR (film): 3066, 2926, 2853, 1608, 1460, 1286 cm^{-1};

^{1}H NMR ($CDCl_3$) ppm: 1.25–1.49 (12H, m), 1.76 (2H, H-10, m), 2.15 (2H, H-3, m), 3.53 (2H, H-11, t, J = 6.8 Hz), 6.15 (2H, H-1 and H-2, m); EI-MS [*m/z* (relative intensity)]: 314 (M^+, 4), 167 (22), 154 (29), 131 (15), 117 (7), 109 (21), 97 (19), 95 (27), 83 (85), 82 (7), 81 (21), 79 (6), 70 (8), 69 (100), 68 (7), 67 (27), 57 (13), 56 (6), 55 (59), 54 (9), 53 (10), 43 (11), 41 (30); anal. calcd. for $C_{11}H_{20}ClI$: C, 41.99; H, 6.41; found C, 41.94; H, 6.32.

11-Chloro-1-iodo-(E)*-1-undecene (6; Figure 1) (Gardette et al., 1984).* To **3** (8.3 g, 0.044 mol) in hexane (50 ml) at room temperature was added DIBAL in hexane (1 M, 50 ml, 0.05 mol). After maintaining the reaction mixture at 50°C for 2 hr, it was cooled to −60°C and I_2 (11.5 g, 0.045 mol) in THF (100 ml) was added dropwise over a period of 20 min. After warming the mixture to −10°C, dilute H_2SO_4 and water was added. The resultant solution was extracted with hexane (3 × 200 ml). Combined hexane extracts were washed with dil. $Na_2S_2O_3$ and sat. NaCl (200 ml), and dried over anhyd. Na_2SO_4. The crude product obtained after solvent evaporation was flash chromatographed with hexane as eluent to yield **6** (9.8 g, 70%); IR (film): 3047, 2927, 2853, 1604, 1460, 1287, 1207, 945 cm^{-1}; H NMR ($CDCl_3$) ppm: 1.25–1.49 (12H, m), 1.76 (2H, H-10, m), 2.05 (2H, H-3, dtd, J = 7.0, 7.0, 1.5 Hz), 3.53 (2H, H-11, t, J = 6.8 Hz), 5.96 (1H, H-1, dt, J = 14.0, 1.5 Hz), 6.51 (1H, H-2, dt, J = 14.0, 7.0 Hz); CI-MS [*m/z* (relative intensity)]: 315 (15), 314 (M^+, 61), 280 (6), 245 (7), 229 (17), 209 (7), 196 (25), 187 (22), 185 (37), 182 (22), 173 (25), 166 (57), 154 (14), 151 (67), 137 (32), 131 (39), 123 (44), 117 (23), 115 (6), 111 (63), 109 (100), 107 (27), 105 (16); anal. calcd. for $C_{11}H_{20}ClI$: C, 41.99; H, 6.41; found C, 42.18; H, 6.55.

14-Chlorotetradeca-(E)*-2-en-4-yne (5; Figure 1).* (*E*)-1-Bromopropene (80% mixture; Wiley Organics, Coshocton, Ohio) was isomerically enriched (Hayashi et al. 1986) and coupled with 11-chloro-1-undecyne, as follows (Gardett et al., 1984): BuLi (4.8 ml, 0.012 mol) was added to **3** (2 g, 0.01 mol) in dry THF (10 mL) at −60°C. The reaction mixture was warmed to −10°C as it was stirred, then was cooled to −30°C and degassed $ZnBr_2$ (2.7 g, 0.012 mol) in dry THF (20 ml) was added. The mixture was stirred 30 min at −30°C to −10°C, and degassed (*E*)-1-bromopropene (1.94 g, 0.016 mol) and tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$, 0.6 g, 0.0005 mol] in dry THF (10 ml) were added. Following a gradual temperature increase to 60°C, the mixture was stirred 4 hr. Aqueous NH_4Cl (100 ml) was added and the mixture extracted with hexane (3 × 200 ml). The organic layer was washed with H_2O and sat. NaCl and dried over anhyd. Na_2SO_4. The organic layer was filtered through a $AgNO_3$–silica gel bed to remove unreacted chloroacetylene. The filtrate was evaporated and the residue flash chromatographed with hexane to yield **5** (2.1 g, 86%); IR (film): 2931, 2854, 2216, 1672, 1463, 1444, 1375, 1300, 952, 690, 625 cm^{-1}; ^{1}H NMR ($CDCl_3$) ppm: 1.20–1.55 (12H, m), 1.75 (3H, H-1, dd, J = 6.8, 1.9 Hz), 1.76 (2H, H-13, m), 2.27 (2H, H-6, td, J =

7.0, 1.9 Hz), 3.53 (2H, H-14, t, J = 6.8 Hz), 5.47 (1H, H-3, dq, J = 15.8, 1.9 Hz), 6.05 (1H, H-2, dq, J = 15.8, 6.8 Hz); EI-MS [*m/z* (relative intensity)]: 226 (M^+, 19), 149 (5), 135 (21), 122 (49), 121 (80), 119 (7), 109 (10), 108 (46), 107 (100), 105 (27), 95 (10), 94 (24), 93 (84), 92 (7), 91 (44), 81 (41), 80 (10), 79 (66), 78 (7), 77 (23); anal. calcd. for $C_{14}H_{23}Cl$: C, 74.15; H, 10.22; found C, 73.94; H, 10.12.

14-Chlorotetradeca-(E)*-4-en-2-yne (7; Figure 1).* Butyllithium (4 ml, 0.01 mol) was added to propyne (2 ml) in dry THF (10 ml) at −78°C and stirred 30 min. Degassed $ZnBr_2$ (2.25 g, 0.01 mol) in dry THF (20 ml) was added and the mixture stirred 30 min at −70°C. The temperature was allowed to increase to −40°C before degassed **6** (1.5 g, 0.0047 mol), $Pd(PPh_3)_4$ (0.3 g, 0.00025 mol) in dry THF (10 ml) was added. The cooling bath was then removed and the mixture stirred 1 hr at room temperature. The reaction was quenched with aq. NH_4Cl and extracted with hexane (3 × 100 ml). The organic layer was washed with H_2O and sat. NaCl and dried over anhyd. Na_2SO_4. Solvent was evaporated in vacuo and the residue chromatographed with hexane as eluent to yield **7** (0.94 g, 88%); IR (film): 2927, 2853, 2223, 1463, 1375, 1308, 1172, 955, 723, 651 cm^{-1}; ^1H NMR ($CDCl_3$); ppm 1.25–1.49 (12H, m), 1.76 (2H, H-13, m), 1.92 (3H, H-1, d, J = 2.2 Hz), 2.06 (2H, H-6, td, J = 7.0, 7.0 Hz), 3.53 (2H, H-14, t, J = 6.8 Hz), 5.42 (1H, H-4, dq, J = 15.8, 2.2 Hz), 6.04 (1H, H-5, dt, J = 15.8, 7.0 Hz); EI-MS [*m/z* (relative intensity)]: 226 (M^+, 19), 149 (6), 135 (23), 121 (24), 109 (7), 108 (13), 107 (43), 105 (8), 95 (23), 94 (28), 93 (83), 91 (44), 81 (24), 80 (19), 79 (100), 78 (19), 77 (86), 69 (14), 68 (7), 67 (29), 66 (97), 65 (16), 55 (19), 53 (12), 51 (7), 41 (20); anal. calcd. for $C_{14}H_{23}Cl$: C, 74.15; H, 10.22; found C, 74.03; H, 10.16.

14-Chlorotetradeca-(Z)*-4-en-2-yne (13; Figure 1).* The procedure used was similar to that for the synthesis of **7** with identical quantities of **12**, instead of **6**, to yield **13** (0.92 g, 88%); IR (film): 3018, 2926, 2854, 1460, 1307, 1201, 1118, 725, 649 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.25–1.49 (12H, m), 1.76 (2H, H-13, m), 1.98 (3H, H-1, d, J = 2.5 Hz), 2.28 (2H, H-6, dtd, J = 7.0, 7.0, 1.5 Hz), 3.53 (2H, H-14, t, J = 6.8 Hz), 5.41 (1H, H-4, dqt, J = 10.5, 2.5, 1.5 Hz), 5.8 (1H, H-5, dt, J = 10.5, 7.0 Hz); EI-MS [*m/z* (relative intensity)]: 226 (M^+, 6), 135 (16), 121 (18), 108 (10), 107 (33), 105 (6), 95 (19), 94 (23), 93 (62), 92 (6), 91 (33), 82 (6), 81 (20), 80 (17), 79 (80), 78 (13), 77 (55), 69 (13), 68 (7), 67 (26), 66 (100), 65 (14), 55 (17), 53 (9), 41 (14); anal. calcd. for $C_{14}H_{23}Cl$: C, 74.15; H, 10.22; found C, 74.09; H, 9.97.

14-Chloro-(Z)*-2,*(E)*-4-tetradecadiene (8; Figure 1).* Reduction was carried out by adding compound **7** (0.75 g, 0.0033 mol) in THF (5 ml) to $(Sia)_2BH$ (0.005 mol) (prepared as for **12**) at 0°C. The reaction mixture was allowed to warm to room temperature and was stirred 2 hr. Subsequently, 99% acetic acid (2 ml) was added, the mixture stirred 24 hr, diluted with water, and extracted with hexane (3 × 50 ml). The hexane layer was washed with H_2O, sat. $NaHCO_3$

and sat. NaCl and dried over anhyd. Na_2SO_4. After solvent evaporation in vacuo, the residue was chromatographed on a $AgNO_3$-silica gel column (hexane) to yield **8** (0.65 g, 87%); IR (film): 3018, 2926, 2854, 1631, 1463, 1443, 1408, 1371, 1278, 981, 945, 863, 711 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.20–1.50 (12H, m), 1.74 (3H, H-1, dd, J = 7.1, 1.3 Hz), 1.76 (2H, H-13, m), 2.09 (2H, H-6, td, J = 7.1, 7.1 Hz), 3.53 (2H, H-14, t, J = 6.8 Hz), 5.37 (1H, H-2, dq, J = 11.0, 7.1 Hz), 5.66 (1H, H-5, dt, J = 14.5, 7.0 Hz), 5.97 (1H, H-3, ddq, J = 11.0, 11.0, 1.3 Hz), 6.32 (1H, H-4, dd, J = 14.5, 11.0 Hz); CI-MS [*m/z* (relative intensity)]: 285 (M^+ + 57, 12), 229 (74), 228 (M^+, 92), 215 (48), 205 (27), 203 (76), 189 (16), 187 (12), 175 (16), 173 (26), 159 (32), 147 (22), 145 (27), 137 (25), 133 (25), 125 (48), 123 (30), 119 (21), 111 (100), 109 (50); anal. calcd. for $C_{14}H_{25}Cl$: C, 73.49; H, 11.01; found C, 73.25; H, 10.85.

14-Chloro-3(E)-*2*,(Z)-*4-tetradecadiene (**10**; Figure 1).* The procedure was equivalent to the synthesis of **8** using identical quantities of **5** instead of **7** to yield **10** (0.60 g, 80%); IR (film): 2952, 2928, 2854, 1463, 1375, 1309, 975, 946, 819, 724, 654 cm^{-1}; ^1H NMR ($CDCl_3$); ppm 1.20–1.45 (12H, m), 1.75 (3H, H-1, d, 7.1 Hz), 1.76 (2H, H-13, m), 2.14 (2H, H-6, td, J = 7.1, 7.1 Hz), 3.53 (2H, H-14, t, J = 6.8 Hz), 5.24 (1H, H-5, dt, J = 10.8, 7.1 Hz), 5.66 (1H, H-2, dq, J = 13.5, 7.1 Hz), 5.94 (1H, H-4, dd, J = 10.8, 10.8 Hz), 6.32 (1H, H-3, dt, J = 13.5, 10.8 Hz); CI-MS [*m/z* (relative intensity)]: 285 (M^+ + 57, 6), 229 (48), 228 (M^+, 91), 215 (43), 205 (20), 203 (56), 189 (11), 187 (12), 175 (36), 173 (26), 159 (48), 147 (20), 145 (37), 137 (25), 133 (25), 125 (43), 123 (30) 119 (21), 111 (100), 109 (50); anal. calcd. for $C_{14}H_{25}Cl$: C, 73.49; H, 11.01; found C, 73.32; H, 10.92.

14-Chloro-(Z)-*2*,(Z)-*4-tetradecadiene (**14**; Figure 1).* The procedure was equivalent to the synthesis of **8** with identical quantities of **13** instead of **7** to yield **14** (0.60 g, 80%); IR (film): 2952, 2854, 1468, 1375, 1309, 978, 946, 819, 727, 654 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.20–1.45 (12H, m), 1.75 (3H, H-1, d, J = 7.0 Hz), 1.76 (2H, H-13, m), 2.15 (2H, H-6, td, J = 7.3, 7.1 Hz), 3.53 (2H, H-14, t, J = 6.8 Hz), 5.41 (1H, H-5, dt, J = 7.8, 7.1 Hz), 5.48 (1H, H-2, dq, J = 7.9, 7.0 Hz), 6.30 (2H, H-3 and H-4, m); CI-MS [*m/z* (relative intensity)]: 285 (M^+ + 57, 2), 229 (54), 228 (M^+, 98), 215 (46), 205 (10), 203 (46), 189 (21), 187 (12), 175 (16), 173 (26), 159 (42), 147 (12), 145 (29), 137 (35), 133 (25), 125 (58), 123 (30), 119 (21), 111 (100), 109 (18); anal. calcd. for $C_{14}H_{25}Cl$: C, 73.49; H, 11.01; found C, 73.22; H, 10.86.

(E)-*10*,(Z)-*12-Tetradecadienyl acetate (**9**; Figure 1).* A mixture of **8** (0.5 g, 0.0022 mol) and KOAc (2.2 g, 0.022 mol) in DMF (20 ml) was heated at 70°C for 36 hr. Water (20 ml) was added, and the mixture extracted with hexane–ether (3 × 50 ml). The organic layer was washed with 10% (v/v) HCl, sat. $NaHCO_3$, water, sat. NaCl, and dried over anhyd. Na_2SO_4. After solvent

evaporation in vacuo, the residue was chromatographed with hexane–ether as eluent system to yield **9** in 99% purity (0.49 g, 89%); IR (film): 3018, 2926, 2854, 1741, 1655, 1461, 1366, 1238, 1038, 981, 946, 711 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.20-1.45 (12H, m), 1.61 (2H, H-2, m), 1.73 (3H, H-14, dd, J = 7.1, 1.6 Hz), 2.04 (3H, $-CH_2OCOC\underline{H}_3$, s), 2.09 (2H, H-9, td, J = 7.0, 7.0 Hz), 4.05 (2H, H-1, t, J = 6.8 Hz), 5.37 (1H, H-13, dq, J = 10.8, 7.0 Hz), 5.65 (1H, H-10, dt, J = 15.0, 7.0 Hz), 5.97 (1H, H-12, ddq, J = 10.8, 10.8, 1.6 Hz), 6.32 (1H, H-11, dd, J = 15.0, 10.8 Hz); EI-MS [*m/z* (relative intensity)]: 252 (M^+, 10), 192 (7), 149 (8), 135 (17), 124 (6), 122 (9), 121 (27), 110 (21), 109 (14), 108 (13), 107 (21), 97 (6.2), 96 (32), 95 (44), 94 (47), 93 (45), 83 (12), 82 (77), 81 (99), 80 (59), 79 (100), 78 (9), 77 (24), 73 (7), 69 (28), 68 (89), 67 (92), 66 (17), 65 (13), 61 (8), 55 (40), 54 (8), 53 (26), 43 (71), 42 (7), 41 (33); anal. calcd. for $C_{16}H_{28}O_2$: C, 76.14; H, 11.18; found C, 76.22; H, 11.39.

(Z)-*10*,(E)-*12-Tetradecadienyl acetate (**11**; Figure 1).* The procedure was equivalent to the synthesis of **9** with identical quantities of **10** instead of **8** to yield **11** in 99% purity (0.50 g, 91%); IR (film); 2927, 2856, 1741, 1462, 1366, 1239, 1040, 974, 722 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.20–1.45 (12H, m), 1.61 (2H, H-2, m), 1.73 (3H, H-14, d, J = 7.1 Hz), 2.04 (3H, $-CH_2OCOC\underline{H}_3$, s), 2.14 (2H, H-9, td, J = 7.1, 7.1 Hz), 4.05, (2H, H-1, t, J = 6.8 Hz), 5.24 (1H, H-10, dt, J = 10.8, 7.1 Hz), 5.66 (1H, H-13, dq, J = 13.5, 7.1 Hz), 5.94 (1H, H-11, dd, J = 10.8, 10.8 Hz), 6.32 (1H, H-12, dt, J = 13.5, 10.8 Hz); EI-MS [*m/z* (relative intensity)]: 252 (M^+, 41), 192 (33), 164 (12), 163 (16), 150 (12), 149 (37), 137 (7), 136 (23), 135 (76), 124 (20), 123 (16), 122 (28), 121 (82), 111 (8), 110 (46), 109 (31), 108 (25), 107 (40), 97 (9), 96 (43), 95 (55), 94 (50), 93 (47), 91 (10), 83 (9), 82 (56), 81 (100), 80 (35), 79 (81), 77 (10), 69 (10), 68 (34), 67 (32), 55 (6), 43 (6); anal. calcd. for $C_{16}H_{28}O_2$: C, 76.14; H, 11.18; found C, 75.98; H, 11.19.

(Z)-*10*,(Z)-*12-Tetradecadienyl acetate (**15**; Figure 1).* The procedure was equivalent to the synthesis of **9** with identical quantities of **14** instead of **8** to yield **15** in 99% purity (0.50 g, 90.9%); IR (film): 2926, 2854, 1741, 1656, 1461, 1366, 1239, 1039, 978, 946, 721 cm^{-1}; ^1H NMR ($CDCl_3$) ppm: 1.20–1.45 (12H, m), 1.61 (2H, H-2, m), 1.75 (3H, H-14, d, J = 7.0 Hz), 2.04 (3H, $-CH_2OCOC\underline{H}_3$, s), 2.15 (2H, H-9, td, J = 7.3, 7.1 Hz), 4.04 (2H, H-1, t, J = 6.8 Hz), 5.41 (1H, H-10, dt, J = 7.8, 7.1 Hz), 5.48 (1H, H-13, dq, J = 7.9, 7.0 Hz), 6.30 (2H, H-11 and H-12, m); EI-MS [*m/z* (relative intensity)]: 252 (M^+, 7), 192 (8), 149 (10), 136 (8), 135 (21), 124 (7) 123 (6), 122 (11), 121 (32), 110 (24), 109 (17), 108 (14), 107 (22), 97 (7), 96 (33), 95 (44), 94 (39), 93 (38), 91 (8), 83 (10), 82 (60), 81 (100), 80 (38), 79 (98), 78 (9), 77 (11), 69 (16), 68 (63), 67 (53), 66 (7), 55 (12), 53 (7), 43 (16), 41 (8); anal. calcd. for $C_{16}H_{28}O_2$: C, 76.14; H, 11.18; found C, 76.24; H, 11.30.

Compounds **9**, **11**, and **15** were purified by high-performance liquid chromatography (HPLC) prior to field testing by employing a Waters LC 6 high-performance liquid chromatograph equipped with a Waters 486 variable-wavelength UV-visible detector set at 220 nm, an HP 3396 series II integrator, and a Nova-Pak C18 (3.9 × 300 mm) column with 1 ml/min of acetonitrile flow. HPLC-purified compounds used for preparation of lures contained no HPLC-detectable isomeric contaminants.

Field Experiments

Candidate Pheromone Components of P. crataegella. Five field experiments were conducted from May to August 1995 in apple orchards in Middlesex County, Connecticut. Wing traps (Pherocon 1C. Trècè Corp. Salinas, California) were suspended from limbs of apple trees 1.5–2 m above ground) and 1 m from the main trunk in randomized complete blocks with traps and blocks at 20-m intervals. Traps were baited with gray rubber septa (The West Company, Lionville, Pennsylvania) impregnated with candidate pheromone components in HPLC-grade hexane. The first experiment (experiment 1) tested *Z*10,*Z*12–14:OAc (10 μg) and *E*10–12:OAc (10 μg) singly and in combinations at 10:1 and 10:0.1 ratios. Experiment 2 tested *Z*10,*Z*12–14:OAc at increasing doses of 0.01, 0.1, 1, and 10 μg. Experiment 3 tested *Z*10,*Z*12–14:OAc (10 μg) singly and in quarternary combinations with *E*10,*E*12–14:OAc, *Z*10,*E*12–14:OAc, and *E*10,*Z*12–14:OAc at 10:0.1, 10:1, and 10:10 ratios. Experiment 4 tested *Z*10,*Z*12–14:OAc (10 μg) singly and in all ternary and quarternary combinations with *E*10,*E*12–14:OAc, *Z*10,*E*12–14:OAc and *E*10,*Z*12–14:OAc at 1 μg each. Experiment 5 tested *Z*10,*Z*12–14:OAc (10 μg) singly and in all binary and quarternary combinations with *E*10,*E*12–14:OAc, *E*10,*Z*12–14:OAC, and *Z*10,*E*12–14:OAc at 1 μg each. In experiments 1–4, captured ABLM males were counted 24 hr after trap placement. In experiment 5, trap positions were rerandomized after 24 hr and captured males counted 24 and 48 hr after experiment initiation.

Effect of P. crataegella *Pheromone Components on* P. mespilella *Pheromonal Communication.* Three field experiments (experiments 6–8) were conducted in September 1995 in the Okanagan Valley, British Columbia. Delta milk carton traps (Gray et al., 1984) were suspended from apple trees 1.5–2 m above ground and 1 m from the main trunk in randomized complete blocks with traps and blocks at 20-m and 40-m intervals, respectively. Traps were baited with gray rubber septa impregnated with candidate pheromone components in HPLC grade hexane. Experiment 6 tested the *P. crataegella* pheromone blend *Z*10,*Z*12–14:OAc (10 μg) plus *E*10,*E*12–14:OAc (1 μg) versus the *P. mespilella* pheromone component (*E*)-4,(*E*)-10-dodecadienyl acetate (*E*4,*E*10–12:OAc) (10 μg) (Gries et al., 1993). Experiment 7 tested *E*4,*E*10–12:OAc

(10 μg) singly and in ternary combinations with *Z*10,*Z*12–14:OAc and *E*10,*E*12–14:OAc at ratios (μg) of 10:10:1, 10:1:0.1, and 10:0.1:0.01. Experiment 8 tested *E*4,*E*10–12:OAc (10 μg) singly and in binary and ternary combinations with *Z*10,*Z*12–14:OAc (10 μg) and *E*10,*E*12–14:OAc (1 or 10 μg). In all three experiments, captured *P. mespilella* males were counted 24 hr after trap placement.

Statistical Analyses.

Statistical analyses were conducted with the SAS statistical package (SAS Institute Inc., Cary, North Carolina). To ensure homogeneity of variance, data in experiment 4 were transformed by $\sqrt{x}$ and subjected to analysis of variance (ANOVA) followed by a Student-Newman-Keuls means separation procedure ($\alpha = 0.05$). Despite transformation, data from other field experiments were not normally distributed and were therefore subjected to nonparametric analysis of variance by ranks (Friedman's test) (Zar, 1984; SAS/STAT User's guide, 1988, release 6.03 edition, SAS Institute Inc.). Cary, NC).

RESULTS

Pheromone Analysis

GC-EAD analysis of female ABLM pheromone gland extracts revealed six antennal responses, with FID-detectable compound E being most EAD-active (Figure 2). Based on its retention index of 1870 and previously reported *Phyllonorycter* sex attractant/pheromone components (Roelofs et al., 1977; Gries et al., 1993), EAD-active compound A was hypothesized to be (*E*)-10-dodecenyl acetate (*E*10–12:OAc). Retention indices of EAD-active compounds C–F were indicative of isomeric, conjugated tetradecadienyl acetates. In GC-EAD analyses of monoene *E* or *Z* tetradecenyl acetates, (*Z*)-10- and (*Z*)-12-tetradecenyl acetates (Figure 2) elicited the strongest antennal responses. Double-bond positions in compounds C–F were therefore hypothesized to be at C_{10} and C_{12}. Because geometrical isomers of homologous 8,10-dodecadienyl acetate eluted in the order of (*Z*)-8,(*E*)-10-,(*E*)-8,(*E*)-10-,(*Z*)-8,(*Z*)-10-, and (*E*)-8,(*Z*)-10-dodecadienyl acetate, EAD-active compounds C–F were hypothesized to be corresponding (*Z*)-10,(*E*)-12-,(*E*)-10,(*E*)-12-,(*Z*)-10,(*Z*)-12- and (*E*)-10,(*Z*)-12-tetradecadienyl acetates, respectively. Identical retention times of synthetic standards and female-produced compounds A and C–F on the DB-210 column supported structural assignments of the candidate pheromone components. Female ABLM-produced E and synthetic *Z*10,*Z*12–14:OAc had corresponding retention indices and comparable EAD-activity on GC columns coated with DB-5, DB-210, and DB-23.

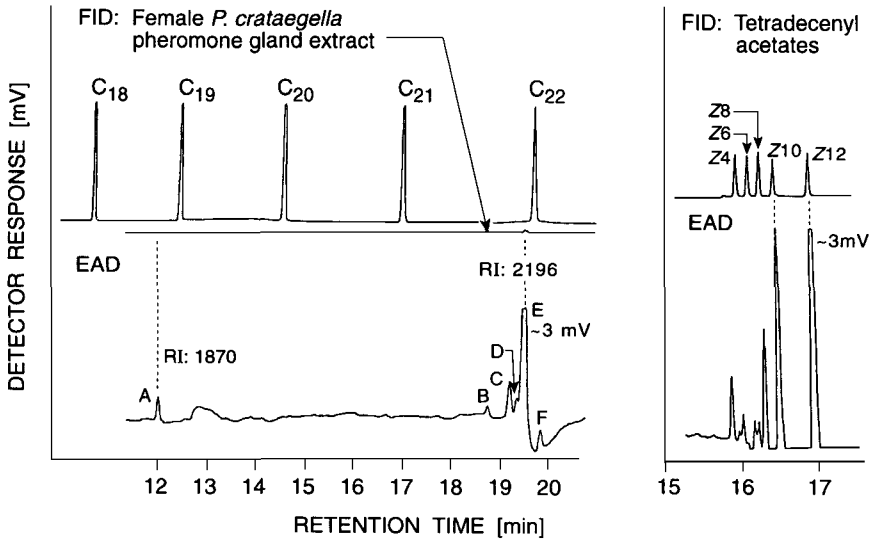


FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *P. crataegella* antenna) responses to one female equivalent of *P. crataegella* pheromone gland extract chromatographed on a DB-210 column (splitless injection; 70°C for 1 min, 20°C/min to 140°C, 2°C/min to 220°C); and to synthetic tetradecenyl acetates chromatographed on a DB-23 column (splitless injection; 50°C for 1 min, 20°C/min to 100°C, 5°C/min to 200°C). RI = retention index. Compounds labeled C_{18-22} represent linear hydrocarbon standards.

Field Experiments

Candidate P. crataegella *Pheromone Components.* Major candidate pheromone component *Z*10,*Z*12-14:OAc at 10 μg was highly attractive to ABLM males, whereas *E*10-12:OAC was behaviorally benign (Figure 3; experiment 1). Addition of *E*10-12:OAc to *Z*10,*Z*12-14:OAC neither enhanced nor reduced attractiveness of the lure (Figure 3; experiment 1). Doses of 10 μg, but not lower, of *Z*10,*Z*12-14:OAc attracted ABLM males (Figure 3; experiment 2). *Z*10,*Z*12-14:OAc (10 μg) in quarternary combinations with all three geometrical isomers at 1 or 0.1 μg each was significantly more attractive than by itself (Figure 4; experiment 3). Testing *Z*10,*Z*12-14:OAc (10 μg) singly and in all ternary (Figure 4; experiment 4), binary (Figure 4; experiment 5), and quarternary combinations (Figure 4; experiments 4 and 5) with geometrical isomers revealed synergistic behavioral activity only of *E*10,*E*12-14:OAc.

Effect of P. crataegella *Sex Pheromone on* P. mespilella *Pheromone Communication.* In the Okanagan Valley, *P. mespilella* pheromone component

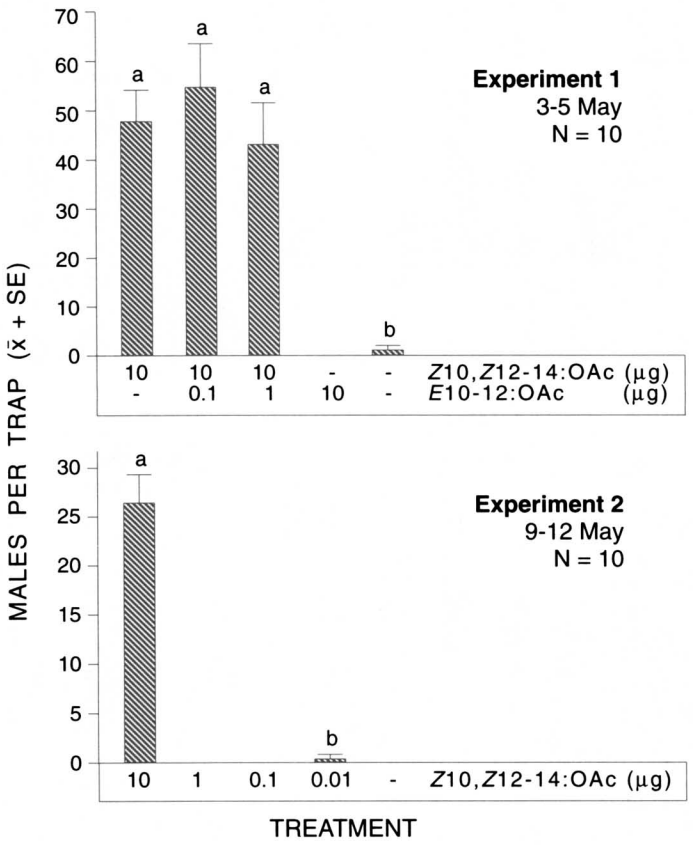


FIG. 3. Numbers of *P. crataegella* males captured in wing traps baited with candidate pheromone components singly and in various combinations and ratios; Middlesex county, Connecticut, 1995; for each experiment, bars with the same letter superscript are not significantly different, $P < 0.05$.

*E*4,*E*10–12:OAC (10 μg) was highly attractive to *P. mespilella* males, whereas the ABLM pheromone blend *Z*10,*Z*12–14:OAc (10 μg) plus *E*10,*E*12–14:OAc (1 μg) was not attractive (Figure 5; experiment 6). Attractiveness of *E*4,*E*10–12:OAC (10 μg) was greatly reduced by the addition of *Z*10,*Z*12–14:OAc (1 μg) and *E*10,*E*12–14:OAC (0.1 μg) (Figure 5; experiment 7). *Z*10,*Z*12–14:OAC (10 μg) was significantly more effective than *E*10,*E*12–14:OAC (10 μg) in reducing attraction of *P. mespilella* males to *E*4,*E*10–12:OAc (10 μg) (Figure 5; experiment 8). *Z*10,*Z*12–14:OAc (10 μg) singly or in combination with *E*10,*E*12–14:OAc (1 or 10 μg) were equally inhibitory (Figure 5; experiment 8).

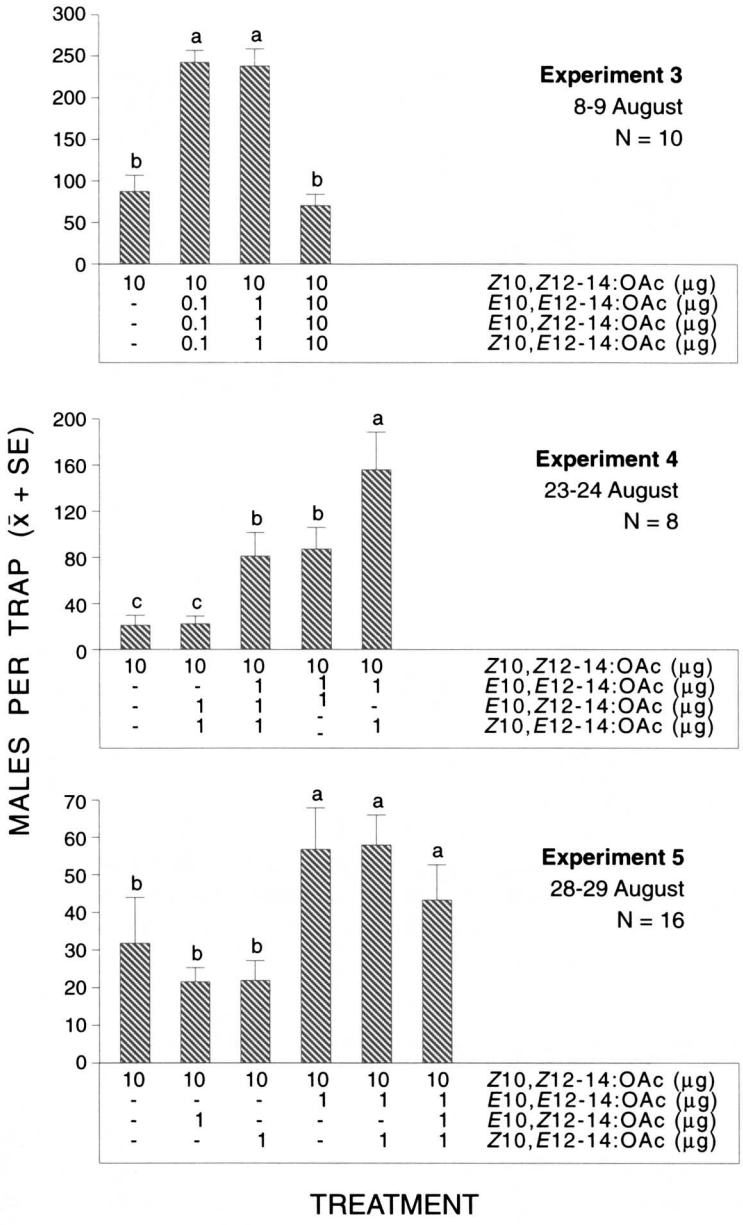


FIG. 4. Numbers of *P. crataegella* males captured in wing traps baited with candidate pheromone components singly and in various combinations and ratios; Middlesex county, Connecticut, 1995; for each experiment, bars with the same letter superscript are not significantly different, $P < 0.05$.

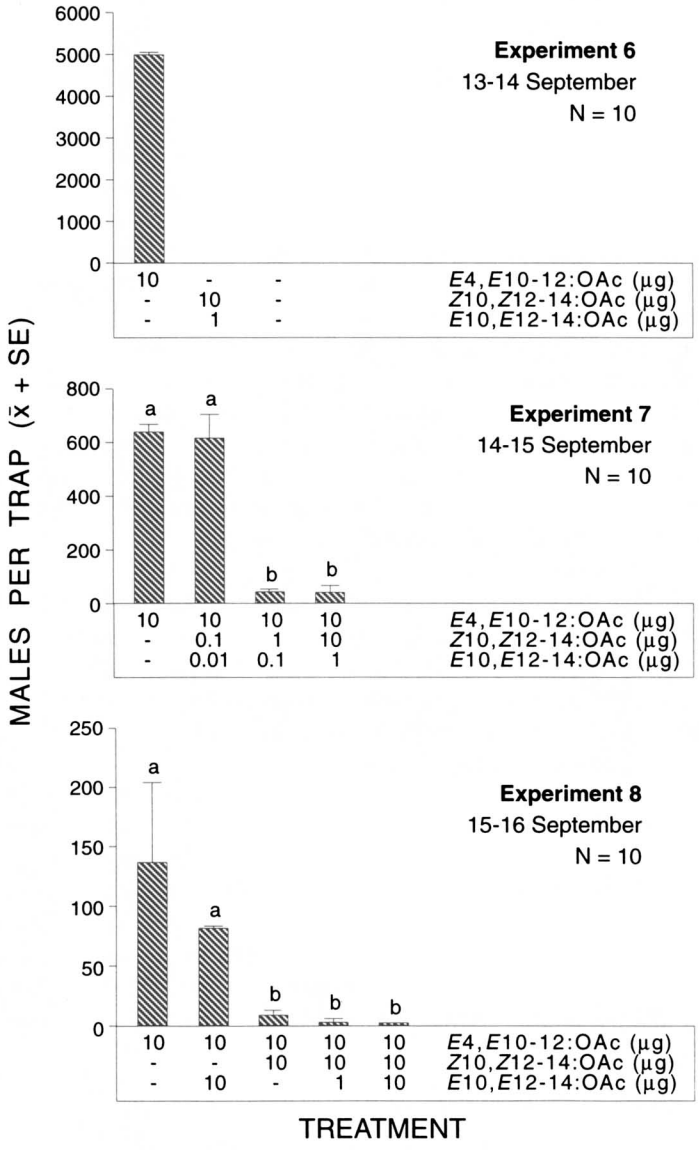


FIG. 5. Numbers of *P. mespilella* males captured in delta traps baited with *P. crataegella* and *P. mespilella* pheromone components singly and in various combinations and ratios; Okanagan Valley, British Columbia, 1995; for each experiment, bars with the same letter superscript are not significantly different, $P < 0.05$.

DISCUSSION

Sex Pheromone of P. crataegella *and Other* Phyllonorycter *Moths*

Evidence that *Z*10,*Z*12–14:OAc is the major ABLM pheromone component includes: (1) strong EAD activity of both synthetic *Z*10– and *Z*12–14:OAC; (2) identical retention characteristics of female-produced compound E and synthetic *Z*10,*Z*12–14:OAc on DB-5, DB-210, and DB-23 columns, respectively; (3) comparable EAD activity of synthetic *Z*10,*Z*12–14:OAc and female-produced compound E; and (4) attractiveness of *Z*10,*Z*12–14:OAc to ABLM males in field experiments.

*Z*10,*E*12–, *E*10,*E*12–, and *E*10,*Z*12–14:OAc cochromatographing with EAD-active compounds C, D, and F, respectively, in female ABLM pheromone extract may be produced by female ABLM or may have resulted from partial isomerization of *Z*10,*Z*12–14:OAc in the injection port (>240°C) of the gas chromatograph. Strong EAD activity of *E*10–and *E*12–14:OAC and synergistic behavioral activity of *E*10,*E*12–14:OAC in field experiments provide evidence that *E*10,*E*12–14:OAC could indeed be produced by ABLM females and be one of their pheromone components.

Equal attractiveness of 0.1:10 or 1:10 ratios of *E*10,*E*12– and *Z*10,*Z*12–14:OAc is indicative that "precise" relative proportions of these two pheromone components are not critical for optimal attraction of ABLM males. The western avocado leafroller, *Amorbia cuneana* (Walsingham), in contrast, responds best to a specific 1:9 ratio of *E*10,*E*12– and *E*10,*Z*12–14:OAc (Bailey et al., 1986). Because attraction of ABLM males to crude, non-HPLC purified *Z*10,*Z*12–14:OAc with >1% of *E*10,*E*12–14:OAC could not be improved by addition of *E*10,*E*12–14:OAC, trace amounts (≤1%) of the *EE* isomer seem sufficient to express synergistic behavioral activity. Other geometrical isomers of *Z*10,*Z*12–14:OAC at ≤10% of the volatile blend did not enhance attractiveness of the lure and are obviously not part of the ABLM sex pheromone. Similarly, *E*10–12:OAc was present in female ABLM pheromone extracts but in field experiments singly or in combination with *Z*10,*Z*12–14:OAc failed to attract ABLM males or to enhance attractiveness of the major pheromone component.

Previously, *Z*10,*Z*12–14:OAC was reported as the major pheromone component in *Notocelia uddmanniana* (L.) (Witzgall et al., 1991); this study reports it for the second time as a major pheromone component. This conjugated diene acetate as well as isomeric and synergistic *E*10,*E*12–14:OAC contrast with other monoene or nonconjugated diene acetate pheromone components reported for *Phyllonorycter* moths. Behaviorally benign in ABLM, *E*10–12:OAc attracts *P. blancardella* (Roelofs et al., 1977) and *P. mespilella* (Gries et al., 1993). *P. mespilella*-produced *E*10–12:OAC and *E*4,*E*10–12:OAC are attractive by

themselves, but unlike the ABLM pheromone components, are not synergistically attractive. Whether *Z*10–14:OAC and *E*4,*Z*10–14:OAC produced by *P. ringoniella* (Matsumura) (Sugie et al., 1986) are singly or synergistically attractive remains to be investigated.

Effect of P. crataegella *Sex Pheromone on* P. mespilella *Pheromone Communication*

Failure of the ABLM pheromone blend to attract any *Phyllonorycter* moths in the Okanagan Valley supports contention that ABLM is not present in the fruit-growing region of British Columbia. Strong attraction of *P. mespilella* males to synthetic *P. mespilella* pheromone *E*4,*E*10–12:OAc and inhibition of pheromonal attraction in the presence of the ABLM pheromone blend clearly indicates recognition of the heterospecific ABLM pheromone blend by *P. mespilella* males. Similarly, response of male oblique-banded leafrollers (OBLR), *Choristoneura rosaceana* (Harris), to the OBLR pheromone is inhibited in the presence of (*Z*)-9-tetradecenyl acetate (*Z*9–14:OAc), a minor pheromone component of the sympatric three-lined leafroller, *Pandemis limitata* (Robinson) (Cardé and Baker, 1984; Evenden, 1998). The effect of ABLM components *Z*10,*Z*10–14:OAC and *E*10,*E*12–14:OAc tested singly indicated that inhibition of *P. mespilella* males is associated with *Z*10,*Z*12–14:OAC. This inhibition supports the hypothesis of a phylogenetic relationship and previous sympatry of ABLM and *P. mespilella*. It would now be of great interest to test possible reciprocal inhibition of ABLM males by *P. mespilella* pheromone components. Moreover, *Z*10,*Z*12–14:OAc may also be a pheromone component of one or more *Phyllonorycter* spp. that are currently sympatric with *P. mespilella* in Europe.

Potential for Pheromone-Based Control of P. crataegella

With the identification of the ABLM pheromone blend there is now an opportunity for pheromone-based ABLM control. While natural pheromone blends may be most effective and may require minimal amounts of pheromone to disorient mate-seeking males (Roelofs, 1978; Minks and Cardé, 1988), there is increasing evidence that off-ratio blends should also be considered for pheromone-based mating disruption. For instance, in mating disruption trials with the codling moth, *Cydia pomonella* (L.), an isomeric mixture of (*E*)-8,(*E*)-10- (61%), (*E*)-8,(*Z*)-10- (20%), (*Z*)-8,(*E*)-10- (14%), and (*Z*)-8,(*Z*)-10- (5%) dodecadienol, or *EZ*- and *ZE*-dodecadienol isomers individually, disrupted *C. pomonella* sexual communication more effectively than did the pure (*E*)-8,(*E*)-10-dodecadienol pheromone component (McDonough et al., 1994). Because *E*10,*Z*12–14:OAC slightly reduced attraction of ABML males to pher-

omonal *Z*10,*Z*12-plus *E*10,*E*12-14:OAC, it may be affective as an ABLM mating disruptant. ABLM pheromone component Z10,Z12-14:OAc greatly inhibited response of *P. mespilella* males to *P. mespilella* pheromone. If ABLM males were reciprocally inhibited by *P. mespilella* pheromone, a generic "disruptive" blend containing ABLM, *P. mespilella* and possibly even *P. blancardella* pheromone could be developed for pheromone-based control of *Phyllonorycter* leafminers in North American apple orchards.

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GEOGRAPHIC VARIATION IN SEX PHEROMONE OF ASIAN CORN BORER, *Ostrinia furnacalis*, IN JAPAN[1]

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Abstract—Geographic variation in the sex pheromone of the Asian corn borer, *Ostrinia furnacalis* (Guenée), was surveyed in populations sampled at four locations ranging from 39.7°N to 32.9°N in Japan. The sex pheromone of the three northern populations was composed of (*E*)- and (*Z*)-12-tetradecenyl acetates with a mean *E* proportion of 36–39%. The southernmost population (Nishigoshi) had the same components but with a significantly higher *E* composition of 44%. The frequency distribution of the *E* ratio in the Nishigoshi population exhibited a small peak near 38% and a major peak near 46%. A family-wise analysis of the sex pheromone of this population confirmed that there were two distinct phenotypes regarding the *E* ratio. An "≈46% *E* strain" inhabits southern parts of Japan, in addition to an "≈38% *E* strain," which seems to be predominant in other regions of Japan.

Key Words—*Ostrinia furnacalis*, sex pheromone, (*E*)-12-tetradecenyl acetate, (*Z*)-12-tetradecenyl acetate, field trap experiment, geographic variation.

INTRODUCTION

The Asian corn borer, *Ostrinia furnacalis* (Guenée) (Lepidoptera: Pyralidae), is one of the most serious pests of maize in Japan and China as well as other

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[1]Studies on sex pheromones of *Ostrinia* spp. in Japan. III.

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Asian countries. Because of its economic importance, the sex pheromone of this species has been intensively studied. Ando et al. (1980) analyzed sex pheromone gland extracts of *O. furnacalis* from Japan, and identified (*E*)- and (*Z*)-12-tetradecenyl acetates (*E*12- and Z12-14:OAc) at a ratio of about 2:3 by gas chromatography (GC) and GC combined with mass spectrometry (GC-MS). Because of the failure of the synthetic compounds to catch males in field-trap experiments, it was suggested that minor component(s) were also present in the sex pheromone communication system of *O. furnacalis* (Ando et al., 1980). Klun et al. (1980) independently reported in the same year that the sex pheromone of *O. furnacalis* (Philippine population) was composed of *E*12- and Z12-14:OAc at a ratio of about 1:1 (combined wash, 53:47). Similar results were subsequently obtained in analyses of the Chinese and Taiwan populations, and the males were successfully captured with the two components at a ratio of 53:47 in field experiments conducted in China and Taiwan (Cheng et al., 1981; Klun et al., 1980; Kou et al., 1992).

In our phylogenetic studies of seven *Ostrinia* species in Japan (*O. furnacalis*, *O. orientalis*, *O. scapulalis*, *O. zealis*, *O. zaguliaevi*, *O. palustralis*, and *O. latipennis*), we have recently started to identify the sex pheromone components and their ratio in each *Ostrinia* species (Huang et al., 1997, 1998). As noted above, the sex pheromone of the Japanese population of the Asian corn borer has not yet been completely determined. It is very important to determine the sex pheromone of Japanese *O. furnacalis*, not only from an agricultural standpoint but also in phylogenetic analyses of *Ostrinia* spp.

In the present study, the sex pheromone of *O. furnacalis* from a geographic population (Matsudo) was first investigated by laboratory analysis of the component ratio and by field trapping tests. Secondly, geographic variation in the component ratio within Japan was investigated because, although the Matsudo population turned out to have an *E*/*Z* ratio near 2:3, all of the other Asian populations examined to date have a ratio near 1:1, and many insects are known to migrate from Southeast Asian countries to the southern parts of Japan every year, thus there is a possibility that a population with a different ratio inhabits that part of Japan.

METHODS AND MATERIALS

Insects. Larvae of *O. furnacalis* infesting maize were collected from the field at Matsudo (35.8°N, 139.9°E) in August–September 1996 (Figure 1). In August–September 1997, the insects were collected at three additional locations: Morioka (39.7°N, 141.1°E), Fukuyama (34.6°N, 133.3°E), and Nishigoshi (32.9°N, 130.7°E). Larvae were reared on a commercial diet for insects (Insecta LF, Nihon Hosan, Yokohama, Japan) in an environmental chamber maintained

FIG. 1. Collection sites of *Ostrinia furnacalis*.

at 25°C and 50–60% relative humidity with a 15-hr light–9-hr dark photocycle. Pupae were sexed based on the morphology of a few terminal abdominal segments and maintained under the same conditions as for larval rearing. For the examination of variation in the sex pheromone component ratio within a family, several newly emerged females and males of the Nishigoshi population were placed in a screen cage (30 × 30 × 30 cm) and allowed to mate freely for two nights. On the next day, the females were individually transferred to a transparent plastic cup with a lid. The egg masses laid on the inner surface of the cup were removed, disinfected in 3% formalin for 15 min, and placed on the artificial diet in a container with the mother's identity marked.

Sex Pheromone Extraction. Sex pheromone was extracted from individual females during the calling period late in the scotophase. The abdominal tip including the sex pheromone gland was excised from a 2- to 4-day-old virgin female, and immersed for 30 min in 8 μl hexane containing tridecyl acetate as an internal standard (1 ng/μl). The extract was concentrated to <2 μl under a nitrogen stream and injected into the gas chromatograph.

Chemicals. Tridecyl acetate (99.7%), used as an internal standard, was prepared from tridecanol and acetic acid anhydride and purified by silica gel chromatography. (*E*)-12-Tetradecenyl acetate (*E*12–14:OAc, 99.5% isomeric purity) and (*Z*)-12-tetradecenyl acetate (*Z*12–14:OAc, 99.7%) were obtained from the Research Institute for Plant Protection (Wageningen, The Netherlands). All other compounds were purchased from Wako Pure Chemical Industries (Kyoto, Japan) or Sigma Chemical Co. (St. Louis, Missouri). Hexane was redistilled before use.

Chemical Analysis. Gas chromatography of the extracts was performed on

a 17-A GC (Shimadzu Corp., Kyoto) fitted with a flame ionization detector (FID), a split/splitless injector and a fused silica capillary column (DB-Wax, 30 m × 0.25 mm ID, J&W Scientific, Folsom, California). The time for splitless injection was 2 min. The oven temperature was maintained at 100°C for 2 min and then programmed at a rate of 5°C/min to 240°C. The injector and detector temperatures were 250°C. The carrier gas was nitrogen. The identification of the compounds was based on the comparison of the retention times with those of authentic standards. The quantity of each component was calculated by comparing the peak area with that of 13:OAc.

Field Trapping. Field-trap experiments were conducted at the experimental farm of Tokyo University at Tanashi (about 40 km southeast of Matsudo, 35.7°N, 139.5°E), Tokyo, Japan. Test chemicals were dissolved in hexane and loaded on rubber septa (Aldrich Chemical Co., Milwaukee, Wisconsin). Sticky traps (Nitolure, Nitto Denko, Osaka, Japan) were hung 50 cm above the ground, and the insects trapped were counted every three days. Traps in an experimental block were set 10 m apart from each other. The distance between the blocks was more than 50 m. Three 2-day-old virgin females were employed as a bait source for comparison, and they were changed every three days. For each dosage, three replicates were used.

RESULTS

Titers and Ratios of Sex Pheromone Components. The analysis of sex pheromone gland extracts from virgin females of *O. furnacalis* showed that the individual variation of the titer was very large (Table 1). For instance, the amounts of *E*12–14:OAc and Z12–14:OAc in a female pheromone gland of

TABLE 1. AMOUNT OF SEX PHEROMONE COMPONENTS AND PERCENTAGE OF *E* ISOMER IN FEMALE SEX PHEROMONE GLAND OF *Ostrinia furnacalis* COLLECTED AT 4 LOCATIONS IN JAPAN

Component	Morioka (N = 11)	Matsudo (N = 39)	Fukuyama (N = 41)	Nishigoshi (N = 100)
*E*12–14:OAc (ng)[a]	1.4 ± 1.4	3.1 ± 1.3	2.2 ± 1.3	3.6 ± 2.7
*Z*12–14:OAc (ng)[a]	2.3 ± 2.4	5.3 ± 3.4	3.5 ± 1.9	4.2 ± 3.0
% *E*[b]	38.6 ± 5.3 ab	35.6 ± 5.5 a	38.8 ± 6.9 a	43.9 ± 6.8 b

[a]Mean ± SD.
[b]Means of % *E* in individual females. Values with the same letters are not significantly different from each other according to Tukey-Kramer multiple range test at the 5% confidence level.

the Matsudo population varied from 0.5 to 4.5 (3.1 ± 1.3) ng, and from 0.3 to 6.8 (5.3 ± 3.4) ng, respectively. Despite these large variations, the ratio of the two components was comparatively stable, varying from 24.0 to 48.1% (35.6 ± 5.5%) *E* in the population (Figure 2). The ratio of the two components in the Matsudo population was close to the ratio reported by Ando et al. (1980).

Field Trapping. Trap catches of *O. furnacalis* males with three virgin females (3 FE) and the binary blend of synthetic *E*12–14 : OAc and Z12–14 : OAc (36 : 64) at various doses were compared. Trap capture increased with the dose range from 1 to 100 μg. A further increase in dose resulted in a decrease of captured males (Figure 3). Lures loaded with 100 μg of the binary blend captured significantly more males than did the virgin females.

Binary blends of *E*12–14 : OAc and *Z*12–14 : OAc with different ratios were tested in the field to determine the optimal ratio for the attraction of males (Figure 4). The ratio of 36 : 64 (*E*/*Z*) was the most attractive, whereas the ratio of 50 : 50 (*E*/*Z*) captured significantly fewer males. Any larger deviation from the range 36 : 64–50 : 50 (*E*/*Z*) lowered the trap catch to a level not significantly different from that of the control (Figure 4). These results indicated that the sex

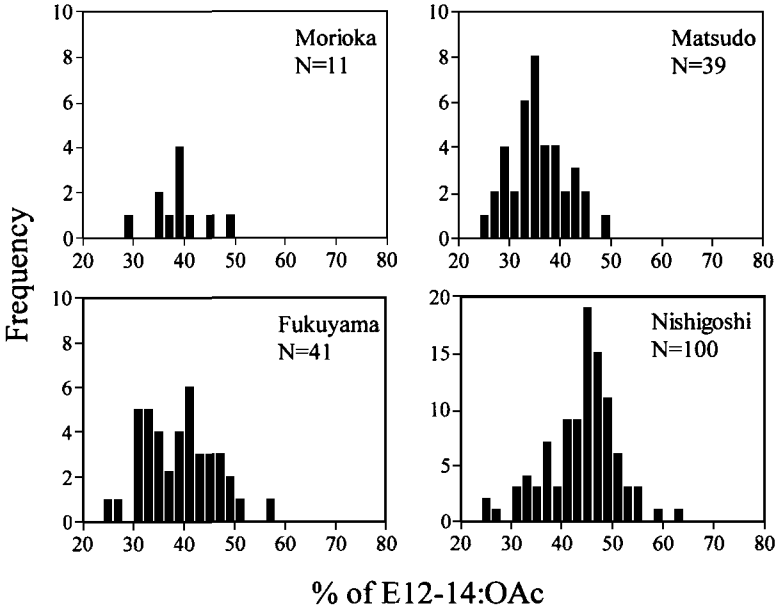

FIG. 2. Distribution of the percentages of *E*12–14 : OAc in the female sex pheromone of *Ostrinia furnacalis* collected at four locations in Japan.

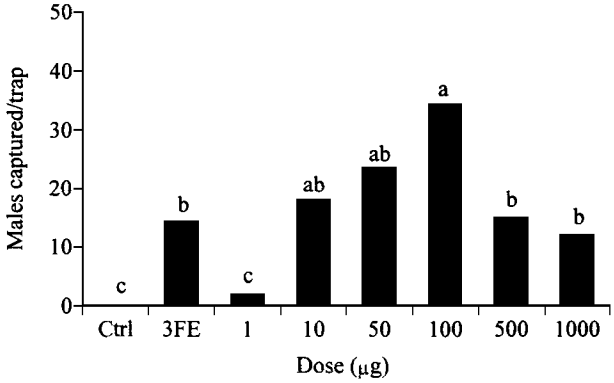

FIG. 3. Trap catches of *O. furnacalis* males with three virgin females (3 FE) and the binary blend of synthetic *E*12–14:OAc and *Z*12–14:OAc (36:64) at various doses. The experiment was conducted at Tanashi, Tokyo, on September 13–21, 1996. Bars with the same letters are not significantly different according to Tukey-Kramer multiple range test at the level of 5%. Ctrl = control.

pheromone of a Japanese population (Matsudo) of the Asian corn borer is composed of *E*12–14:OAc and *Z*12–14:OAc at the ratio of 36:64.

Geographic Variation. There was a significant difference in the proportion of *E*12–14:OAc (%) among the populations (Table 1, *F* test, $P < 0.001$). The

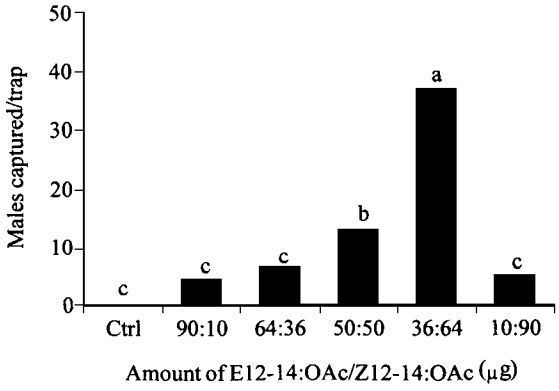

FIG. 4. Attractiveness of the different blends of *E*12–14:OAc and *Z*12–14:OAc in the field. The experiment was conducted at Tanashi, Tokyo, on May 29–June 18, 1997. Bars with the same letters are not significantly different according to Tukey-Kramer multiple range test at the level of 5%. Ctrl = control.

TABLE 2. AMOUNT OF SEX PHEROMONE COMPONENTS AND PERCENTAGE OF *E*. ISOMER IN FEMALE SEX PHEROMONE GLAND OF NISHIGOSHI POPULATION OF *Ostrinia furnacalis* (N = 20 EACH)

	Family				
Component	NG1	NG2	NG3	NG4	NG5
*E*12–14:OAc (ng)[a]	3.3 ± 2.2	2.9 ± 1.8	5.1 ± 3.5	1.3 ± 1.6	3.6 ± 2.5
*Z*12–14:OAc (ng)[a]	3.8 ± 2.3	3.2 ± 1.8	5.9 ± 4.1	1.9 ± 1.9	3.8 ± 2.4
% *E*[b]	43.6 ± 5.0 a	46.2 ± 4.0 a	45.2 ± 6.5 a	37.6 ± 8.0 b	46.8 ± 6.2 a

[a]Mean ± SD.
[b]Means of % *E* in individual females. Values with the same letters are not significantly different from each other according to Tukey-Kramer multiple range test at the 5% confidence level.

population obtained from Nishigoshi showed a significantly higher *E* ratio (44%) compared with the other three populations (36–39%) (Table 1). Analyses of the *E* ratio in each individual revealed that the Fukuyama and Nishigoshi populations had greater variations in the *E* ratio compared with the other two northern populations (Figure 2). The distributions of the *E* ratio in the Fukuyama and Nishigoshi populations were not unimodal.

Variation of Component Ratio Within a Family and Among Families. To examine whether the shift of composition in the Nishigoshi population has a genetic basis, we analyzed the intra- and interfamily variation of the pheromone composition (Table 2). Among the five families investigated, four families (NG1–NG3, NG5) showed a sex pheromone composition with *E* ratios at 44–47%, and one family (NG4) showed a significantly lower *E* ratio of 38%.

DISCUSSION

Based on laboratory analyses and field trap experiments, we conclude that the sex pheromone of *O. furnacalis* (Matsudo population) is composed of *E*12–14:OAc and *Z*12–14:OAc at the ratio of 36:64, confirming the results of Ando et al. (1980). The binary blend at a dose of 100 μg attracted nearly two times more males than did virgin females in our field experiment. The reason why Ando et al. (1980) failed to capture males in their field experiments cannot be answered decisively, because there is no description about the field-trap experiments in their report except that "In the field trials with two synthetic compounds, we did not succeed in attracting the male moths, although the crude extract from the virgin females possessed attractive activity." Since the sex

pheromone components and the ratio were correctly identified by Ando et al. (1980), it is possible that the dose of the synthetic lure was not in the optimum range and/or the type of trap and/or lure dispenser utilized were inappropriate.

The present results indicated that sex pheromone polymorphism exists in the Asian corn borer in Japan. The differences in pheromone blend cannot be ascribed to differences in climate of the original habitats, since all the larvae were brought to the laboratory and reared under the same conditions. A strain with $\approx 46\%$ *E* inhabits the southernmost part of Japan in addition to a strain with $\approx 38\%$ *E*, which seems to be predominant in other regions of Japan. The frequency distribution of *E* ratios in the Fukuyama population also was not unimodal. A family-wise analysis of this population is required to determine whether the distribution of the 46% *E* strain extends to Fukuyama.

The sex pheromone blends of *O. furnacalis* in Taiwan, the Philippines, and the south part of the People's Republic of China have all been reported to be 53:47 (Cheng et al., 1981; Klun et al., 1980; Kou et al., 1992) and are slightly different from the Japanese 46:54 blend. However, the blend ratio reported for the former Asian countries is based on the combined wash of the sex pheromone glands, rather than individual analyses. Klun et al. (1980) reported that the *E* ratio in the Philippine population was 47.7:52.3 when seven females were analyzed individually. At present, whether the 46% *E* strain in Japan is equivalent to the 53% *E* strain in Southeast Asia is open to question. This question must be answered before addressing interesting and important questions such as whether the 46% *E* strain is established in the southern parts of Japan or is replenished annually by immigration.

Field experiments conducted in Taiwan indicated that a 40% *E* lure (Japan formulation) was as attractive as a 53% *E* lure (Taiwan formulation) (Kou et al., 1992). However, in the present field-trapping test conducted at Tanashi, Japan, the 50% *E* lure was significantly less attractive than the 36% *E* lure.

The European corn borer *O. nubilalis* uses (*E*)- and (*Z*)-11-tetradecenyl acetates as sex pheromone components and is known to be polymorphic with respect to female sex pheromone composition: *E*-strain females produce an *E*:*Z* blend of 99:1, whereas *Z*-strain females produce a 3:97 blend (Cardé et al., 1978; Klun and cooperators, 1975; Kochansky et al., 1975). In *O. nubilalis*, the genetic control of the sex pheromone blend has been clarified; the sex pheromone blend is basically determined by an autosomal locus with two codominant alleles (Roelofs et al., 1987).

The establishment of pure 38% *E* and 46% *E* strains and crossing experiments with these two strains are required to determine whether the pheromone blend in *O. furnacalis* is also genetically determined. In this species, however, detection of the hybrid is very difficult compared with *O. nubilalis*, because of the large individual variation in the *E* ratio in each strain and a smaller difference

in *E* ratios between the two strains (≈99% *E* and ≈3% *E* in *O. nubilalis*, and ≈38% *E* and ≈46% *E* in *O. furnacalis*).

A sex pheromone polymorphism very similar to that of *O. nubilalis* was recently found in the adzuki bean borer, *O. scapulalis* (Huang et al., 1997). In this species, the genetic mechanism controlling the pheromone blend also seems to be similar to that of *O. nubilalis* (Takanashi, unpublished data). The presence of sex pheromone polymorphism in the three species (*nubilalis*, *scapulalis*, and *furnacalis*) in the genus *Ostrinia* may indicate that polymorphic pheromones are not unusual in this genus.

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ROLE OF ISOFLAVONOIDS IN RESISTANCE OF SUBTERRANEAN CLOVER TRIFOLIATES TO THE REDLEGGED EARTH MITE *Halotydeus destructor*

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Abstract—Mature leaves of variety SE014 of *Trifolium subterraneum* are resistant to the redlegged earth mite (*Halotydeus destructor*). Compounds with feeding deterrent activity present in the leaves were isolated and identified by using a membrane bioassay technique. The free isoflavones, formononetin, genistein, and biochanin A, their corresponding 7-*O*-glucosides, and biochanin A 7-*O*-glucoside-6″-*O*-malonate and genistein 7-*O*-glucoside-6″-*O*-malonate, were active. In addition, the biosynthetic precursors of formononetin and genistein, 2,5,7-trihydroxy-4′-methoxyisoflavanol and 2,5,7,4′-tetrahydroxyisoflavanol, also were deterrent to the redlegged mites. The relative activity of these compounds was determined and some structure–activity correlations are noted. The constitutive defense mechanism that contributes to the resistance of subclover trifoliates to the mite contrasts with the induced chemical mechanism previously found for subterranean clover cotyledons.

Key Words—*Trifolium subterraneum*, trifoliates, *Halotydeus destructor*, free isoflavones, bound isoflavones, resistance, deterrent activity.

INTRODUCTION

Redlegged earth mite, *Halotydeus destructor* Tucker (Acarina: Penthaleidae), is a destructive pest of pasture legumes, particularly *Trifolium subterraneum* L.

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(subterranean clover; subclover) in southern Australia. Considerable effort has been invested in developing resistant plant varieties of subclover, for medics (*Medicago* sp.) and for canola (*Brassica napus*) to reduce the economic losses caused by the mite (Ridsdill-Smith, 1997).

In previous work, we have shown that resistance of subclover cotyledons to feeding by redlegged earth mite was correlated with the amount of 1-octen-3-one produced by the cotyledons (Jiang et al., 1996a) and with cotyledon strength (Jiang and Ridsdill-Smith, 1996a,b). The production of the volatile compound 1-octen-3-one, formed by lipid peroxidation of linoleic acid, is induced during damage of the cotyledons by mite feeding. In continuation of this work, we have investigated the factors involved in the resistance of trifoliates of subclover to the mite. Separate studies (Ridsdill-Smith, in preparation) have shown that resistance in trifoliates, while still significant, was less obvious than that observed with cotyledons. Moreover, preliminary studies indicated that the induced resistance found for cotyledons was less important for trifoliates and that constitutive resistance might play a significant role.

The aims of the present work are to identify the individual metabolites with deterrent activity from the trifoliates of the resistant subclover variety SE014 and to investigate the contribution of these compounds to the resistance of trifoliates to redlegged earth mite.

METHODS AND MATERIALS

Plant Material. Seeds of subclover (resistant variety SE014) were sown in pots with standard soil mix (Ridsdill-Smith and Gillespie, 1993). The pots were moved to a glasshouse after seed germination. The mature leaves (four to five weeks after seeding) were used for the experiments and analyses.

Redlegged Earth Mites. Mites were cultured in the laboratory on vetch (*Vicia sativa* cv. Blanchefleur, Leguminosae) in summer and collected from pasture near Perth, Western Australia, in winter. Mites were staged, and young adults, starved for 2 hr in a humid vial (15°C), were used for experiments.

Deterrent Assay. Extracts from the clover trifoliate leaves were bioassayed for feeding deterrence towards redlegged earth mites. The membrane sachet technique previously described (Jiang et al., 1996b) was used. Extracts to be tested were solubilized in 5% Tween 80 containing 1% glucose, and control solutions contained only 5% Tween 80 and 1% glucose. Mites were given a choice of a sachet containing a feeding stimulant (1% aqueous glucose) with or without the test sample (extract, samples from chromatography fractions, or pure compounds). Membrane sachets were made with stretched Parafilm. For each sachet, a membrane was stretched over a 2-cm-diameter plastic ring, 35 μl of test solution added, and a second membrane stretched over the top. In

choice experiments, two membrane sachets were buried, nearly touching, so as to be just above the surface of the soil (4:1 sand to loam) which was moistened close to field capacity, in a three-quarters filled plastic jar. Twenty mites were released by gently tapping them from a vial onto the soil beside the membrane sachets in the plastic jar, which was then sealed with stretched parafilm to contain the mites. Experiments were carried out at 15°C under fluorescent light in a room with no natural light. Mites were observed through the parafilm membrane, and the number of mites on each membrane sachet was counted at 20- or 30-min intervals for 3 hr. Deterrence in the choice test for each experiment was calculated from the average of seven observation as follows: (mite number on treatment-mite number on control)/(total number of mites) × 100. A negative value is taken to refer to deterrence. Differences between means were examined with paired *t* tests.

General Analytical Methods. ^{1}H and ^{13}C NMR spectra were obtained for CD_3OD, acetone-d_6, DMSO-d_6, or D_2O in a Bruker AM-500 instrument. Mass spectra were measured with a VG Autospec Mass Spectrometer (70 eV). Flash column chromatography was performed with 40–60 μm Si gel (BDH) and Si gel 100 C-18-reverse phase (Merck) as the stationary phase. For TLC, precoated AL SIL G/UV (Whatman) plates were used, and the compounds were detected by spraying with $CeSO_4$ in concentrated H_2SO_4. For HPLC, an HP 1050 instrument equipped with an HP ODS hypersil column (5 μm, 250 × 4 mm) was used. A gradient from water (2% AcOH)–MeOH (4:1) to MeOH was used as the solvent.

Extraction and Isolation. The bioassay technique described above was used to guide the extraction and fractionation of the plant extract and to determine the activity of the pure compounds. Trifoliates of subclover (600 g) were frozen in liquid nitrogen, powdered, and extracted with MeOH at room temperature (4×). The combined MeOH solutions were concentrated under reduced pressure to yield 28 g of extract. A portion (10 g) of this extract was partitioned between C_6H_{14} (15% of extract), CH_2Cl_2 (22%), EtOAc (8%), *n*-BuOH (22%), and H_2O (33%). Samples of each of these fractions were tested for antifeedant activity. The active C_6H_{14} and CH_2Cl_2 fractions were combined, dissolved in $CHCl_3$, and extracted with 1% $NaOH_{aq}$. The basic solution was acidified with 2.5 M HCl_{aq} to pH 5, extracted with EtOAc, and dried over Na_2SO_4. The extract was subjected to flash column chromatography with hexane–EtOAc to give seven fractions (A1–A7).

Fraction A3 consisted of biochanin A (**1**; Figure 1), and fraction A4 contained genistein (**2**) and formononetin (**3**). Fraction A5 was separated by repeated flash column chromatography to give 2,5,7-trihydroxy-4′-methoxyisoflavanol (**4**). The purification of fraction A6 by flash column chromatography yielded 2,5,7,4′-tetrahydroxyisoflavanol (**5**).

The EtOAc fraction was subjected to column chromatography on Sephadex

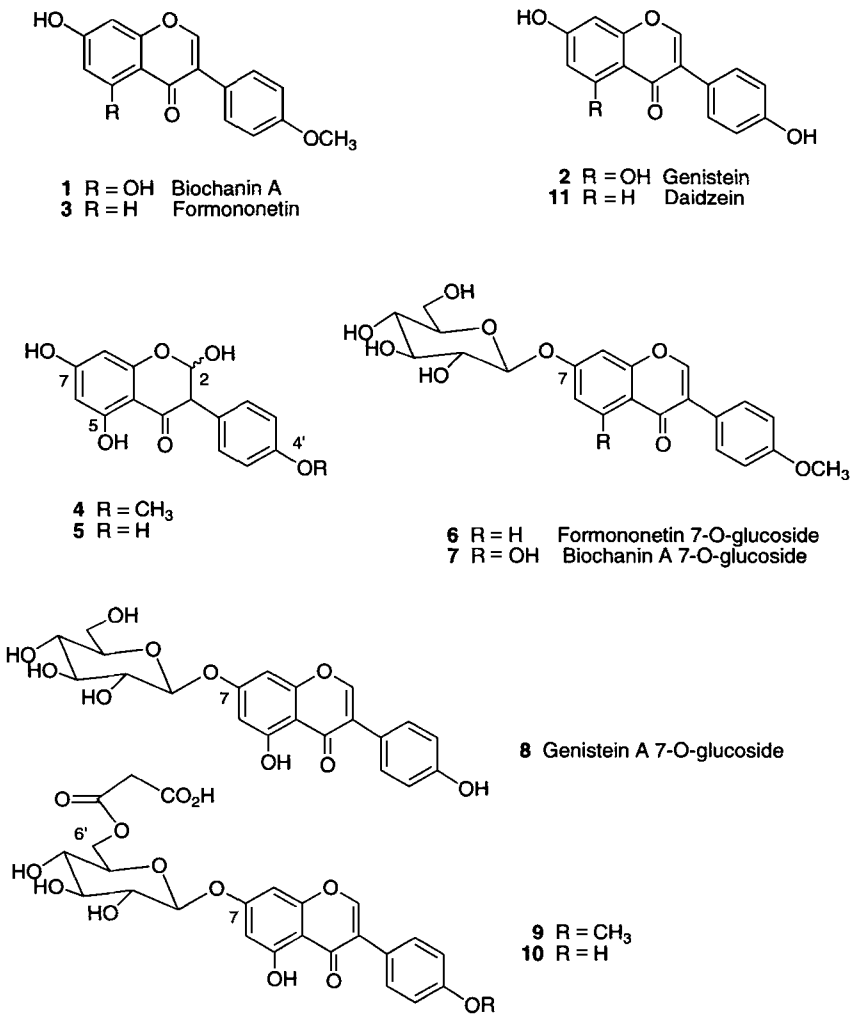

FIG. 1. Compounds isolated from the leaves of *Trifolium subterraneum* (variety SE014). The number **1–11** refer to the compounds described in the text.

LH-20 (MeOH) to give six fractions (B1–B6). Formononetin-7-*O*-glucoside (**6**), biochanin A-7-*O*-glucoside (**7**), and genistein-7-*O*-glucoside (**8**) were obtained by flash column chromatography (Si gel; gradient elution; CH_2Cl_2 in MeOH) from fractions B3 and B4. Fraction B5 was purified by reverse-phase column chromatography (C-18) by a gradient of MeOH in H_2O to yield biochanin A 7-*O*-glucoside-6″-*O*-malonate (**9**) and genistein 7-*O*-glucoside-6″-*O*-malonate (**10**).

Biochanin A (**1**), genistein (**2**), and formononetin (**3**) were identified by comparison (TLC and HPLC) to authentic samples. Their ^{1}H-NMR spectral data were in accordance with those reported by Beck and Knox (1971).

2,5,7-Trihydroxy-4′-methoxyisoflavanol (**4**) and 2,5,7,4′-tetrahydroxyisoflavanol (**5**) were identified as described in Wang et al. (1997). The spectral data of the remaining compounds **6–10** were compared with those described in the literature (Beck and Knox, 1971; Köster et al., 1983). Updated NMR spectral data for compounds **6**, **7**, and **9** are given below.

Formononetin-7-O-Glucoside (6). ^{1}H NMR (CD_3OD, 500 MHz): 8.23 (1H, s, H-2), 8.14 (1H, d, J = 8.9 Hz, H-5), 7.22 (1H, dd, J = 2.3, 8.9 Hz, H-6), 7.26 (1H, d, J = 2.3 Hz, H-8), 7.48 and 6.98 (4H, AA′BB′, H-2′, 6′ and H-3′, 5′), 5.12 (1H, dd, J = 7.5 Hz, H-1″), 3.53 (1H, ddd, J = 2.2, 5.8, 9.6 Hz, H-5″), 3.42–3.52 (3H, m, H-2″–4″), 3.92 (1H, dd, J = 2.2, 12.0 Hz, H-6a″), 3.72 (1H, dd, J = 2.2, 12.0 Hz, H-6b″), 3.83 (3H, s, OMe).

Biochanin A-7-O-Glucoside (7). ^{1}H NMR (CD_3OD, 500 MHz): 8.12 (1H, s, H-2), 6.64 (1H, d, J = 2.2 Hz, H-6), 6.44 (1H, d, J = 2.2 Hz, H-8), 7.43 and 6.91 (4H, AA′BB′, H-2′, 6′ and H-3′, 5′), 5.00 (1H, d, J = 7.5, H-1″), 3.42–3.55 (4H, m, H-2″–5″), 3.93 (1H, dd, J = 2.2, 12.0 Hz, H-6a″), 3.73 (1H, dd, J = 5.5, 12.0 Hz, H-6b″), 3.82 (3H, s, OMe).

Biochanin A 7-O-Glucoside-6″-O-Malonate (9). ^{1}H NMR (DMSO-d_6, 500 MHz): 8.44 (1H, s, H-2), 7.53 and 7.02 (4H, AA′BB′, H-2′, 6′ and H-3′, 5′), 6.72 (1H, d, J = 2.2 Hz, H-6), 6.48 (1H, d, J = 2.2 Hz, H-8), 5.12 (1H, d, J = 7.5 Hz, H-1″), 3.15–3.45 (4H, H-2″–H5″), 4.37 (1H, dd, J = 1.8, 11.8 Hz, H-6a″), 4.13 (1H, dd, J = 7.0, 11.8 Hz, H-6b″), 3.38 (1H, s, H-2″), 3.78 (3H, s, OMe); ^{13}C NMR (125 MHz): 154.8 (C-2), 122.6 (C-3), 180.3 (C-4), 161.5 (C-5), 99.4 (C-6), 162.6 (C-7), 94.5 (C-8), 157.1 (C-9), 106.1 (C-10), 122.1 (C-1′), 130.1 (C-2′, 6′), 113.6 (C-3′, 5′), 159.1 (C-4′), 99.4 (C-2″), 72.8 (C-3″), 73.6 (C-4″), 69.5 (C-5″), 76.0 (C-6″), 63.9 (C-7″), 167.9 (C-1‴), 41.4 (C-2‴), 166.8 (C-3‴), 55.1 (OMe).

Chemicals. Genistein (**2**) and daidzein (**11**) were purchased from Sigma and ICN. The other compounds tested were isolated from the mature leaves of subclover as described.

RESULTS

Bioassays of Extract and Fractions from Subclover Variety SE014. Bioassays of the methanol extract obtained from the trifoliates of the resistant varieties SE014 showed feeding deterrence to the redlegged earth mites (Figure 2). The fractions obtained by solvent partitioning of the methanol extracts with hexane, dichloromethane, ethyl acetate, *n*-butanol and water were then tested for deterrence. For the first three fractions, fewer mites gathered on the membrane sach-

ets containing test solution than the control solution (Figure 2). The hexane fraction appeared to have the strongest effect (80% deterrence), and the ethyl acetate and dichloromethane fractions showed 73% and 64% deterrence, respectively. The extracts from the more polar fractions, *n*-butanol, and water showed no activity.

Identification of Active Components from Subclover Variety SE014. Separation of the components of the first three fractions mentioned above was conducted by bioassay-guided fractionation. The acidic portion of the hexane and dichloromethane fractions were combined because of their similar composition (TLC) and after chromatography yielded biochanin A (**1**), genistein (**2**), and formononetin (**3**), which were identified by comparison with authentic samples. In addition, the two isoflavanols 2,5,7-trihydroxy-4′-methoxyisoflavanol (**4**) and 2,5,7,4′-tetrahydroxyisoflavanol (**5**) also were isolated. Evidence for the structure of these two isoflavanols, isolated for the first time as natural products, is described elsewhere (Wang et al., 1997).

The ethyl acetate fraction was separated on Sephadex LH-20 with MeOH as eluent to give six fractions (B1–B6) and repeated column chromatography of

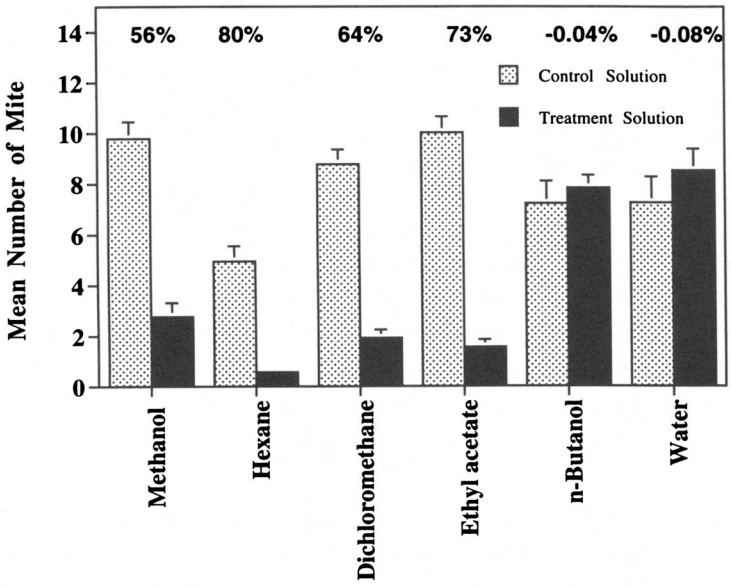

FIG. 2. Deterrent activity of methanol extract obtained from variety SE014 and portions of the extract soluble in various solvents. The values given above each treatment–control combination refer to the percentage deterrence calculated.

individual fractions gave the 7-*O*-glucosyl derivatives of biochanin A (**6**), genistein (**7**), and formononetin (**8**), and the 7-*O*-glucoside-6″-*O*-malonate derivatives of biochanin A (**9**) and genistein (**10**).

Bioassays of Isoflavones. The free isoflavones biochanin A (**1**), genistein (**2**), formononetin (**3**), and daidzein (**11**) were tested at concentrations between 0.5% and 0.01% (w/v) (Table 1). Generally, mites avoided gathering on the membrane sachets containing treatment solution. At lower concentrations of the isoflavones 0.01% genistein, 0.015% formononetin, and 0.1% daidzein, more mites gathered on test solutions. The level of deterrence of isoflavones to the mites was positively correlated with the logarithm of their concentrations, as illustrated for formononetin (Figure 3). The lowest concentration at which deterrence was observed for genistein (**2**) was 0.045% (68%), for biochanin A (**1**) 0.15% (80%), and for formononetin (**3**) 0.15% (49%). In contrast to the other three isoflavones, daidzein (**11**)) was active only at high concentration (0.5%)

TABLE 1. MEAN NUMBER OF MITES ON CONTROL AND TREATMENT MEMBRANE SACHETS CONTAINING BIOCHANIN A, GENISTEIN, FORMONONETIN, AND DAIDZEN DURING 3 HOURS[a]

Concentration (%)	Control	Treatment	Deterrence (%)[b]
Genistein			
0.08	8.68 ± 0.469	0.31 ± 0.0840	93.2***
0.045	3.20 ± 0.348	0.60 ± 0.197	68.4***
0.01	1.77 ± 0.327	3.37 ± 0.508	−31.0
Biochanin A			
0.5	7.78 ± 1.14	0.36 ± 0.103	91.1***
0.15	4.08 ± 0.479	0.44 ± 0.086	80.5***
0.05	2.04 ± 0.204	2.83 ± 0.410	−16.2
0.01	2.43 ± 0.497	2.74 ± 0.354	−5.9
Formononetin			
0.5	8.55 ± 0.719	0.56 ± 0.137	87.8***
0.15	3.81 ± 0.506	1.30 ± 0.143	49.1**
0.1	4.65 ± 0.572	3.23 ± 0.431	18.0
0.05	2.28 ± 0.557	2.98 ± 0.353	−13.3
0.015	2.83 ± 0.416	4.30 ± 0.447	−20.0*
Daidzein			
0.5	5.86 ± 0.489	3.04 ± 0.350	32.7**
0.1	3.17 ± 0.401	5.58 ± 0.738	−27.5*
0.05	3.93 ± 0.427	6.73 ± 0.485	−26.3**

[a] Both control and test membrane sachets contained 1% glucose and 5% Tween 80.
[b] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

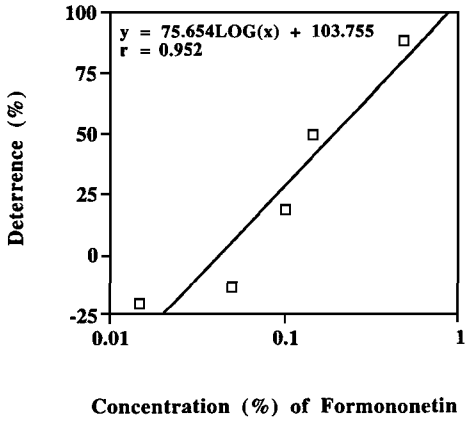

FIG. 3. Correlation of deterrence and log concentration for formononetin.

TABLE 2. MEAN NUMBER OF MITES ON CONTROL AND TREATMENT MEMBRANE SACHETS CONTAINING ISOFLAVONE-7-*O*-GLUCOSIDES AND 7-GLUCOSIDE-6″-*O*-MALONATE DURING 3 HOURS[a]

Concentration (%)	Control	Treatment	Deterrence (%)[b]
Biochanin A-7-O-glucoside			
1	9.61 ± 0.788	4.45 ± 0.597	36.7***
0.5	7.05 ± 0.645	4.53 ± 0.658	21.8
Formononetin-7-*O*-glucoside			
0.5	5.20 ± 0.423	2.01 ± 0.229	44.2***
Genistein-7-*O*-glucoside			
0.5	7.20 ± 0.488	2.14 ± 0.347	54.2***
0.1	4.00 ± 0.434	3.65 ± 0.661	4.6
Biochanin A 7-*O*-glucoside-6″-*O*-malonate			
1.0	9.53 ± 1.058	2.21 ± 0.303	62.4***
0.5	5.83 ± 0.918	1.78 ± 0.305	53.2**
0.1	5.41 ± 0.598	6.98 ± 0.410	−12.7
Genistein 7-*O*-glucoside-6″-*O*-malonate			
1.0	9.86 ± 0.317	4.07 ± 0.507	41.6***
0.5	6.60 ± 0.577	4.97 ± 0.557	14.1
0.1	3.40 ± 0.417	2.97 ± 0.438	6.7
0.05	6.81 ± 0.502	6.23 ± 0.674	4.4

[a]Both control and test membrane sachets contained 1% glucose and 5% Tween 80.
[b]$^{**}P < 0.01$; $^{***}P < 0.001$.

(Table 1). Daidzein was not detected in this variety but is known to occur in *T. subterraneum* (Vetter, 1995).

Bioassays of Isoflavone 7-O-Glucosides. The 7-*O*-glucoside and 7-*O*-glucoside-6″-*O*-malonate derivatives of the isoflavones also were tested for deterrent activity towards the redlegged earth mite, but the amounts of the three 7-*O*-glucoside isoflavones available allowed only a limited number of concentrations to be tested (Table 2). The glucosides of formononetin and genistein showed significant deterrence to mites at 0.5% concentration; formononetin-7-*O*-glucoside, 44% deterrence; and genistein-7-*O*-glucoside, 54% deterrence). Biochanin A-7-*O*-glucoside showed 37% deterrence at 1%. Biochanin A 7-glucoside-6″-*O*-malonate (53% deterrence at 0.5%) showed higher deterrence to mites than the 42% deterrence shown by the corresponding genistein derivative at 1%.

Bioassays of Isoflavanols. The two isoflavanols, 2,5,7-trihydroxy-4′-methoxyisoflavanol (**4**) and 2,5,7,4′-tetrahydroxyisoflavanol (**5**) showed significant deterrence to mites at 0.05% (Table 3).

For ease of comparison, the minimum concentrations at which significant deterrence was observed for the various compounds are listed in Table 4. The approximate concentrations of each of the compounds in the fresh leaves of the resistant variety SE014 and the susceptible variety Dalkeith, determined by HPLC, are included also.

TABLE 3. MEAN NUMBER OF MITES ON CONTROL AND TREATMENT MEMBRANE SACHETS CONTAINING 2,5,7-TRIHYDROXY-4′-METHOXYISOFLAVANAL AND 2,5,7,4′-TETRAHYDROXYISOFLAVANOL DURING 3 HOURS[a]

Concentration (%)	Control	Treatment	Deterrence (5)[b]
2,5,7-Trihydroxy-4′-methoxyisoflavanol			
0.5	2.34 ± 0.344	1.26 ± 0.347	30.0**
0.1	4.16 ± 0.780	2.84 ± 0.289	19.1
0.05	4.18 ± 0.369	2.77 ± 0.327	20.0*
0.01	6.14 ± 0.744	4.58 ± 0.769	14.6
2,5,7,4′-Tetrahydroxyisoflavanol			
0.5	6.60 ± 0.852	1.58 ± 0.216	61.4***
0.1	5.29 ± 0.575	3.16 ± 0.552	25.2*
0.05	7.33 ± 0.744	4.55 ± 0.659	23.4*
0.01	2.50 ± 0.438	7.01 ± 0.721	−47.4**

[a]Both control and test membrane sachets contained 1% glucose and 5% Tween 80.
[b]*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 4. MINIMUM CONCENTRATIONS FOR DETERRENT ACTIVITY OF ISOFLAVONOID DERIVATIVES TO MITES, AND CONCENTRATIONS OF COMPOUNDS IN TRIFOLIATE LEAVES OF RESISTANT VARIETY SE014 AND SUSCEPTIBLE VARIETY DALKEITH

Compound	MPC (%)[a]	Concentration in leaves (%)	
		SE014	Dalkeith
Biochanin A (**1**)	0.15	0.17	0.01
Genistein (**2**)	0.05	0.05	0.03
Formononetin (**3**)	0.10	0.02	0.002
Daidzein (**11**)	0.50		
2,5,7-Trihydroxy-4′-methoxyisoflavanol (**4**)	0.05	0.02	trace
2,5,7,4′-Tetrahydroxy isoflavanol (**5**)	0.05	0.02	trace
Biochanin A 7-*O*-glucoside-6″-*O*-malonate (**9**)	0.50	0.01	
Genistein-7-*O*-glucoside-6″-*O*-malonate (**10**)	0.50	0.01	0.007

[a]Minimum percentage concentration exhibiting significant activity ($P < 0.05$). The isoflavone-7-*O*-glucosides were not available in sufficient amounts for the MPC to be determined.

DISCUSSION

Bioassay of the methanol extract from trifoliates of the subclover resistant variety SE014 revealed the presence of strong deterrents to the mite, which have the effect of reducing feeding. The deterrence was associated with the nonpolar and medium polar components of the extract. Bioassay-guided fractionation led to the identification of 10 isoflavone derivatives that showed deterrent activity. These compounds include three free isoflavones—biochanin A, genistein, formononetin—their 7-*O*-glucosides and their 7-*O*-glucoside-6″-*O*-malonate derivatives, as well as two novel isoflavanols. Isoflavones have long been recognized as major secondary metabolites of subclover species (Bradbury and White, 1951; Beck, 1964; Beck and Knox, 1971; Vetter, 1995). The results presented here identify a number of isoflavone derivatives as deterrents to the redlegged earth mite and indicate that the resistance of subclover trifoliates can be attributed, at least in part, to the presence of these compounds. Interestingly, differences in the deterrent activity between individual isoflavones were also observed. Genistein (**2**) is the most active; 68% deterrence was detected at 0.045% concentration, which is equivalent to the amount of this compound in 1 g of fresh mature leaves (Table 4).

Generally, it was found that the isoflavones were deterrent at high concentrations but attractive at lower concentrations. For example, genistein showed 93% deterrence at 0.08%, decreasing to 68% at 0.045%, and tending to attrac-

tiveness to the mite at 0.01%. This is not an uncommon observation. For example, in the study of the resistance mechanisms operating in subclover cotyledons, mites were found to be repelled by high concentrations of 1-octen-3-one and attracted by low concentrations (Jiang et al., 1996a, 1997).

The greater activity of the free isoflavones compared to those of the isoflavone glucosides is noteworthy. The latter group displayed deterrence to mites at more than 0.5% concentration and showed rapid loss of activity with decreasing concentrations. The fact that genistein and biochanin A are more deterrent than their precursors, isoflavanols **4** and **5**, suggests that the extra OH group in ring B of the isoflavones reduces the antifeedant activity. On the other hand, the presence of a hydroxyl group at C-5 in genistein and biochanin A increases activity. Methylation at the C-4′ position decreases activity of both the isoflavone and isoflavanol compounds.

One of the more interesting results arising from this work relates to the different resistance mechanisms operating in subclover cotyledons compared to those apparently contributing to the resistance in trifoliates. In cotyledons, the production of the deterrent volatile compound 1-octen-3-one is an induced response of cotyledons to mite feeding (Jiang et al., 1996a,b, 1997). In contrast, trifoliates present a constitutive defense mechanism that is determined by the relative levels of isoflavone derivatives. As a caveat, accurate determination of the relative amount of free to glucosylated isoflavones requires special precautions in extraction to minimize hydrolytic processes that can lead to loss of the malonyl unit and the glucose at C-7 (Köster et al., 1983). In the present study, the trifoliates were frozen in liquid nitrogen before extraction.

The insect antifeedant activity of some isoflavonoids has been observed previously (Lane et al., 1985). Coumestrol is a feeding deterrent to *Epilachna varivestis* (Burden and Norris, 1992) and vestitol, isolated from the root of *Lotus pedunculatus* and other legumes, showed feeding deterrence to larvae of the beetle *Costelytra zealandica* (Russell et al., 1978, 1979). Biochanin A, genistein, and formononetin are inactive to *C. zealandica*. However, biochanin A and genistein show feeding deterrence to *Heteronychus arator* at concentrations of 50 μg/ml (Sutherland et al., 1980).

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RESPONSE OF CABBAGE SEED WEEVIL (*Ceutorhynchus assimilis*) TO BAITS OF EXTRACTED AND SYNTHETIC HOST-PLANT ODOR

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Abstract—The effect of extracted and artificial oilseed rape (*Brassica napus* ssp. *oleifera*) odors on the behavioral response of male and female cabbage seed weevils (*Ceutorynchus assimilis*) was investigated in a wind tunnel. Odor-mediated upwind anemotaxis was induced by leaf extract and its artificial equivalent. Omission of two isothiocyanates from the artificial extract significantly reduced the upwind movement of females. Increasing the wind speed within the tunnel significantly reduced upwind movement in response to the odor of leaf and flower extracts. The artificial baits proved less attractive than simple extracts from oilseed rape. Field trapping confirmed that extracted leaf material was more attractive than artificial equivalents.

Key Words—Anemotaxis, *Ceutorhynchus assimilis*, *Brassica napus*, host-plant extracts, wind tunnel, isothiocyanates, α-farnesene, trapping.

INTRODUCTION

In field experiments, cabbage seed weevils (*Ceutorhynchus assimilis* Payk.), pests of oilseed rape, have been shown to respond positively to the odor of extracts of oilseed rape plants (*Brassica napus* L.), in baited yellow water traps (Finch, 1977; Free and Williams, 1978; Evans and Allen-Williams, 1989, 1993). This technique has been widely used to monitor the presence of insect pests of cruciferous crops and relies on the combination of odor and color to attract the

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insects (Lerin, 1984; Seidel and Daebeler, 1986). Behavioral responses to odor and color also have been investigated using laboratory olfactometer studies (Evans and Allen-Williams, 1989; Bartelt et al., 1993).

The most common flight behavior reported in wind tunnel studies is odor-mediated positive (upwind) anemotaxis, in which perception of odor molecules induces insects to move upwind. In this study, an inflatable wind tunnel (Jones et al., 1981) was used to investigate the anemotactic responses of weevils to the odors of extracts of oilseed rape leaves and flowers in comparison with artificially produced extracts, with and without certain key components at different wind speeds. Their behavior in a wind tunnel has not been investigated previously, although a study by Kjaer-Pedersen (1992), in which a small (39-cm-long) field cage was used, has indicated upwind flight in the presence of oilseed rape odor.

METHODS AND MATERIALS

Design and Construction of Wind Tunnel. The inflatable wind tunnel was based on a design by Jones et al. (1981), that utilized plastic tubing, which, when inflated, formed a cylindrical tunnel 21 cm in diameter and 168 cm in length. The tunnel was inflated by a modified glasshouse fan heater (Findlay Irvine Ltd, Penicuik, Edinburgh) whose heating element had been removed, while a second, similar fan was used to draw the air out of the tunnel. The speeds of both fans were controlled by variable voltage regulators. The air entering the tunnel passed through an outer and inner honeycomb mesh of 1.7 and 0.6 mm, respectively. This evened out the turbulence in the airflow as it passed into the test chamber of the tunnel. A fine mesh (0.5 mm) acted as a barrier to any weevils attempting to leave the tunnel at the downwind section where they were released and also acted to reduce the air turbulence (Jones et al., 1981). Access to the tunnel was provided by slits in the plastic walls, which were resealed with clear sticky tape. A 2 m length of 0.4-m-wide white paper with transverse 2-cm-wide black stripes at 25-cm intervals was placed beneath the tunnel to provide a uniform ground pattern for flying weevils. To overcome the problem of contamination of the walls of the tunnel, new plastic tubing was used for each new odor tested. The tunnel was housed in a darkroom with no natural light; overhead fluorescent lighting gave an even illumination of 230 lux. Room temperature was 21–24°C for all tests.

Measurement of Airflow and Odor Distribution. Airspeed was measured by using a microanemometer (Furness Controls Ltd., Bexhill, England) connected to a Thurlby 1504 RMS multimeter. Wind velocity was varied by increasing or decreasing the speed of the fans. Odor plumes at different wind velocities were visualized within the tunnel by generating ammonium chloride smoke from

concentrated hydrochloric acid and ammonia. Ammonia was drawn into the tunnel by the fan and, upon contacting the vapor of the HCl emitted by the bait dispenser, a grey smoke was formed, easily seen against a black background.

Sources of Odor. Oilseed rape leaf extracts were obtained by shredding rape leaves (cv. Ariana) and standing them in 70% industrial methylated spirit (IMS) for an hour (50% w/v). The mixture was filtered (1-mm mesh) and the filtrate used. Rape flower extracts were obtained by standing flowers (cv. Ariana) in 70% IMS for an hour (100% w/v) and filtered as above. These extracts were chosen for their ease of production and previously shown attractiveness to seed weevils when used as baits in yellow water traps during field experiments (Evans and Allen-Williams, 1989, 1993).

Artificial equivalents of rape leaf and flower extracts were made up from compounds identified by GC-MS in leaf and flower odor (Tollsten and Bergstrom, 1988; Evans and Allen-Williams, 1992). Their appropriate proportions and composition is detailed in Table 1. An artificial leaf extract was also made up without the compounds 3-butenyl and 4-pentenyl isothiocyanate, both of which elicited strong responses in electroantennograms and single-cell recordings of seed weevil antennal receptors (Blight et al., 1989, 1995; Evans and

Table 1. Composition of Artificial Rape Extracts (Made up to 500 ml by 70% IMS)

Artificial rape leaf			Artificial rape flower		
Source[a]	Compound (ml)	Purity (%)	Source[a]	Compound (ml)	Purity (%)
1	3-Butenyl ITC[b] (0.012)	96	6	Carophyllene (0.022)	97
1	4-Pentenyl ITC (0.034)	96	6	Cineole (0.021)	99
2	cis-3-Hexen-1-ol (2.500)	98	2	Dimethyl sulphide (0.008)	99
4	cis-3-Hexenyl acetate (0.900)	98	4	cis-3-Hexenyl acetate (0.025)	98
2	Dimethyl sulphide (0.010)	99	6	Limonene (0.100)	98
2	Hexanol-1 (0.200)	98	2	Linalool (0.021)	97
2	Pent-2-en-1-ol (0.020)	95	6	Myrcene (0.075)	98
2	Pentan-1-ol (0.005)	99	6	Phenylacetaldehyde (0.010)	95
6	Phenylacetaldehyde (0.008)	95	6	Phenylethanol (0.008)	97
6	Phenylethanol (0.008)	97	2	Sabinene (0.163)	98
3	Phenylethyl ITC (0.025)	98	2	β-Pinene (0.005)	99
5	sec-Butyl ITC (0.200)	99	3	α-Cedrene (0.021)	99
2	trans-2-Hexenal (0.300)	98	7	α-Farnesene (1.000)	90
2	trans-3-Hex-3-enol (0.200)	98	2	α-Pinene (0.023)	99

[a]Sources of chemical compounds: 1, AFRC Food Research Institute, Norwich, Norfolk, UK; 2, Aldrich, Poole, UK; 3, Fluka, Glossop, UK; 4, International Flavours & Fragrances, Haverhill, UK; 5, Maybridge Chemical Co., Tintagel, UK; 6, Sigma, Poole, UK; 7, Prepared using method described by Murray (1969).
[b]ITC: isothiocyanate.

Allen-Williams, 1992). An artificial flower extract was prepared, without α-farnesene, which is one of the major constituents of rape flower odor (Tollsten and Bergstrom, 1988; Evans and Allen-Williams, 1992). This compound elicits significant electroantennogram responses from seed weevils (Evans and Allen-Williams, 1992; Blight et al., 1995). The α-farnesene (90% purity) was prepared by the method of Murray (1969) and was used at a concentration of 1% in 70% IMS.

Extracts were placed in a bait dispenser consisting of a glass bottle with plastic lid with a wick extending into the extract. A muslin hood covered the exposed wick above the lid of the dispenser. These were identical to dispensers used successfully in the field trapping of the weevils (Evans and Allen-Williams, 1989, 1993). Two dispensers were placed side by side in the wind tunnel on a small stand so that the wicks were in the airflow from the input fan. Control tests were carried out with dispensers containing only 70% IMS in order to calibrate the evaporation rate at the three wind speeds.

Bioassay Procedure. Weevils (at least 25 for each treatment with three replicates) were placed on a raised platform (10 cm) in the downwind section of the wind tunnel. The position of the weevils was noted at 5-min intervals for 30 min after their introduction. Analysis of the results was based on the number of weevils in the downwind, middle, and upwind sections of the tunnel (each 56 cm long). Male and female weevils were tested separately.

Weevils were obtained from the field by sweep netting and kept individually at 20°C, and a 16L:8D light regime in the laboratory and fed on rape plant materials for 12 hr prior to use. New weevils were used for each replicate.

Field Trapping. Baited yellow water traps (Evans and Allen-Williams, 1993) were placed along each field edge of a crop of winter wheat, which the previous year had grown winter oilseed rape. Traps at this emergence site were baited with seven different extracts in IMS; extracted rape leaf and flower extracts, artificial rape flower extract, artificial rape flower extract excluding α-farnesene, 1% α-farnesene, 1% allyl isothiocyanate, and IMS as the control bait. Traps were placed 20 m apart with two replicates along each edge, giving eight traps per bait. The full range of traps was placed at the emergence site when weevils began to be caught in baited traps so that traps were present when the peak of weevil emergence was reached. This occurred over a three day period between April 28 and May 1.

RESULTS

Airflow and Odor Distribution. The inflation and exhaust fans were calibrated so they could produce three different wind speeds. This gave evenly

dispersed plumes within the tunnel as visualized by the NH_4Cl smoke. The plume distribution visualized was similar to that described by Jones et al. (1981), in that as wind speed decreased, plume diameter increased. The minimum speed that kept the tunnel inflated and gave an evenly distributed plume was 0.446 m/sec. Other wind speeds used were 0.682 and 1.062 m/sec. Mean evaporation rate for IMS was 0.76, 1.02, and 1.5 ml/hr ($N = 4$) for the three respective speeds. This was equivalent to releasing 0.38, 0.51, and 0.75 ml bait, respectively, during the experimental period.

Bioassays. Distribution of weevils within the tunnel was dependent on time (analysis of variance, $P < 0.05$); weevils did not attempt flight during the first 5–10 min within the tunnel, after which they began to fly in short hops of approximately 10 cm. In the presence of rape extract odor, the majority faced upwind before take-off. This orientation upwind was less common when no rape extract odor was present. Because of this period of "settling," the weevil distribution after 30 min in the tunnel was used as the criterion for response to a particular test odor. Distributions of weevils within the downwind, middle, and upwind sections were converted to percentages and transformed using arcsin square root (Snedecor, 1956) to meet the assumptions of the analysis of variance (Sokal and Rohlf, 1981). The percentage of weevils in the middle and upwind thirds was used as a measure of the degree of upwind movement demonstrated to a particular odor.

Increasing wind speed did not affect the percentage of weevils moving into the upwind third of the tunnel in the control tests, but did have an effect in tests with leaf and flower extracts. In leaf extract tests, the percentage of male weevils moving into the upwind section was significantly reduced ($P < 0.025$) when the wind speed increased from 0.446 to 0.682 m/sec. When leaf extract was used, the percentage of females moving upwind was reduced ($P < 0.01$) as wind speed increased from 0.682 to 1.062 m/sec.

At wind speeds of 0.446, 0.682, and 1.062 m/sec, the upwind distribution of both males and females in the tests with flower extract was significantly greater ($P < 0.05$) than that in corresponding control tests (Figure 1, only 0.682 m/sec shown). In tests with leaf extract, the percentage of males moving upwind was greater ($P < 0.025$) than the conrols at wind speeds of 0.446 and 1.062 m/sec. With the same extract, more females moved upwind at wind speeds of 0.446 and 0.682 m/sec compared to controls (Figure 1b). At all wind speeds, higher percentage of weevils moved into the upwind section of the tunnel in tests with flower extracts compared to leaf extracts (Figure 1). This difference was significant ($P < 0.05$) between males at speeds of 0.682 and 1.062 m/sec and between females at 1.062 m/sec. There was greater female response to leaf extract odor at 0.682 m/sec and greater male response to flower odor at 1.062 m/sec ($P < 0.05$).

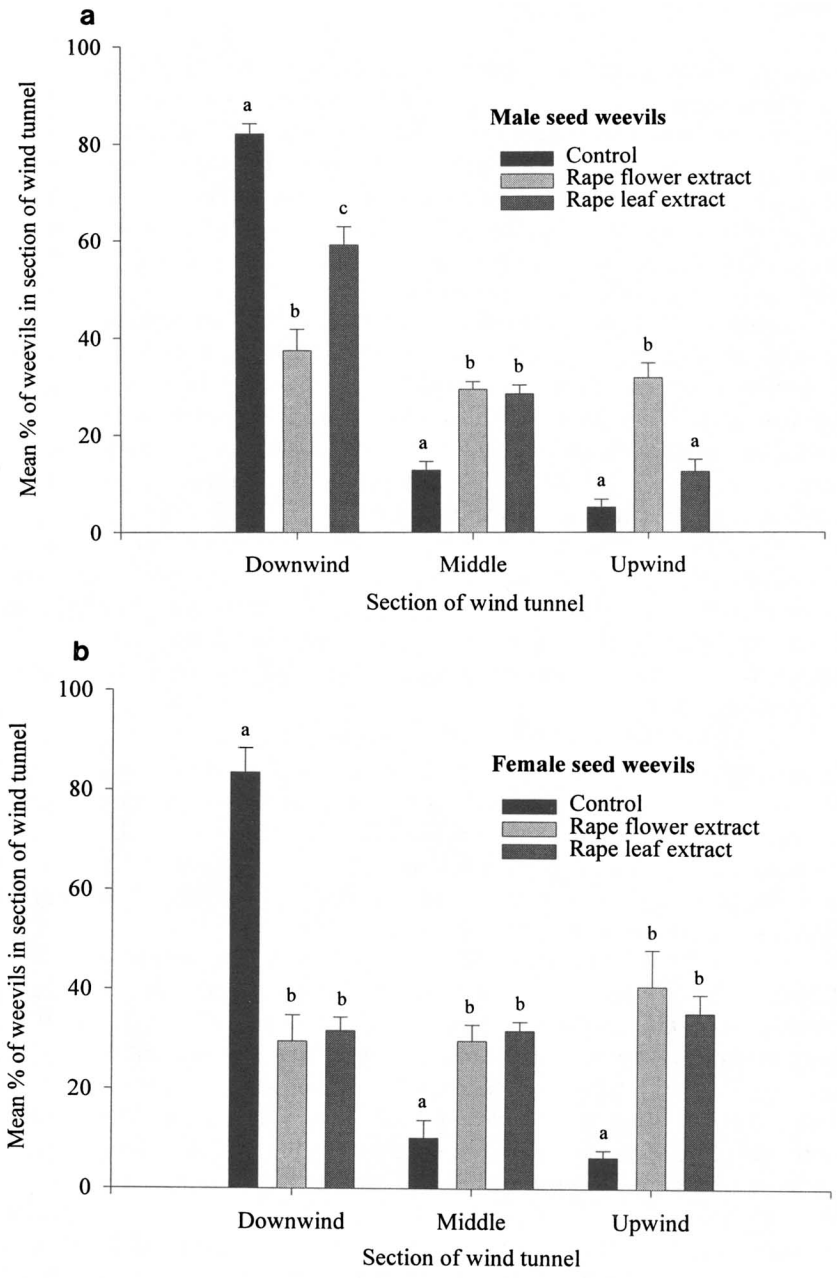

FIG. 1. The mean percentage distribution (±SEM, three replicates) of male (a) and female (b) seed weevils in a wind tunnel in the presence of rape flower extract and rape leaf extract (wind speed of 0.682 m/sec). Columns for each section of the wind tunnel with different letters indicate significant differences in response for individual comparisons (analysis of variance, $P < 0.05$).

Tests with artificial leaf and flower extracts were carried out at a wind speed 0.682 m/sec, as the largest percentage of weevils in the upwind section in tests with plant extracts were obtained at this speed (Figure 1). In tests with artificial leaf extract, both males and females moved in greater numbers ($P < 0.025$) into the upwind and middle sections compared to controls (Figure 2). However, when artificial leaf extract with no 3-butenyl or 4-pentenyl isothiocyanate was used, the percentages upwind were no different from controls, although there were more weevils ($P < 0.01$) in the middle third of the wind tunnel (Figure 2). The upwind response to artificial leaf extracts ($\pm$isothiocyanates) showed no differences in male responses, but a decrease ($P < 0.05$) in female responses in the absence of isothiocyanates (Figure 2).

In tests with artificial flower extracts, only the male upwind response was greater ($P < 0.05$) than the control (Figure 3). The percentage of males in the middle of the tunnel in tests with artificial flower extract, both with and without α-farnesene, was greater ($P < 0.01$) than in controls, as was the response of females ($P < 0.05$) in tests with artificial flower extract with no α-farnesene (Figure 3). Comparison of the upwind responses of weevils to artificial flower extract with or without α-farnesene indicated no statistically significant differences between the extracts (Figure 3).

The upwind movement of weevils to α-farnesene was no different from controls, but significantly less ($P < 0.05$) than the response to artificial flower odor with α-farnesene (Figure 3). There was no difference between sexes in response to any of the artificial leaf or flower extracts tested.

At a wind speed of 0.632 m/sec, the percentage of males and females moving to the upwind section of the tunnel in response to flower extract was greater ($P < 0.05$) than the response to the artificial equivalent: male 31.7% vs. 17.7%; female 41.4% vs. 16.9%. The upwind movement of females was also greater ($P < 0.05$) to leaf extract (35.9%) than to artificial leaf odor (21.5%). Males were more responsive ($P < 0.05$) to the artificial odor (23.8%) than to leaf extract (12.4%).

Field Trapping. Over a three-day period at the peak of emergence, more weevils were caught in the yellow water traps baited with extracted flower and leaf material than in those of their artificial equivalents or individual compounds (Figure 4). Extracted flower baits were the most attractive, followed by leaf extracts, artificial flower extracts, and allyl isothiocyanate (Figure 4). None of the artificial flower extract equivalents, α-farnesene, or allyl isothiocyanate caught significantly more weevils than IMS control baited traps.

DISCUSSION

Both sexes of cabbage seed weevil exhibit significant upwind movement in a wind tunnel in the presence of rape extract odor. This movement can be

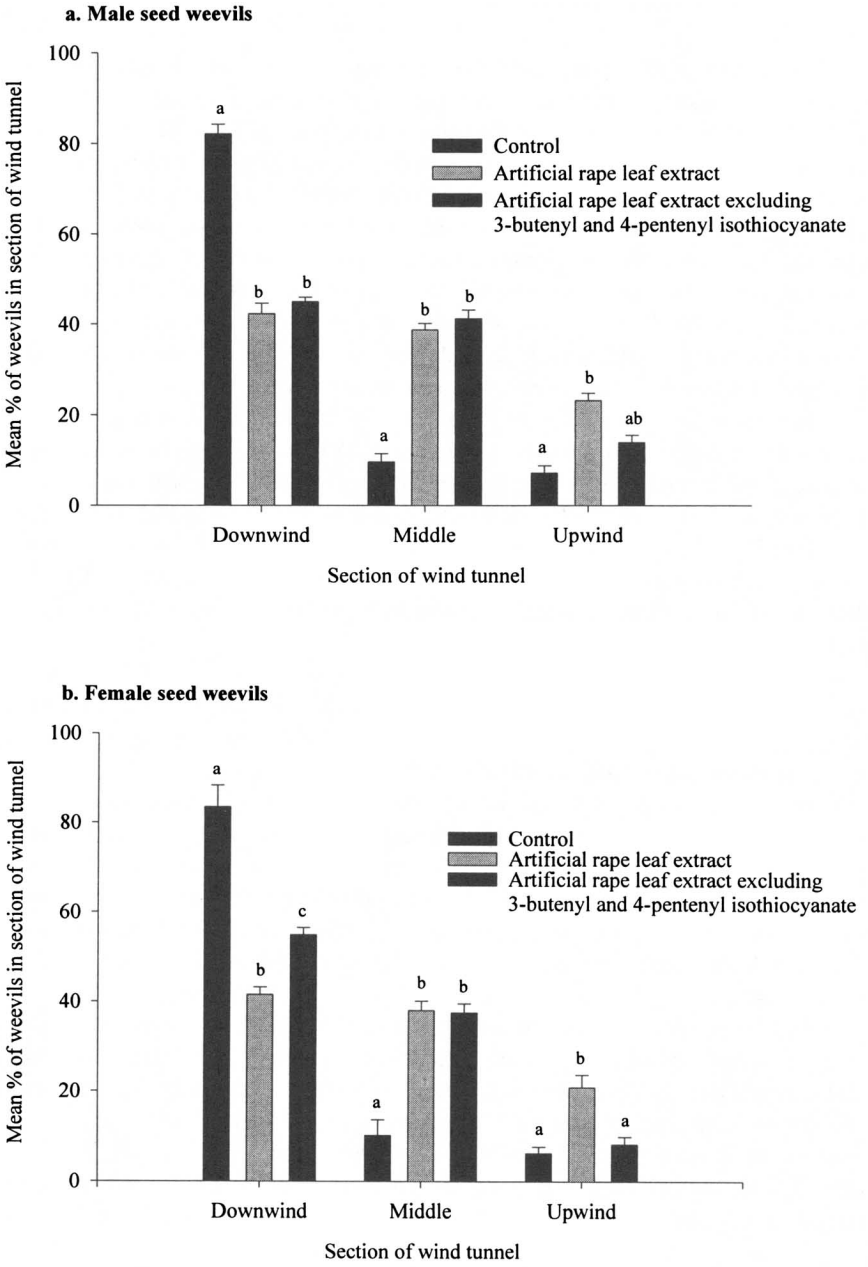

FIG. 2. The mean percentage distribution (±SEM, three replicates) of male (a) and female (b) seed weevils in a wind tunnel (wind speed 0.682 m/sec) in the presence of artificial rape leaf extract ± 3-butenyl and 4-pentenyl isothiocyanate. Columns for each section of the wind tunnel with different letters indicate significant differences in response for individual comparisons (analysis of variance, $P < 0.05$).

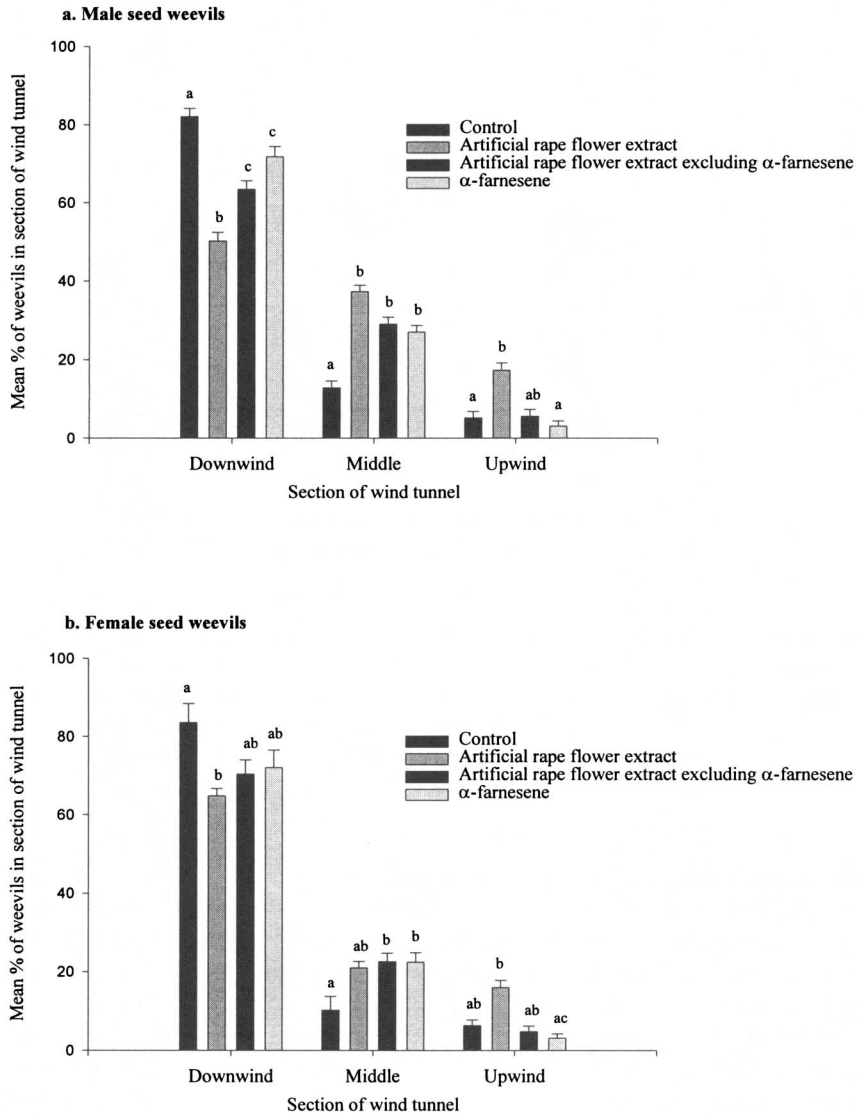

FIG. 3. The mean percentage distribution (±SEM, three replicates) of male (a) and female (b) seed weevils in a wind tunnel (wind speed 0.682 m/sec) in the presence of artificial rape flower extract ± α-farnesene and α-farnesene alone. Columns for each section of the wind tunnel with different letters indicate significant differences in response for individual comparisons (analysis of variance, $P < 0.05$).

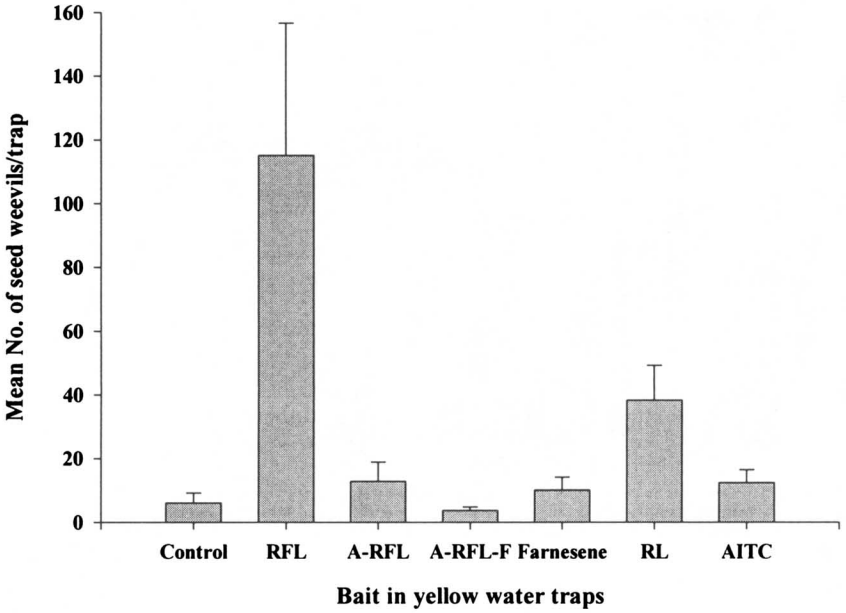

FIG. 4. The mean number (±SEM) of seed weevils caught in baited yellow water traps ($N = 8$) over a three-day period during the peak of seed weevil emergence at an overintering site. The baits were IMS (control), rape flower extract (RFL), artificial rape flower extract (A-RFL), artificial rape flower extract excluding α-farnesene (A-RFL-F), 1% α-farnesene (farnesene), rape leaf extract (RL), and 1% allyl isothiocyanate (AITC).

interpreted as odor-mediated positive anemotaxis (Kennedy, 1977), as there is no significant movement upwind in the absence of extract odor. Anemotactic movement upwind was reduced at higher wind speeds. In the field, traps baited with rape plant extracts were more attractive than those baited with artificial equivalents or individual components of plant odor.

The short-hop upwind flight and the orientation upwind of the majority before take-off suggests that weevils do not utilize odor-modulated, optomotor-guided anemotaxis (Nottingham and Coaker, 1985). The lack of significant movement upwind in the absence of odor indicates that weevils probably do not move upwind in search of an odor plume. This conforms to models proposed by Sabelis and Schippers (1984) and Dusenbery (1989), who suggest that crosswind or downwind searching of an odor plume is the best explanation.

In the presence of extracts, the highest wind speed reduced the upwind movement of the females. Kennedy (1990) has demonstrated that take-off of winged black bean aphids in a flight chamber was inhibited by relatively high

wind speeds and may also occur in mosquitoes and locusts (Kennedy, 1940, 1951) and cabbage root flies (Nottingham, 1987). Inhibition of take-off may explain the reduction in weevil upwind movement at the upper range of the wind speeds.

Leaf and flower extracts induced upwind movements as did artificial equivalents. Omission of 3-butenyl and 4-pentenyl isothiocyanates from artificial extract reduced upwind movements by females. Isothiocyanate compounds, the breakdown products of glucosinolates, characterize plants in the family Cruciferae (Fenwick et al., 1983). Blight et al. (1989, 1995) have identified specific receptors for 3-butenyl and 4-pentenyl isothiocyanates on the antennae of seed weevils. Electroantennogram studies (Evans and Allen-Williams, 1992) have demonstrated significant responses to these compounds by both sexes, with females being more sensitive to 3-butenyl isothiocyanate. In a linear olfactometer study that used both sexes of the cabbage seed weevil, Bartlet et al. (1993) found that 3-butenyl and 4-pentenyl isothiocyanate were attractive to weevils, and Smart et al. (1996, 1997) demonstrated attraction to yellow traps baited with mixtures of these and other isothiocyanates. The omission of the 3-butenyl and 4-pentenyl isothiocyanates from artificial leaf extract caused a reduction in the upwind movement of females. This may have been due to an alteration in sensory perception of odors by the insect antennae (Blight et al., 1989, 1995), which changed the subsequent behavioral response. As these compounds individually evoke strong electroantennogram responses from seed weevil antennae and also reduce responses to artificial leaf and flower odors when they are absent (Evans and Allen-Williams, 1992), they may play a role in host-plant location by cabbage seed weevils.

α-Farnesene is a major constituent of rape flower odor (Tollsten and Bergstrom, 1988; Evans and Allen-Williams, 1992), and the omission of α-farnesene from artificial odor reduced, to some extent, the numbers of weevils moving upwind in the wind tunnel. Electroantennogram studies (Evans and Allen-Williams, 1992; Blight et al., 1995) have demonstrated a strong antennal sensory response to α-farnesene by seed weevils, but olfactometer studies (Evans, 1991) have not shown a behavioral response to the odor of α-farnesene. While α-farnesene is not an attractant compound, it may act synergistically with other components of rape odor to elicit upwind flight. This complements the observation that electroantennograms from weevils presented with artificial rape flower extract excluding α-farnesene were significantly reduced compared to those obtained when α-farnesene was present in the extract (Evans and Allen-Williams, 1992).

When baited yellow water traps were used in the field at an emergence site where there would be no interference from odor of oilseed rape plants, extracted plant baits were more attractive than their artificial equivalents or individual compounds. The numbers caught in traps at the emergence site were three to

four times greater than those caught in similar traps at nearby oilseed crops (Evans, 1991), and the timing of peak catches in traps at emergence sites and crops was identical over two years (Evans, 1991).

An extraction of host-plant material is, at least for female seed weevils, more attractive in wind-tunnel and field conditions than pure compounds or artificial mixtures. Pure compounds, such as isothiocyanates (Smart et al., 1996, 1997), while catching more weevils than unbaited yellow sticky traps, do not catch as many weevils as yellow water traps baited with simple IMS extracts of oilseed rape plant material.

For practical use in monitoring cabbage seed weevil and possibly other pests of oilseed rape such as pollen beetles (*Meligethes aeneus* F.) and brassica pod midge (*Dasineura brassicae* Winn.), a crude extract of rape leaves or flowers is sufficient to detect their appearance, particularly if placed at their emergence sites rather than at the crop itself where there is competition between the bait and the odor of the crop. Seed weevils and pollen beetles are attracted to rape leaf and flower extracts from distances of at least 20 m (Evans and Allen-Williams, 1993, 1994), and if the detection of weevil movement into oilseed rape crops is the ultimate aim of field trapping, then detecting the peak of emergence at their overwintering sites is preferable to having baited traps at the oilseed rape crop, as dates of peak trap catches of weevils at emergence sites and rape crops are virtually identical (Evans, 1991).

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SEASONAL PATTERNS OF PLANT FLAMMABILITY AND MONOTERPENOID CONTENT IN *Juniperus ashei*

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Abstract—The susceptibility of *Juniperus* communities to prescribed fires can vary greatly throughout the year. The objective of this project was to determine the relationship between the seasonal concentration and composition of volatile oils and plant flammability in two Ashe juniper (*Juniperus ashei*) populations. Total monoterpenoid concentration was significantly affected by season and by plant population. Mean monoterpenoid concentration of a population from each central Texas was 9.16 mg/g fresh weight of juniper needles while the mean concentration of a west central Texas population was 11.62 mg/g of fresh weight. Monoterpenoid concentrations were typically lowest during the summer and highest during the spring and winter in the western population, but there was no seasonal pattern in the eastern population. The eastern population of trees was slightly (4.8%) more flammable than the western population, and male trees were slightly (3.8%) more flammable than female trees. The concentration of limonene was positively related to plant flammability and could increase flammability by 30% over the range of cencentrations found in this species. Bornyl acetate was negatively related to flammability with each 1 mg/g increase in concentration resulting in a 2% decrease in flammability. Caloric energy content and percent leaf moisture were not significant factors in determining the percentage of the Ashe juniper plant actually burned. Secondary chemicals, usually considered as antiherbi-

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vore mechanisms, may also serve an important role in determining the likelihood of a plant being consumed by fire.

Key Words—Prescribed fire, volatile oils, juniper, monoterpenoids, flammability.

INTRODUCTION

The physiognomy of the Edwards Plateau region of Texas has changed from an open savanna with scattered trees to an almost closed woodland during the last 200 years (Smeins, 1980). Ashe juniper (*Juniperus ashei* Buchholz), a native tree of the Edwards Plateau, is not one of the dominant species in this area. This species may have been limited originally to rocky outcrops and steep canyons, but it has increased in density and range since the suppression of natural wildfires (Foster, 1917). Ashe juniper is vulnerable to fire-induced mortality when the tree is young (<2 m tall), but the susceptibility decreases with age (Bryant et al., 1983). As the plant matures, herbaceous production under the tree canopy decreases and consequently there is little fine fuel available for maintaining fire continuity (Fulhendorf et al., 1996).

Disturbances historically have been via either man-made or lightning-caused fires. Lightning-caused fires often originated during the hot, dry summer months whereas man-made fires (prescribed fires) are usually conducted in early to mid-winter for increased safety. Besides the obvious differences in environmental conditions between summer and winter, plant flammability changes with season (Rodríguez Añón et al., 1995), potentially resulting in different fire intensities. Plant moisture (Wright and Bailey, 1982) and heat content of plant tissues (Rodríguez Añón et al., 1995; Philpot, 1969) vary seasonally and directly influence plant flammability. Plant phytochemicals, particularly monoterpenoids, may also increase the combustibility of plant material by increasing the probability of ignition (White, 1994).

Seasonal changes in monoterpenoids have been well documented for many coniferous species (Adams, 1987; Nerg et al., 1994; Riddle et al., 1996) but there does not appear to be a well-defined seasonal pattern in either the total concentration or the composition of individual monoterpenoids. Monoterpenoid concentrations were greatest in spring for Douglas fir [*Pseudotsuga menziesii* (Mirb). Franco] (Zou and Cates, 1995) and redberry juniper (*Juniperus pinchotii* Sudw.) (Riddle et al., 1996), but were greatest at the end of the growing season and lowest in the spring for western juniper [*Juniperus osteosperma* (Torr.) Little] and one-seeded juniper [*J. monosperma* (*Engelm.*) *Sarg.*] (Adams, 1987). These seasonal patterns may result from environmental variables such as soil moisture that vary seasonally and that may increase the concentration of some oils (e.g., α-pinene) while having no effect or even decreasing the concentration

of other monoterpenoids (e.g., β-pinene, myrcene, limonene) (Gilmore, 1977). Other environmental factors such as soil fertility (Muzika et al., 1989; Mihaliak and Lincoln, 1985) and light (Klepzig et al., 1995) can also significantly affect the concentration and composition of monoterpenoids in plants.

To develop effective fire management systems for juniper communities, we need to understand the relationship between environmental factors, plant secondary chemicals, and flammability. The objectives of this study were to document the seasonal changes in secondary chemistry in Ashe juniper and to relate the observed changes in monoterpenoid concentrations and composition to plant flammability.

METHODS AND MATERIALS

Collection of Material. The study was conducted at the Sonora Research Station (31°N, 100°W) located 45 km southeast of Sonora, Texas, and on the Annandale Ranch (29°29′N; 99°44′W) near Concan, Texas. The two sites are approximately 120 km apart. Long-term average precipitation for both sites is approximately 60 cm/yr. Detailed site descriptions can be found in Riddle et al. (1996) and Owens (1996) for the Sonora and Annandale sites, respectively. Twenty Ashe juniper trees were permanently marked at each site in March 1993 and sampled approximately every six weeks for 16 months. At each site, 10 of the trees were male and 10 were female.

Approximately 80 g fresh weight of mature juniper foliage and twigs were collected at each sample date. Juvenile foliage was not collected because oil concentration is generally greater in mature foliage than in young foliage (Maarse and Kepner, 1970). Samples were collected from around the entire tree without regard to spatial location because spatial location within the canopy does not significantly affect monoterpenoid concentrations in leaf tissue (Hall and Langenheim, 1986). Twenty grams of sample were immediately placed in liquid nitrogen in the field and frozen for later analysis of oils and gross energy. Sixty grams were placed in a paper bag in a cooler for later analysis of flammability. Xylem water potential was measured at each sampling date to estimate water stress by using a pressure bomb (Scholander et al., 1965).

Chemical Extractions and Identification. Fifteen grams (fresh weight) of frozen juniper needles and small twigs were steam distilled in 150 ml water for 8 hr (Owens et al., 1998). The distillate was collected in 5 ml hexane, and tetradecane was added as an internal standard. After distillation, a 2-ml aliquot was withdrawn from the sample. Samples were stored at −80°C until lab analysis was complete. Analysis was performed on an HP 5890 gas chromatograph by using a methyl silicon capillary column (0.25 μm × 25 m) with a nitrogen carrier. The temperature program was 70°C for 2 min, a 1.5°C/min increase to

97°C, then a 6°C/min increase to 187°C, followed by a 15°C/min increase to 262°C with a 5-min plateau at 262°C. Injector temperature was 280°C, and the detector temperature was 240°C. Terpenes were identified by comparison with retention times of known external standards. Commercially available external standards were tricyclene, α-pinene, camphene, β-pinene, sabinene, myrcene, cymene, limonene, γ-terpinene, linalool, fenchyl alcohol, camphor, citronellol, borneol, terpinen-4-ol, terpineol, carvone, and bornyl acetate.

Energy Content and Flammability. Gross energy content was determined by using a bomb calorimeter with 1 g fresh weight of juniper needles. Wet weight, rather than dry weight, was used to determine the gross energy content of flammable tissue under seasonal conditions. Ten grams of fresh juniper needles were weighed, oven-dried at 60°C for 48 hr, and reweighed to determine gravimetric plant moisture.

Flammability was determined by placing 50 g of juniper needles and small twigs on a screen above an open flame. The open flame was placed under the sample for 60 sec and then removed. Four thermocouples were placed in the sample and one was placed between the sample and the open flame. Temperatures were recorded every 5 sec and averaged every min on a Campbell 21X data logger. After 5 min, the remaining sample was reweighed to determine the percentage of the plant material actually burned. If the sample was still burning at the end of the 5-min observation, it was extinguished and the remaining tissue was weighed.

Data Analysis. Seasonal changes in total concentration of the monoterpenoids and the concentration for each major compound were analyzed using split-plot models with site in the main plot and time in the split plot. The error term for the main plot was tree nested within site.

The relationship between plant flammability and plant characteristics was analyzed by using a growth curve model. Independent variables were the concentrations of the three dominant oils (camphor, bornyl acetate, and limonene), percent plant moisture, caloric content on a fresh weight basis and on a dry weight basis, site, tree sex, and xylem water potential. The dependent variable was the percentage of the plant that burned. Five different structures of the variance/covariance matrix were tested. The models were: (1) simple structure—where a single variance estimate was used over all time intervals and no correlation between time periods was assumed; (2) banded main diagonal—where the variance was allowed to vary with each sample date but there was still an assumption of no correlation between time periods; (3) compound symmetry—where a constant covariance between time periods was assumed but variance was allowed to vary by time period; (4) first-order autoregressive—where the variances were the same and the covariance was held constant between time periods, and (5) Toeplitz—where variances were the same and the covariances

in one line up and down the diagonal were the same. This last model is similar to model 4 but allows a different rate of covariance in different time periods. The best model was selected using a likelihood ratio analysis with $\alpha = 0.05$.

RESULTS

Monoterpenoids. Total concentration of volatile oils was significantly greater ($P = 0.075$) in the Sonora population than in the trees at the Annandale Ranch (11.92 vs. 9.16 mg/g fresh weight, respectively). There was a strong seasonal effect ($P < 0.001$) on the volatile oil concentration at Sonora, where concentrations were greatest during the winter and spring months (January through May) and lowest during the summer and fall (Figure 1). There was no seasonal effect at the Annandale site, where monoterpenoid concentrations remained constant throughout the year.

Eighteen different monoterpenoids have been identified in Ashe juniper (Owens et al., 1998), but only 12 were present in sufficient quantity to analyze in this study. The remaining six were present in trace amounts and were not present in every month. The concentration of limonene, cymene, γ-terpinene, and myrcene were affected by the tree population ($P < 0.05$) with a greater concentration found in Sonora than in Annandale. The concentration of these four compounds was also affected by a significant interaction between the season

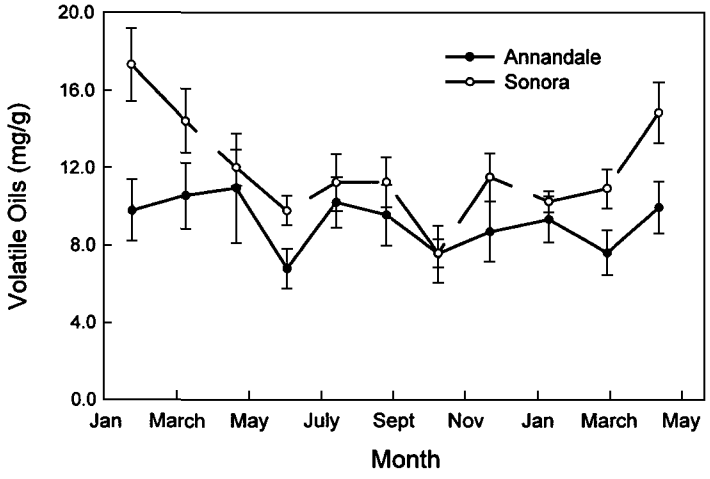

FIG. 1. Seasonal concentrations of monoterpenoids in Ashe juniper at two sites. Bars represent ± 1 SE.

of the year and the tree population (Figure 2, cymene not shown). An additional eight compounds (β-pinene, borneol, bornyl acetate, camphene, camphor, carvone, citronellol, and tricyclene) exhibited a seasonal change in concentrations that paralleled the changes observed for the total concentrations (Figure 2, only bornyl acetate shown). These monoterpenoids were not affected by the different populations of trees.

Plant Parameters. Percent moisture in the needles and fine twigs averaged about 44% on both sites throughout the year. There was a strong seasonal effect demonstrated by high plant moisture in winter and spring and low gravimetric plant moisture during the summer (Figure 3A). Gravimetric plant moisture ranged from 36 to 48% on the Annandale site and from 38 to 48% on the Sonora site.

Trees on the Sonora site exhibited significantly lower plant water potential ($\bar{X} = 2.1$ mPa) than trees on the Annandale site ($\bar{X} = 1.7$ mPa) throughout the year. On a seasonal basis, plant water potentials varied with low water stress during the winter months and increasing stress during the summer (Figure 3B). During the summer months of July and September, water potential dropped below the -3.5 mPa sensitivity of the pressure bomb. Xylem water potential was greater at the Sonora site than at the Annandale site during the second winter, which probably reflects the greater precipitation received in Sonora during the November to January time period (Figure 3C). Whenever precipitation at the Annandale site was greater than at the Sonora site (e.g., May, 1994), then xylem water potential at Annandale was also greater.

Mean caloric content of fresh needles and twigs was not significantly different between the two sites ($\bar{X} = 2.6$ kcal/g fresh weight), although there was a significant interaction between the site and season. Energy content was least during the winter and spring months and greatest during the summer (Figure 3D). Energy content on a fresh weight basis was least at whichever site had the greatest precipitation.

Flammability. Plant flammability, as measured by the percentage of fresh needle and twig material burned, did not vary across the two research sites. Over the course of the year, an average of 78.8% of the fresh foliage burned, but there was a highly significant effect of the season of the year (Figure 4). During the peak spring growing period, only about 50% of the material burned, while almost 90% burned in the late growing season and the dormant winter period. During the summer, fire temperatures increased rapidly and reached almost 500°C within 2 min after the flame was placed under the sample (Figure 5A). The majority of the sample had burned after 3 min and temperatures dropped rapidly. Peak temperatures were over 500°C. Winter fires were less intense with peak temperatures reaching only 450°C after 3–4 min (Figure 5B).

The growth model analysis of plant flammability and plant characteristics was best described by the banded main diagonal model. Although all five models provided some degree of fit to the data, only the model allowing the variance

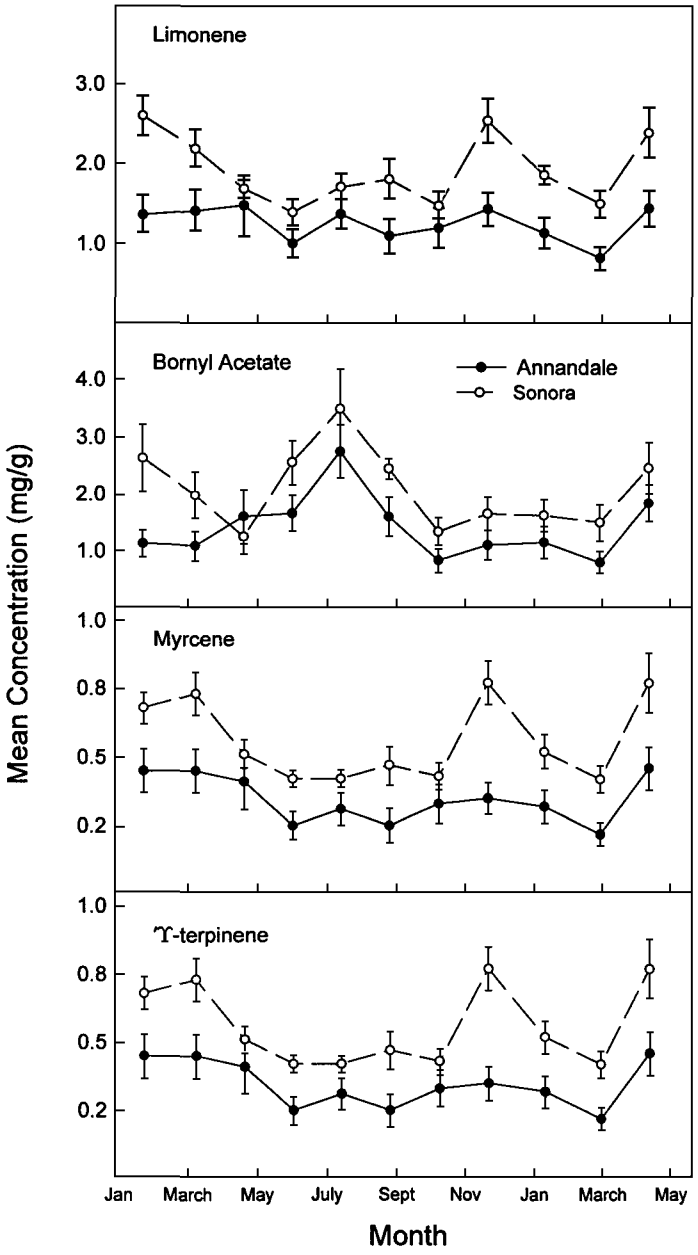

FIG. 2. Seasonal concentrations of limonene, bornyl acetate, myrcene, and γ-terpinene in Ashe juniper at two sites. Bars represent ± 1 SE.

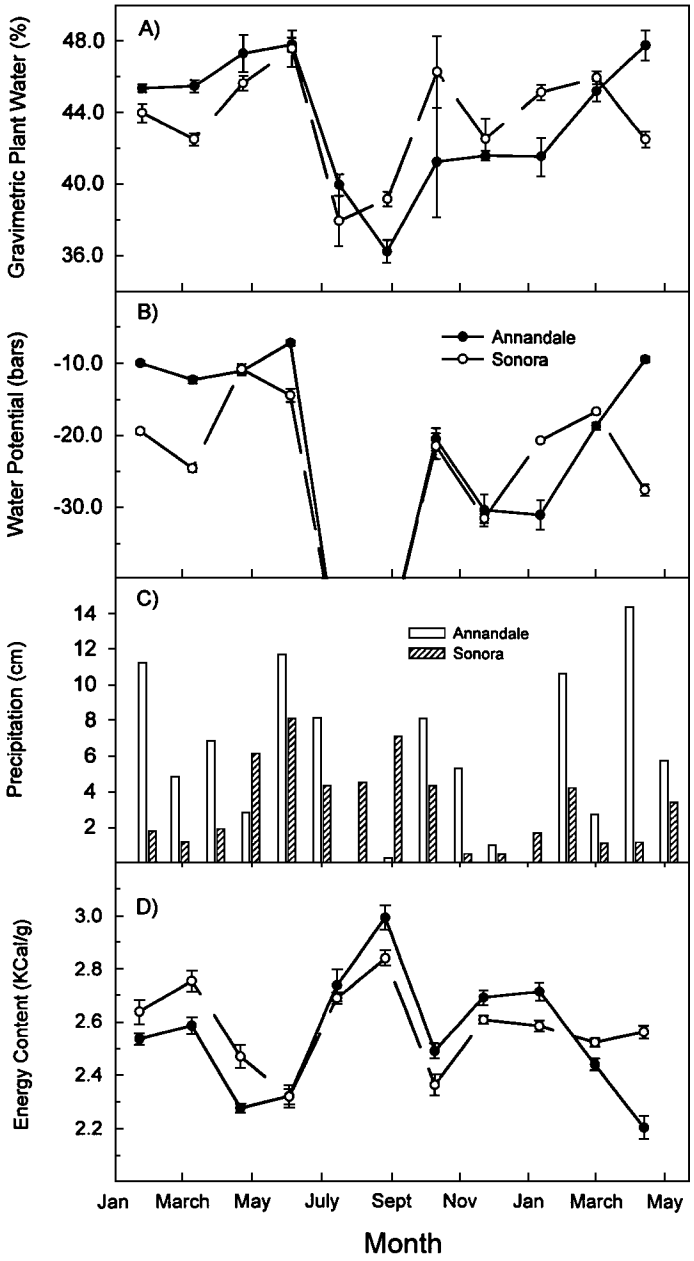

FIG. 3. Seasonal plant moisture characteristics and energy content for two populations of Ashe juniper and precipitation received at Annandale and Sonora sites over a 16-month period.

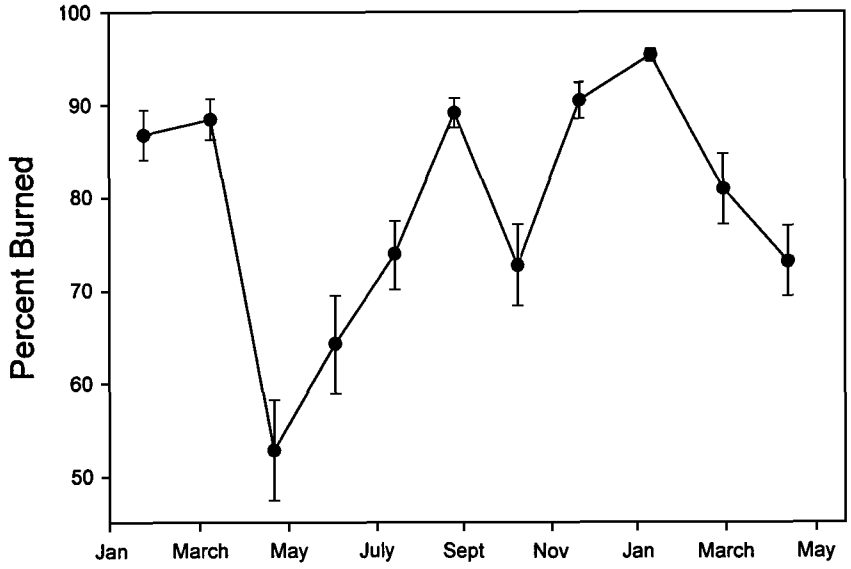

FIG. 4. Seasonal pattern of flammability of Ashe juniper. Bars represent ± 1 SE.

to vary across time periods and assuming no correlation between time periods (banded main diagonal) provided a better fit than the simple model that assumed a single variance across all time periods ($\chi^2 = 69.16$, $P \leq 0.001$). The compound symmetry ($\chi^2 = 1.73$, $P \leq 0.188$), first-order autoregressive ($\chi^2 = 1.83$, $P = 0.176$), and Toeplitz ($\chi^2 = 14.99$, $P = 0.132$) models were not significantly different than the simple model assuming a single variance. The plant population, sex of the tree, and the concentrations of bornyl acetate and limonene had a significant effect on the amount of the plant that burned (Table 1). Since the sex of the tree and the plant population were coded as binary variables, their net effect was to adjust the intercept of the model. Male trees were slightly (3.8%) more flammable than female trees, and trees from Annandale were slightly (4.8%) more flammable than trees at Sonora. The concentration of limonene has a gross positive effect on flammability, whereas the concentration of bornyl acetate had a gross negative effect. Over the concentrations of these two compounds actually found in the plants, limonene could increase flammability by nearly 30% while bornyl acetate decreased flammability by about 10%

DISCUSSION

Ashe juniper trees at Sonora contained about 30% more monoterpenoids than trees at the Annandale Ranch. This is in contrast to other reports, where

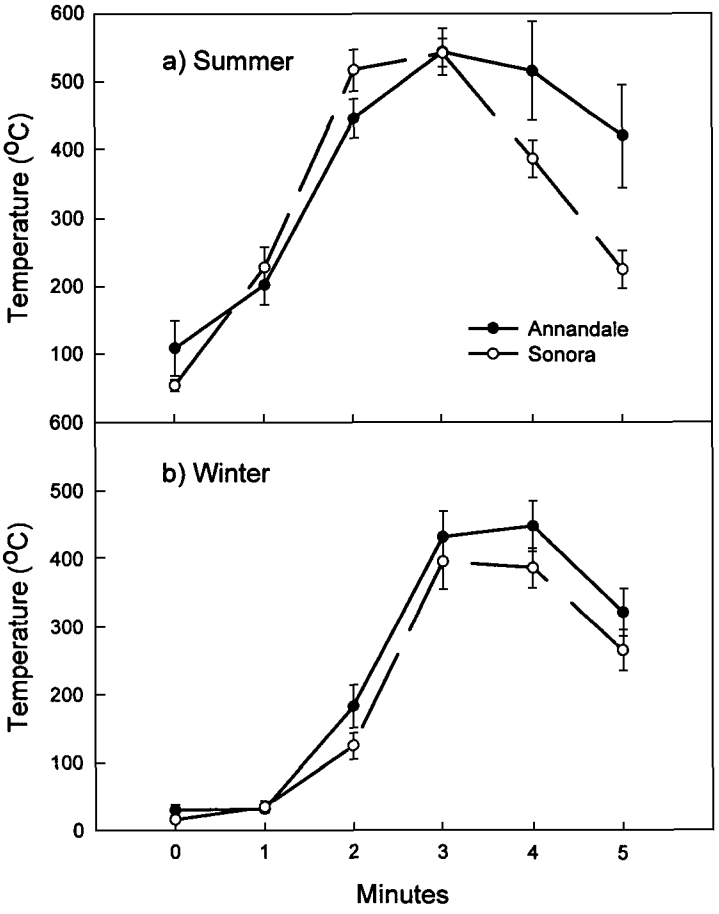

FIG. 5. Temperature profile of 50-g samples of Ashe juniper burned during summer (a) and winter (b). Bars represent ± 1 SE.

little variation was found in the concentration and composition of monoterpenoids between different populations of a single species, including Ashe juniper (Adams and Turner, 1970), Scots pine (*Pinus sylvestris L.*) (Nerg et al., 1994), or alerce (*Fitroya cupressoides* D. Don) (Cool et al., 1991). Our populations of trees were geographically distant (about 120 km apart), and we hypothesize that the greater concentrations found on the Sonora site resulted from environmental differences between Sonora and Annandale. Precipitation was sporadic at both sites, but the Annandale site received more precipitation on average than

TABLE 1. REGRESSION COEFFICIENTS OF BANDED MAIN DIAGONAL MODEL OF PLANT FLAMMABILITY ASSOCIATED WITH PLANT AND SITE CHARACTERISTICS

Factor	Regression coefficient	Standard error	*t* value	*P*
Site	4.84	1.81	2.67	0.011
Tree sex	−3.87	1.51	−2.57	0.021
Camphor	−0.1	0.46	−0.22	0.828
Bornyl acetate	−2.08	0.47	−4.42	0.001
Limonene	6.78	1.57	4.30	0.001
Energy content (wet weight basis)	0.03	0.04	0.84	0.45
Gravimetric plant moisture	0.37	1.65	0.22	0.83
Energy content (dry weight basis)	0.002	0.02	0.11	0.92

Sonora over the 16 months of this study (5.9 vs 3.3 cm/month, Figure 3C). The trees at Sonora responded to the lower precipitation by exhibiting greater water stress (e.g., lower xylem water potential) than the trees at Annandale (Figure 3B). Drought stress has been shown to increase the concentrations of monoterpenoids in other coniferous species (Kainulainen et al., 1992; Gilmore, 1977) and may have increased the concentration in the Sonora trees. Other environmental factors, such as soil fertility, may have affected monoterpenoid concentrations (Muzika et al., 1989) but could not be investigated in this study. Ashe juniper is a drought-tolerant species that can access soil moisture, and presumably nutrients, from the fractured limestone bedrock. During prolonged dry periods, Ashe juniper remains photosynthetically active and transpires water even though surface soils are completely dry (Owens and Schreiber, 1992), which indicates that deeper sources of water within the substrate are being tapped.

Most monoterpenoids (12 of 14) exhibited seasonal differences in concentration with the greatest concentrations in the winter and spring. Riddle et al. (1996), on the other hand, found the greatest concentrations of monoterpenoids in Ashe and redberry juniper (*J. pinchottii*) in the spring and the least concentrations in the winter. The season of peak concentrations seems to be species-specific with reports of spring maximums for Scots pine and some junipers (Adams, 1970) and summer maximums for Douglas fir (Gambliel and Cates, 1995; Zou and Cates, 1995), rubber rabbitbrush [*Chrysothamnus nauseosus* (Pursh) Britt.] (Halls et al., 1994), and other coniferous species (Wagner et al.,

1990). Monoterpenoid content at the lowest concentrations may be under genetic control (Welch and McArthur, 1981; Adams, 1970), while the environment controls the monoterpenoid content at other times.

Flammability. Extracting ether-soluble substances (including monoterpenoids) from flammable Mediterranean shrubs can decrease flammability by increasing time to ignition, raising ignition temperature, decreasing flaming time, and decreasing flame height and intensity (Montgomery, 1976). The extracted material contains about twice the heat content of the remaining fuel (Philpot, 1969), and heat content has been shown to positively affect plant flammability (Rodríguez Añón et al., 1995). In our case, the percentage of the plant that burned was dependent not only on the heat content on a fresh weight basis but also by the concentration of specific monoterpenoids (Table 1). As the concentration of limonene increased during the fall and late winter (Figure 2), plant flammability increased (Figure 4). Flammability did not increase at the maximum linear rate because the concentration of bornyl acetate was increasing at the same time. Bornyl acetate has a net negative effect on flammability. Limonene naturally occurs in a liquid state and has a boiling point of 80°C, whereas bornyl acetate occurs as a crystal and has a boiling point of 107°C. The higher boiling point and crystal structure of bornyl acetate implies a greater volatilization temperature, and hence a lower degree of flammability.

The seasonal pattern of plant flammability was best modeled using a banded main diagonal variance/covariance matrix. The variance estimate was least when a large percentage of the plant burned and greatest when the plants were not very flammable (Figure 4). The narrow range of responses of plant flammability suggests that the environment may be regulating flammability when the plants are stressed (Figures 3B and 4). The much greater range of responses seen when plants are less stressed (e.g., greater xylem water potentials) indicates that characteristics of individual plants are regulating flammability under unstressed conditions. This model also allowed the covariance to change according to the sampling period, which suggests that the percentage of the plant that burned during any sample was independent of the amount that burned during another time of the year. This supports other observations that plant flammability changes seasonally.

The concentration and composition of monoterpenoids in plant tissue has been used extensively to separate species taxonomically via chemosystematics (Adams, 1977, 1994; Adams et al., 1984), to explain plant defenses against herbivory (Raffa and Smalley, 1995; Zou and Cates, 1995; Duncan et al., 1994), and to describe altered ecosystem processes such as nutrient cycling (White, 1994). Monoterpenoids may also play an additional role in plant communities by altering plant flammability (Bond and Midgley, 1995; Zedler, 1995). A current hypothesis suggests that being flammable may benefit a species if resultant fires kill neighboring nonflammable plants and increase the fitness of the

flammable species (Bond and Midgley, 1995). Ashe juniper does not support this hypothesis because, although flammability changes, neighboring species are resprouting shrubs and grasses, which are rarely affected by fires (Reineke, 1996). Fitness is not increased because most Ashe juniper seedlings and most of the seed bank occur under the canopy of mature trees (Owens and Schliesing, 1995), so fires can selectively remove Ashe juniper from the plant community. It would appear that flammability on an individual level has developed in conjunction with tolerance to other stresses such as herbivory and water stress (Troumbis and Trabaud, 1989).

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ALLELOPATHIC POTENTIAL OF *Acacia confusa* AND RELATED SPECIES IN TAIWAN

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Abstract—*Acacia confusa* (an endemic species) and other introduced species, namely *A. aulacocarpa*, *A. auricumiformis*, *A. cincinnata*, *A. crassicarpa*, *A. leptocarpa*, *A. margium*, *A. polystachya*, and *A. torfilis* were evaluated for allelopathic potential. Among these, *A. confusa* is widely distributed on the hills and lowlands of Taiwn and often exhibits a unique pattern of weed exclusion under stands. Four study sites were selected. Field observations and measurements were carried out at sites that exhibited relatively pure stands of *A. confusa*. Although the diversity of understory species was comparatively higher in *Acacia* stands than in adjacent weedy sites, the total coverage and biomass of understory plants was significantly lower than in control sites. Aqueous extracts (0.5%, 1%, 2%, 3%, 4%, and 5%) of dry leaves and litter of *Acacia confusa* and other species collected from various sites and dates were bioassayed using lettuce, alfalfa, and Chinese cabbage to examine their phytotoxicity. Most extracts exhibited phytotoxicity even at a concentration as low as 0.5%. Inhibition of radicle growth of test plants varied with sampling sites and dates. Bioassay with 5% extracts or above produced more than 85% inhibition of test plants regardless of habitat. In addition, surface soils collected from the upper 20 cm layer of *Acacia* stands revealed significant inhibition as compared with adjacent grassland control soils. The most inhibitory compounds isolated from the ether fraction of aqueous extracts were identified as: ferulic, vanillic, caffeic, gallic, *m*-hydroxybenzoic, and *m*-hydroxyphenylacetic acids. Unidentified flavonoids were also found. Chromatographic bioassays of compounds isolated from both ethyl acetate and water fractions of

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methanolic extracts of *Acacia* leaves also showed significant phytotoxicity but none was found in the fractions of chloroform and hexane, suggesting the phytotoxic compounds present in *Acacia* plants are water soluble.

Key Words—*Acacia confusa*, allelopathy, phytotoxicity, phytotoxin, phenolics, flavonoids, herbicide, sustainable agriculture.

INTRODUCTION

Acacia confusa Merr, an endemic species, is widely distributed on the hills and lowland areas of Taiwan and in the subtropical regions of Asia (Kuo et al., 1989). The plant has been extensively used in many ways, including as a feedstock, for charcoal-making, and as construction material, and it is particularly important for conservation of soil and water in Taiwan. It often exhibits a unique pattern of lower amounts of understory plants under the canopy. Observations and extensive studies in the field since 1986 have concluded that the pattern is not due to physical competition for light, soil moisture, or nutrients and suggests that the phenomenon could be due to a detrimental biochemical effect (allelopathy) of the *Acacia* plant on its understory plants (Kuo et al., 1989; Fu, 1995). Allelopathic research of subtropical vegetation in Taiwan, including *Phyllostachys edulis* (Chou and Yang, 1982), *Leucaena leucocephala* (Chou and Kuo, 1986), *Delonix regia* (Chou and Leu, 1992), and *Zelkova formosana* (Chou et al., 1989), has been extensive. These plants show allelopathic potential, but have not yet been used as biological control herbicides.

In recent years, biologically active compounds released from plants, such as *Azadirachta indica* and *Agrostemma githago* L., have been used as naturally occurring herbicides, fungicides, and nematicides (Parmar and Singh, 1993; Welte and Szabolcs, 1987). From the view point of sustainable agriculture, allelopathic compounds are particularly meaningful as natural agrochemicals, with potential to protect our environment from pollution and maintain better ecosystem balance. Since there is a substantial quantity of *Acacia* plants in Taiwan, our goal is to use the plants, which possess phytotoxic substances, as natural herbicides. In a series of studies, we focused on the following tasks: (1) evaluating the phytotoxic activity of leaves, litter, pods, and twigs of the *Acacia* plants; (2) examining how the phytotoxicity of plants collected varied with seasons and habitats; (3) comparing the phytotoxicity among species of *Acacia* plants; (4) isolating and identifying phytotoxins from various parts of *Acacia* plants; and (5) confirming the potential use of phytotoxic compounds as natural herbicides in agricultural practice. Some findings of the aforementioned studies are presented in this paper.

METHODS AND MATERIALS

Study Sites. Four sites representing different weather types were situated at: (1) Chaoushi in Ilan County and (2) Linkow in Taipei County, both belonging to the northeast humid zone; (3) Tachia in Taichung County, belonging to the central west summer rain zone; and (4) Fengkang and Liukua in Pingtung County in the southern tip of Taiwan with a long drought season in winter. In addition, a site chosen for comparison was on the slopes of Nankang mountain in the vicinity of the Institute of Botany, Academic Sinica, Taipei. The climatic patterns of these sites were described by Fu (1995). In general, precipitation in Taiwan is usually concentrated in the summer season. In particular, frequent typhoons hit Taiwan during the period from August to October. Northeastern Taiwan has a substantial amount of rainfall in winter in addition to summer rain; however, in the south, it is often dry in the autumn and winter.

Field Measurement. In each site, four plots, 10×10 m^2 each, were selected in a stand of *Acacia confusa* and in the vicinity adjacent to *A. confusa.* The coverage of each species per plot was measured, and each species present was identified.

Sampling. Leaves, litter, pods, and twigs of *A. confusa* were sampled from all four sites in June, September, and December 1994 and March 1995. In addition, leaves and litter of nine *Acacia* species, namely, *A. anlacocarpa*, *A. auricumiformis*, *A. cincinnata*, *A. confusa*, *A. crassicarpa*, *A. leptocarpa*, *A. mangium*, *A. polystachya*, and *A. torfilis* growing in the experimental farm of Liukua were also collected in October 1996. Samples were brought back to the laboratory and allowed to air dry at room temperature. The dry matter was ground into powder (60 mesh screen) for water and methanol extraction.

Soils from the upper 20 cm layer of *Acacia* floor and that of control grassland area were collected and brought to the laboratory. All samples including soils were collected from the field after a dry period of at least two weeks. They were allowed to air dry before use.

Preparation of Aqueous Extracts. Materials were allowed to dry, then ground to a powder. A series of aqueous extracts, 0.5%. (0.5 g plant sample + 99.5 ml distilled water), 1%, 2%, 3%, 4%, and 5%, of each sample was prepared by using techniques described by Chou and Muller (1972).

Bioassays. Aqueous extracts of all six concentrations of each sample were separately biossayed as described by Muller (1966) and Chou (1997). Seeds of radish (*Raphanus acanthiformis*), lettuce (*Lactuca sativa*), Chinese cabbage (*Brassica chinensis*), and alfalfa (*Medicago sativa*) were used as test species. In addition, a chromatographic bioassay was employed to determine biologically active compounds present in fractions of hexane, chloroform, ethyl acetate, and water of a methanolic extract of *Acacia confusa* leaves. Lettuce seeds were used as test plants (McPherson et al., 1971; Chou, 1997).

Determination of Phytotoxicity of Decomposed Acacia *Leaves in Soils.* *Acacia* leaf powder in amounts of 0.5, 1.0, and 2.0 g was separately mixed with 100 g loamy soil, which was obtained from the experimental farm of Academia Sinica, placed in pots, and allowed to decompose for 2, 4, and 12 days, respectively. After the decomposition time, 15 lettuce seeds were planted in the soil mixture. Seven days later, the number of seeds that germinated and radicle growth of lettuce in millimeters was measured. The experiment was replicated twice.

Identification of Phytotoxic Compounds. Paper strip chromatography of the ether fraction of aqueous extracts of *Acacia* leaves was employed as described by Wang et al. (1967), and thin-layer and column chromatography of the ethyl acetate and water fractions of methanolic extract of the plant leaves were employed as described by Mabry et al. (1970). A solvent of 2% acetic acid was used for paper strip descending chromatography to separate and identify phenolic compounds, while two other solvent systems, *t*-butanol–acetic acid–water (3:1:1, v/v/v) and 15% HOAc were employed in two-dimensional paper chromatography to separate flavonoids. Additional solvent systems for thin-layer and column chromatography followed the systems of Mabry et al. (1970). Compounds isolated from column chromatography were further purified by affinity column chromatography by using Sephadex LH20. Phenolic compounds from *Acacia* were cochromatographed with phenolic standards on TLC (Chou, 1997) and HPLC (Chou and Leu, 1992). Flavonoids were identified by UV-visible and mass spectrophotometry.

Statistics. Bioassay data were analyzed by the Student's *t* test or Duncan's multiple range test (Gomez and Gomez, 1976).

RESULTS

Comparison of Vegetation Composition Under Acacia *and Adjacent Grassland.* Based on long-term field observation (more than 10 years), we found that there is usually bare ground beneath *Acacia* stands, and the density of the understory plants is low. This phenomenon is particularly pronounced in the drought area with low winter precipitation at Fengkang in Pingtung County in southern Taiwan (Figure 1, top), and on the hillsides along the Chung-Shan Freeway in the Linkow area about 40 miles south of Taipei city (Figure 1, bottom).

Although *Acacia confusa* is distributed island-wide, and four study sites were chosen for the field experiments, we only present data from two distinctly different sites, namely Linkow (representing northern Taiwan) and Fengkang (representing the south). The floristic composition and coverage of each species per plot between the *Acacia* stands and adjacent grassland area are given in

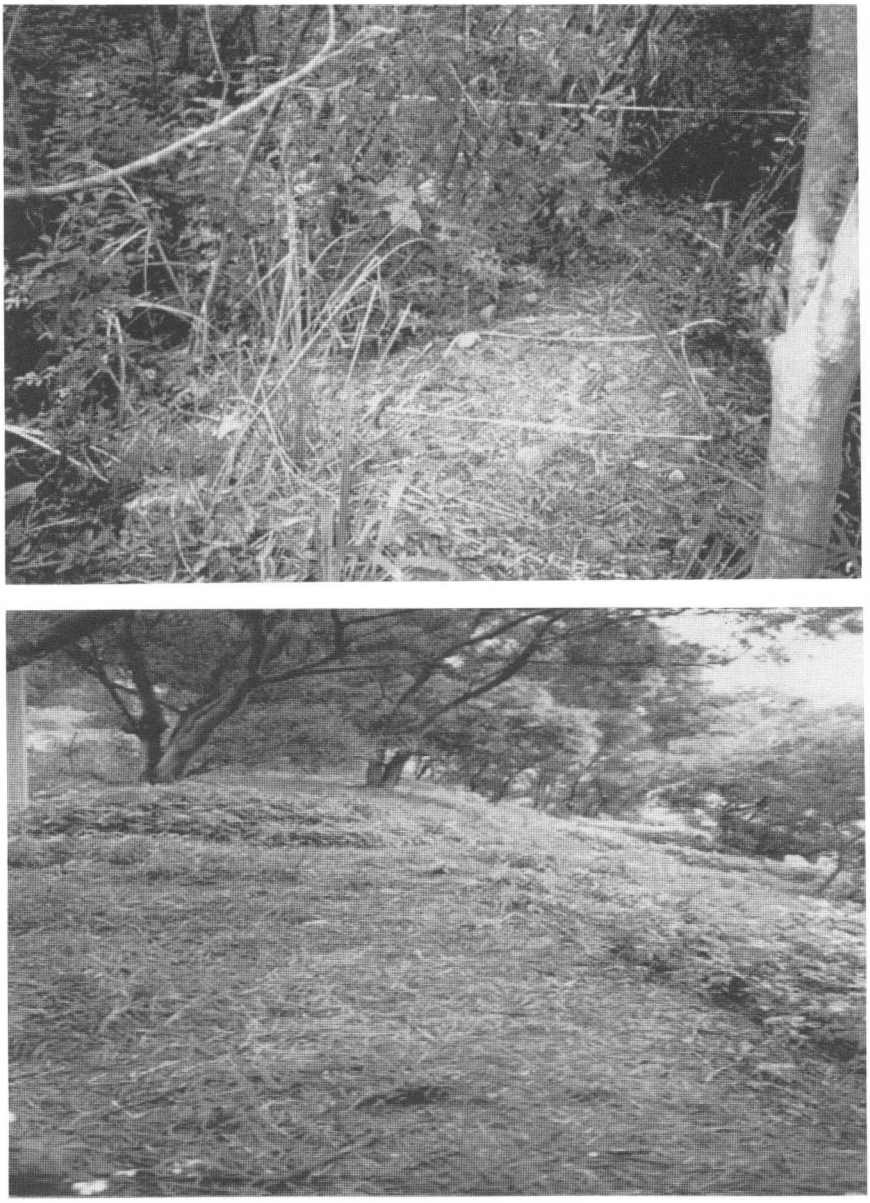

FIG. 1. Relatively bare surface under the canopy of *Acacia confusa*; (top) at the Tachia site and (bottom) from the roadside along Chungshan freeway at Linkow area.

Table 1. The vegetation composition on the *A. confusa* floor was quite different between the two sites. Similarly, the floristic composition in each grassland area was also different, perhaps due to weather patterns. The Linkow site has a subtropical climate with a high annual rainfall, around 3000 mm, and is humid most of year; the Fengkeng site has a tropical climate with a long dry winter season and a comparatively low amount of annual rainfall, around 2000 mm.

Both sites were formerly abandoned fields. After several years of *Acacia* plantation, the number of species was relatively higher in the *A. confusa* stand than in the grassland area. However, the total coverage of species present in these sites was significantly lower on the *Acacia* floor than in the grassland area. It was more pronounced at the Fengkang site; for example, the relative coverage was only 43.6% on the *Acacia* floor as compared with 100% in the grassland area. Understory species coverage was reduced 21.4% at the Linkow site and 56.4% at the Fengkang site. Seed germination of understory was not, however, noticeably suppressed. Growth performance of plants in the grassland area was better than that in *Acacia*. For example, in the grassland control plot at the Fengkang site, the percent coverage of the two dominant plants, *Ipomoea acuminata* and *Lantana camara*, reached 50% and 70%, respectively; however, in the *Acacia* plot, the coverage was nearly zero. Although the phenomenon was not pronounced at the Linkow site, the percent coverage of dominant species in

TABLE 1. FLORISTIC COMPOSITION AND COVERAGE OF EACH SPECIES PRESENT IN LINKOW AND FENGKANG

	Coverage (%)/species			
	Linkow		Fengkang	
Species	Grassland	*Acacia*	Grassland	*Acacia*
Adiantum capillus-veneris L.		2.0		
Aleurites fordii	10.0	3.5		
Buddleia asiatica	3.0	1.0		
Clerodendrum crytophyllum		0.5		
Dianella ensifolia	2.0	0		
Dicranopteris lineanis	2.0	2.5		
Ecdysanthern rosea	0	1.0		
Ficus formosana		1.5		
Gordonia axillaris		0.5		
Ilex asprella	2.0	1.5		
Ilex ficoidea		1.0		
Itea oldhamii		0.5		
Lonicera japonica	6.0	4.5		
Lygodium japonicum		1.5		

TABLE 1. CONTINUED

Species	Coverage (%)/species			
	Linkow		Fengkang	
	Grassland	*Acacia*	Grassland	*Acacia*
Miscanthus floridulus	4.0	2.0		
Mollotus paniculatus		1.0		
Passiflora edulis	8.0	2.0		
Pleuchea indica		0.5		
Psychstria rubra	4.0	5.0		
Randia cochinchinensis		0.5		
Sarcandra glabra	5.0	2.5		
Schefflera octophylla	3.0	3.5		
Smilax china	2.0	1.0		
Thea sinensis	5.0	2.5		
Zanthoxylum nitidum		1.5		
Acronychia pedunculata			0	2.0
Agave sislana			0	8.0
Albizzia julibrissin			7.0	9.0
Breynia afficinalis			0	2.5
Clausena excavata			0	5.0
Crotalaria pallida			3.0	
Eleusine indica			8.0	
Euphorbia hirta			5.0	
Gelonium aequoreum				1.0
Geodorum densiflorum				1.5
Ipomoea acuminata			50.0	2.5
Jasminum hemsleyi				3.0
Lantana camara			70.0	
Lophantherum gracile				0.25
Lygodium japonicum				15.5
Maytenns diversifolia			5.0	5.0
Melothria formosana			2.0	3.0
Passiflora foetida				0.5
Passiflora subnosa				4.0
Phyllanthus urinaria			5.0	
Securinega virosa				4.0
Severinia baxifolia			3.0	
Sida heterophylla				5.0
Tabernaemontana divaricata			3.0	
Trichanthes kirilowii				2.5
Tridax procumbens			15.0	
Vitex negundo				7.5
Average total coverage (%)	56.0	44.0	176.0	76.75
% of grassland control	100.0	78.6	100.0	43.6
Number of species	13.0	24.0	12.0	18.0

the grassland area was relatively high; e.g., *Aleurites fordii*, *Buddleia asiatica*, *Miscanthus floridulus*, *Sarcandra glabra*, *Smilax china*, *Thea sinensis*, *Passiflora edulis*, and *Lonicera japonica* (Table 1) as compared with that found in the *Acacia confusa* stand.

Phytotoxicity of Acacia *Leaves Varies with Habitats and Seasons.* The aqueous extracts, 0.5%, 1%, 2%, 3%, 4% and 5%, of *A. confusa* leaves collected in June, September, and December 1994 and March 1995 at the Fengkang, Tachia, Linkow, and Chiaushi sites were bioassayed by using lettuce seeds as test species. In general, the radicle growth of lettuce was suppressed by the extracts at concentrations as low as 0.5%, and the inhibition increased with increasing concentration. To simplify the presentation of all bioassay data, we have selected only representative results. For example, for 0.5% leaf extracts of samples obtained in May 1995, the inhibition ranged from 60% to 70%, while for 5% extracts the inhibition reached 95% regardless of sampling site (Figure 2). The variation in phytotoxicity was not significantly different between sites when the concentration was above 2%. By using the findings of 2% extracts for comparison among sampling times, the inhibition varied significantly with sampling seasons. Except for samples from Tachia, those obtained in March 1995 revealed higher inhibition (Figure 3), suggesting that phytotoxic metabolites accumulated in leaves during the winter and early spring (from November to April in southern Taiwan, which was under a severe drought).

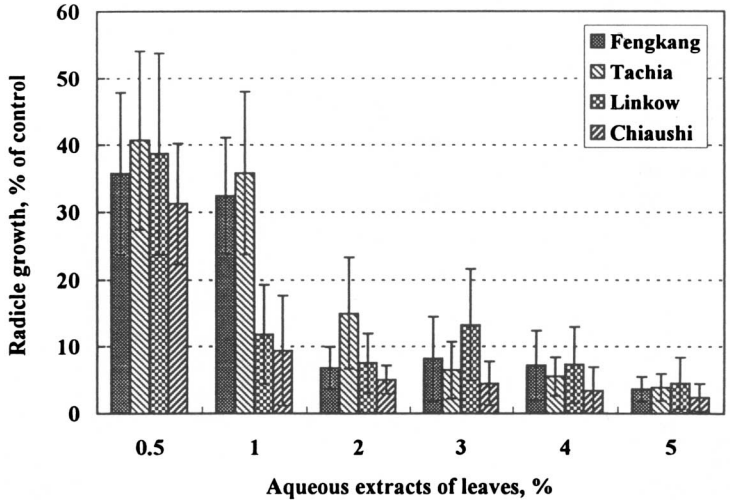

FIG. 2. Effect of aqueous extracts (0.5–5%) of *A. confusa* leaves on radicle growth of lettuce. The samples were collected from four sites in March 1995.

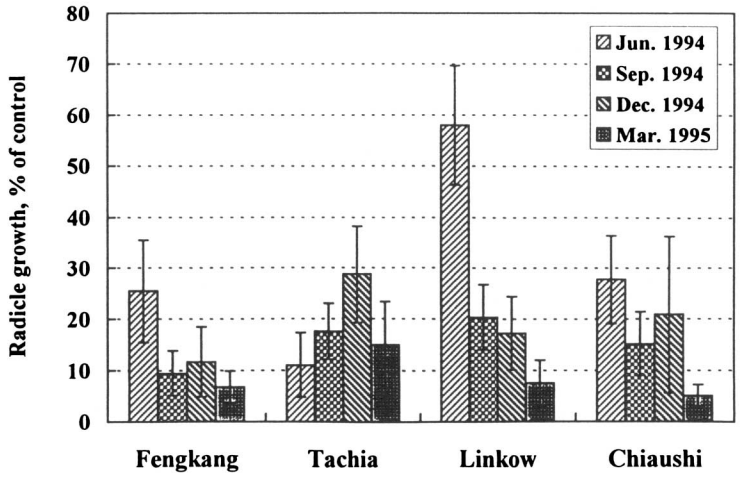

FIG. 3. Effect of 2% aqueous extracts of *Acacia confusa* leaves on radicle growth of lettuce.

One might question whether the inhibition may be due to an osmotic effect. The osmotic concentration of the 0.5% extracts was below 5 mosmol, while that of 5% extracts ranged from 40 to 50 mosmol (Chou, unpublished data). Chou and Young (1974) indicated that osmotic concentrations of extracts below 30 mosmol would not cause a significant osmotic inhibition. Normally, when the osmotic concentration of extracts exceeds 50 mosmol, the concentration of extract may cause 20–30% inhibition. In a case in which the osmotic concentration of extract is 50 mosmol and the inhibition is above 50%, there is at least a 20% inhibition due to a phytotoxic effect. In the present study, even with the 5% extract, the inhibition ranged from 80% to 90%, indicating that significant phytotoxic effects from the *Acacia* plants occurred. We also conclude that the phytotoxic activity of *Acacia* plants growing in southern Taiwan was higher than that in the north, suggesting that drought increases phytotoxic activity. Generally, the phytotoxicity of *Acacia* leaves collected in various seasons was in an ascending order of spring < summer < autumn < winter.

Phytotoxicity of Acacia confusa *Twigs Varies with Habitats and Seasons.* Aqueous extracts of 0.5%, 1%, 2%, 3%, 4%, and 5% *Acacia* twigs collected from four seasons—summer (June), autumn (September), and winter (December) of 1994 and spring (March) of 1995—at the four sites were bioassayed against lettuce. The results, represented by the samples of March 1995, showed that at the 0.5% aqueous extracts, inhibition of lettuce varied from 25% to 65%. The inhibition generally increased with extract concentration and reached

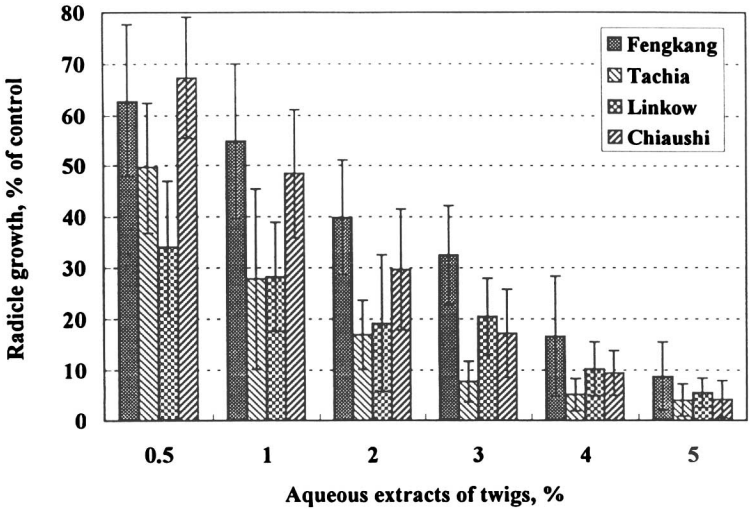

FIG. 4. Effect of aqueous extracts, 0.5–5%, of *A. confusa* twigs on radicle growth of lettuce.

90–95% inhibition for 5% extracts (Figure 4). Using bioassay results from 2% extracts from four seasons and four sites for comparison, the inhibition of Fengkang samples was generally higher than that of other sites; however, the inhibition seems to be variable with sampling times with no general trend (Figure 5).

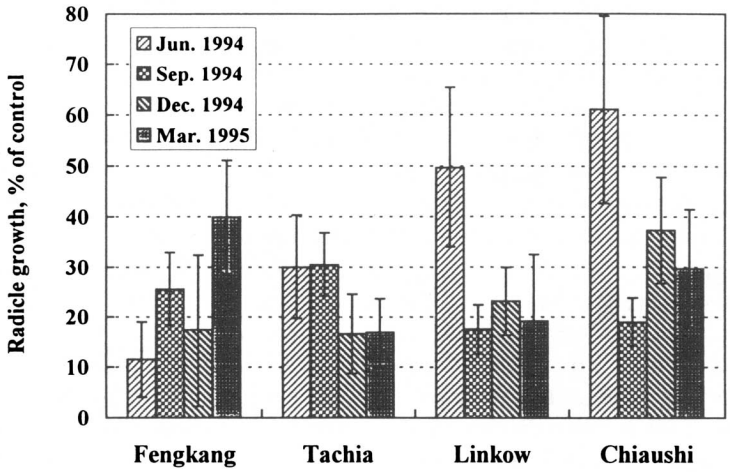

FIG. 5. Effect of 2% aqueous extracts of *A. confusa* twigs on radicle growth of lettuce.

In the overall comparison of phytotoxicity between leaves and twigs, we conclude that phytotoxicity is higher in leaves; in addition, in drought areas like Fengkang and Tachia, the phytotoxic metabolites may not be leached out during the winter season, resulting in markedly high inhibition later.

Phytotoxicity Varied with Acacia *Species Grown in Liukua.* Different con-

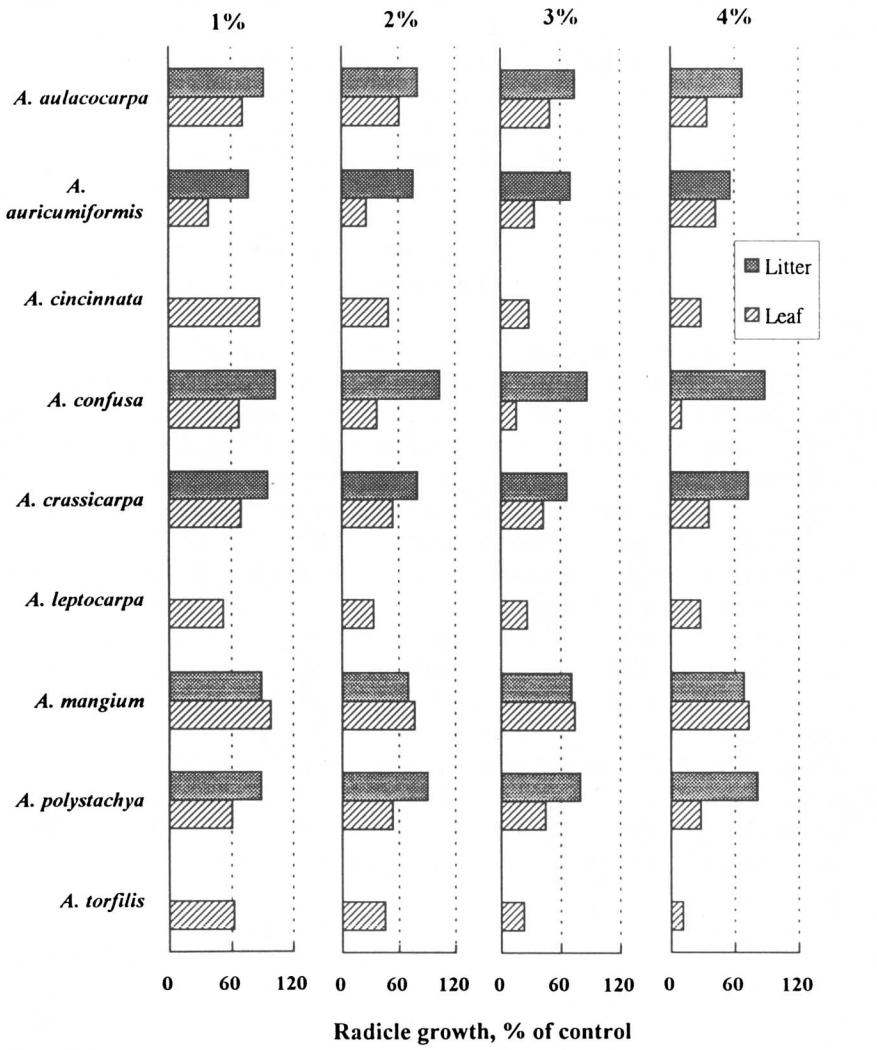

FIG. 6. Effect of aqueous leaf and litter extracts (1–4%) of different *Acacia* species from the Liukua experimental farm on radicle growth of lettuce.

centrations of aqueous extracts (1%, 2%, 3%, and 4%) of leaves (F) and litter (L) of *Acacia* plants grown in Liukua were bioassayed separately by using germination and radicle growth of seeds of lettuce, Chinese cabbage, and alfalfa. Only the lettuce findings are presented (Figure 6). In general, phytotoxicity in leaves was significantly higher than in litter. For example, comparing the 1% extracts, the inhibition of radicle growth was highest in *A. auricumiformis* (60%), followed by *A. leptocarpa*, *A. polystachya*, *A. torfilis*, *A. crassicarpa*, *A. confusa*, *A. aulacocarpa*, and was lowest in *A. mangium*. Patterns of inhibition shown by the remaining extracts from 2% to 4% were similar to that of the 1% extract; normally, inhibition increased with extract concentration (Figure 6). By comparing the effects of 5% aqueous extracts of nine *Acacia* species on radicle growth of Chinese cabbage, alfalfa, and lettuce, we demonstrated that most litter extracts had no significant inhibition (Chou, unpublished data). However, leaf extracts of the aforementioned *Acacia* species did exhibit inhibition (Figure 7). Among the nine species, phytotoxicity was markedly higher in *A. torfilis* and *A. confusa*. However, with the alfalfa bioassay material, the inhibition was much lower in extracts of *A. auricumiformis*, *A. crassicarpa*, *A. leptocarpa*, and *A. mangium*. We conclude that phytotoxicity of *Acacia* varies with test species and the inhibition is usually higher in leaves than in litter.

Effect of Aqueous Extract of Seed Pods of A. confusa *on Plant Growth.* The aqueous extracts at 1–5% of seed pods were bioassayed against Chinese cabbage, lettuce, and radish. Inhibition ranged from 45% (1% extract) to 65% (5% extract) and was not statistically different between Chinese cabbage and lettuce (Figure 8). Seed pods, thus, possess some phytotoxic activity.

Effect of Aqueous Extracts of Acacia *Soils on Plant Growth.* Aqueous extracts of soil samples from the *Acacia* floor and adjacent open areas as control at the Tachia and Fengkang sites were obtained. The extracts were bioassayed against two test species, radish and lettuce. Results of the bioassay are given in Figure 9A and B. Generally, the growth of test species was affected by soil extracts obtained from both depths of sampled soil regardless of soil habitat. There was no significant difference between depths. In general, the suppression of radicle growth of lettuce reached more than 50% in the *Acacia* soil, but radish was not affected at all. However, with open ground soils, the radicle growth of lettuce was 40% suppressed by Fengkang soil, but was not affected by Tachia soil (Figure 9). Thus, *Acacia* soil can possess both stimulatory and inhibitory properties.

Degradation of Phytotoxicity of Decomposed Leaves in Soils. Results are presented in Figure 10. They show that phytotoxicity was below 40% in the early two- and four-day period of decomposition and lasted for 12 days. The inhibition was not significantly different among treatments that increased the amount of leaves mixed with the soil. The inhibition gradually disappeared

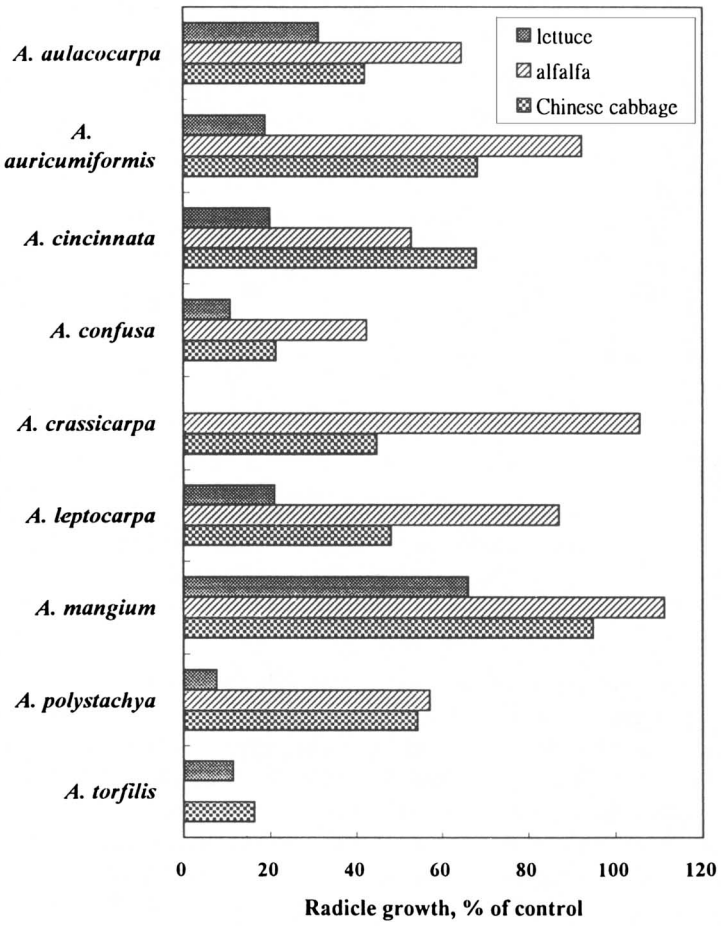

FIG. 7. Effect of 5% aqueous extracts of leaves of *Acacia* plants from the Liukua site on radicle growth of three tested species.

during 4–12 days of decomposition even when the amount of leaf powder mixed in the soil was increased. This suggests that allelopathic activity of *Acacia* plant parts might not persist once the leaves have fallen to the ground. It also suggests that the phytotoxicity of *Acacia* leaves does not last long when decomposed in soil. This finding supports the low phytotoxicity finding revealed from the bioassay of *Acacia* litter (Figure 6).

Phytotoxicity of Compounds in Methanolic Extracts of Acacia *Leaves.* Four fractions—hexane, chloroform, ethyl acetate and water—of a methanolic extract

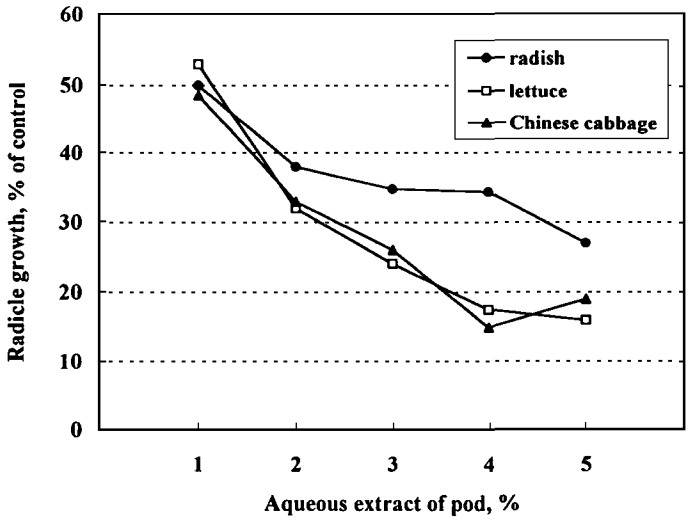

FIG. 8. Effect of aqueous extracts at 1–5% of *A. confusa* pod on radicle growth of radish, lettuce, and Chinese cabbage.

of *Acacia* leaves were chromatographed with Whatmann 3MM chromatographic paper. Spots on a two-dimensional paper chromatogram of the four fractions were cut out and bioassayed against lettuce seeds, using chromatographic bioassay techniques (Chou, 1997). Results of the bioassay showed that only compounds isolated from the fractions of ethyl acetate and water produced significant inhibition of lettuce growth (Figures 11 and 12). For example, among 14 isolated spots from the ethyl acetate fraction, nine revealed an inhibitory effect on lettuce. Spots D, I, M, and N exhibited inhibition from 30% to 40% (Figure 11). On the other hand, nine spots in the water fraction revealed an inhibitory effect, and three spots exhibited a stimulatory effect (Figure 12). In particular, spots F, G, H, J, K, L revealed 25–60% phytotoxicity, and spots G and J were distinguished (Figure 12). The spots were isolated and purified, but the amounts were too small for further assays.

Identity of Phytotoxic Compounds. Since the aforementioned results showed that potential allelopathic compounds were water-soluble, the aqueous extract was extracted with anhydrous ethyl ether. The ether fraction was chromatographed, and phytotoxic phenolics were identified by PC and HPLC. The compounds identified are: ferulic, vanillic, gallic, caffeic, *m*-hydroxybenzoic, and *m*-hydroxyphenylacetic acids (Table 2). Several unidentified compounds are thought to be flavonoids.

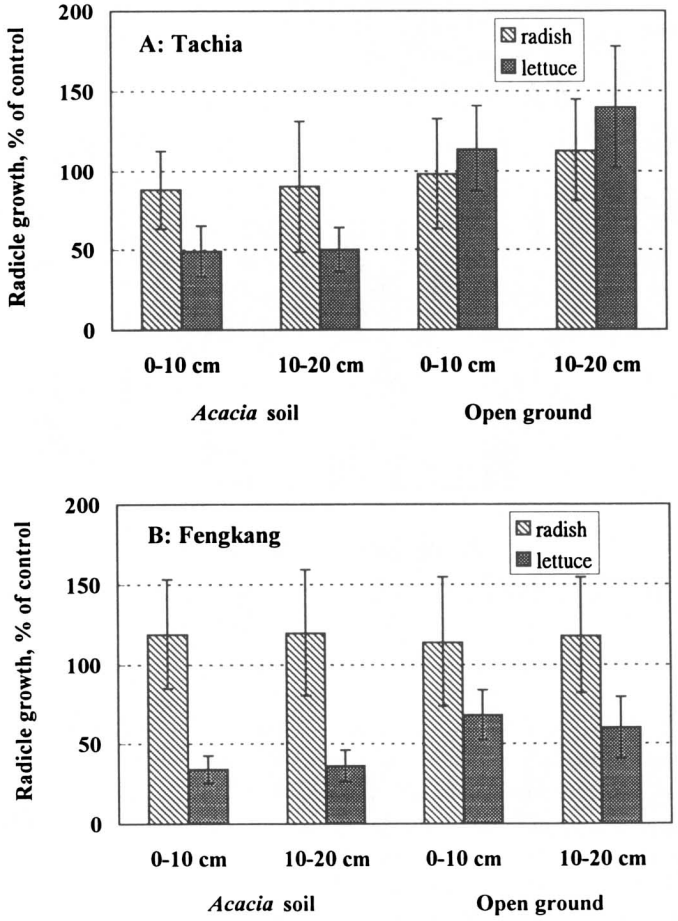

FIG. 9. Effect of aqueous extracts of soils on radicle growth of radish and lettuce. The soils were sampled from two depths of both *Acacia* and nearby weed dominated soils at two sites: (A) Tachia, (B) Fengkang.

DISCUSSION

As mentioned, field measurements previously revealed that *Acacia* may suppress the growth of understory plants without much involvement of physical competition for light, soil moisture, and nutrients (Kuo et al., 1989; Fu, 1995). Based on our results, we suggest that allelopathy plays a significant role in

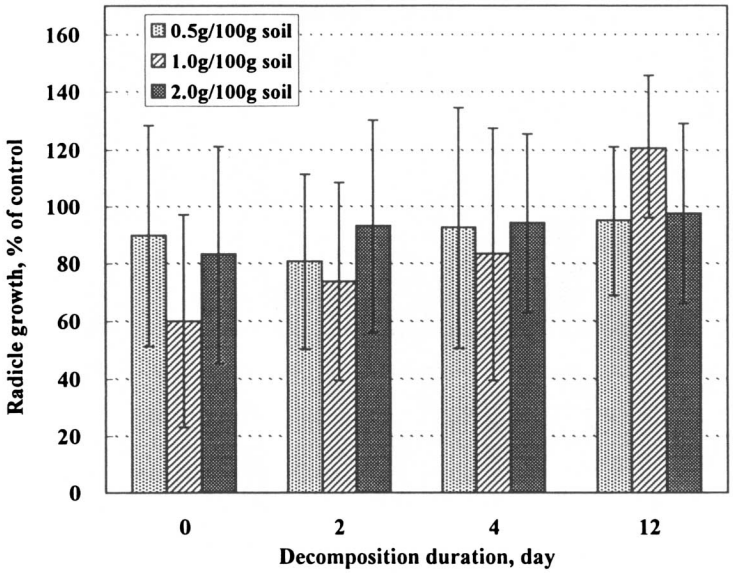

FIG. 10. Effect of decomposing *Acacia* leaves mixed with loam soil on radicle growth of lettuce.

regulating the species diversity, coverage, and dominance of *Acacia* associated vegetation in the field. This pattern of weed exclusion is not noticeable in some areas where seasonal monsoons take place. The phytotoxicity of fallen *Acacia* leaves seems to dissipate after heavy rainfall, and potential allelopathic compounds are easily degraded when the leaves are incorporated into the soil, as shown in Figure 10. Miles (1979) stated that the existence of phytotoxic substances in a particular plant does not necessarily mean they are released into the environment of another plant in concentrations sufficient to suppress its growth in some aspect. This may explain why leachates of entire *Acacia confusa* leaves revealed significant phytotoxicity but not in high toxic levels, possibly due to the thick cuticle layer of the leaf; however, aqueous extracts of ground leaves did exhibit a high toxicity of 95% against tested plants. Our evidence shows that phytotoxins present in *Acacia* leaves are being decomposed within a few days in loamy soil. Allelopathic compounds released into the soil environment could be bound with humic acid or polymerized, thus, rapidly losing their toxicity (Wang et al., 1978).

One of our major interests in *Acacia* is to use the compounds present in its plant parts as natural herbicides. Evidence of the role of allelopathy in weed control has been cited in many publications (Chou, 1995; Rice, 1995; Rizvi

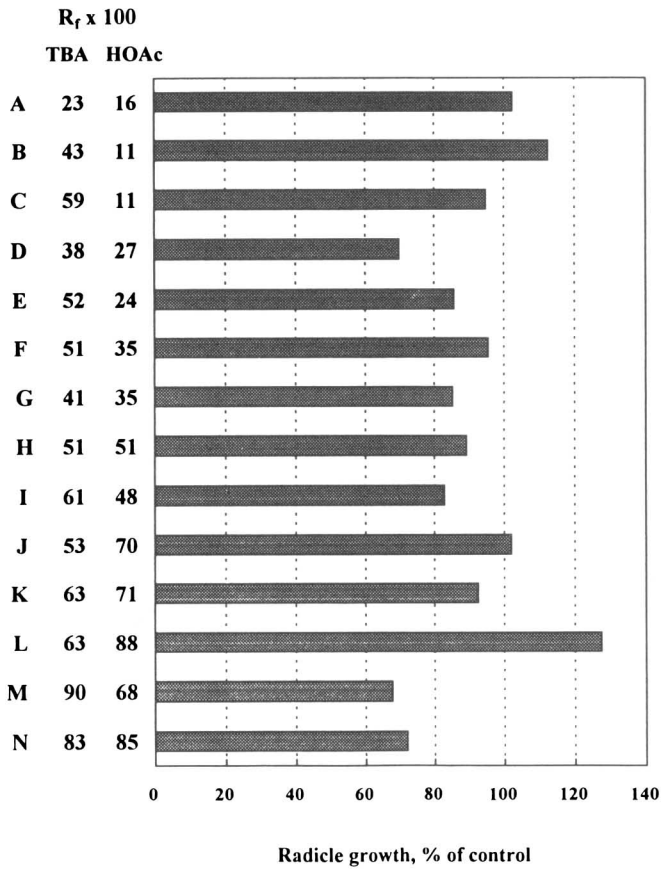

FIG. 11. Effect of compounds isolated from paper chromatograms of the ethyl acetate fraction of methanol extracts of *A. confusa* on the radicle growth of lettuce. Solvent systems are: TBA = *t*-butyl alcohol–acetic acid–water (3:1:1, v/v/v), and 15% HOAc = acetic acid–water (15:85, v/v).

and Rizvi, 1992; Waller, 1987). It is agreed that the proper use of allelopathic plants or natural products produced by plants or microorganisms can decrease the use of expensive synthetic herbicides that deteriorate environmental quality. However, they also point out that this goal is difficult to achieve due to the lack of economic incentive. However, there are several examples of successful use in agricultural practice. For example, the neem plant (*Azadirachta indica* A) has been widely used in India as a fungicide, and nematocide (Parmar and Singh, 1993). A natural product of *Agrostemin*, obtained from corn cockle of *Agros-*

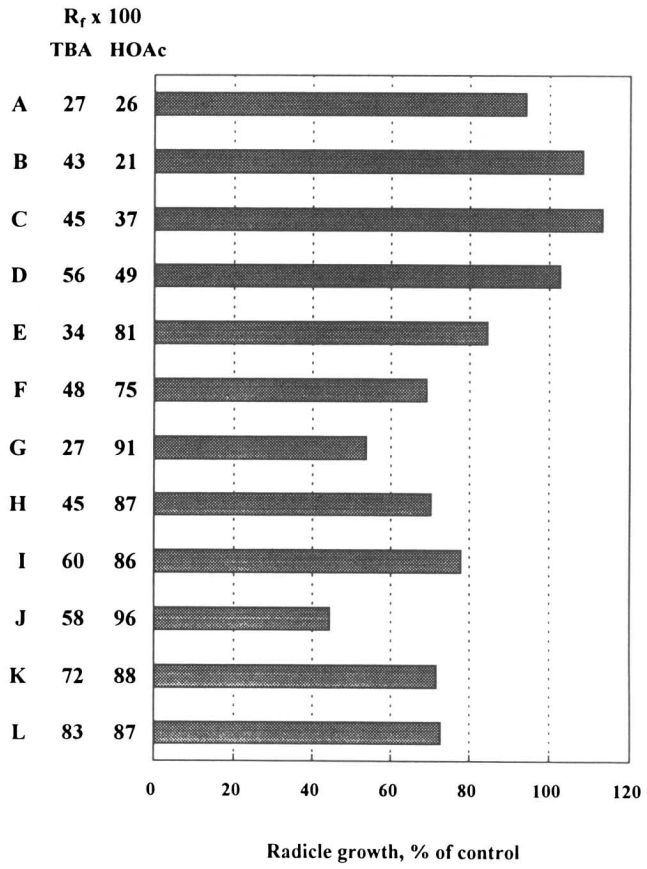

FIG. 12. Effect of compounds isolated from the water fraction of methanol extracts of *A. confusa* on radicle growth of lettuce.

temma githago L, has been widely used in eastern European countries to increase the productivity of many crops; it also suppresses weed growth (Welte and Szabolcs, 1987). In Taiwan, Chou et al. (1989) demonstrated a case study of a grass–forest intercropping system. A grass, *Pennisetum clandestinum*, was planted on the deforested land of a coniferous tree, *Cunninghamia lanceolata*. After planting, the grass became predominant and suppressed the growth of many weeds in situ. The bioassay of the aqueous extract of grass revealed significant phytotoxicity; however, the grass was not harmful to the growth of coniferous trees or to the hardwood forest plants. Moreover, the grass could be used as a forage crop for livestock.

TABLE 2. ALLELOPATHIC SUBSTANCES FOUND IN AQUEOUS EXTRACTS OF LEAVES OF *Acacia confusa*[a]

Compound	PC	HPLC
Caffeic acid	+	−
m-Hydroxybenzoic acid	+	−
m-Hydroxyphenylacetic acid	+	+
Ferulic acid	+	+
Vanillic acid	+	+
Gallic acid	+	+

[a]The compounds were identified by paper chromatography (PC) and high performance liquid chromatography (HPLC). + = Presence, − = Not found.

From the aforementioned findings, we conclude that *Acacia confusa* is an allelopathic plant, the leaves of which contain the major phytotoxic agent. In addition, the phytotoxins present are water soluble. Some of the responsible allelopathic compounds have been isolated and some of the phytotoxic phenolics have been identified. The isolation and identification of more compounds, such as flavonoids, are in progress.

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EFFECTS OF PLANT DIET ON DETOXIFICATION ENZYME ACTIVITIES OF TWO GRASSHOPPERS, *Melanoplus differentialis* AND *Taeniopoda eques*

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Abstract—The polyphagous grasshoppers *Melanoplus differentialis* and *Taeniopoda eques* use different foraging patterns: over time *M. differentialis* tends to reduce the variety of host plants it feeds on and specialize on particular plants (diet components), whereas *T. eques* mixes host plants to achieve a very diverse diet. We tested the hypothesis that these differing behaviors are correlated with differing patterns of detoxification enzymes. The activities of midgut, fat body, and malpighian tubule detoxification enzymes were determined in last instars of the two grasshoppers, reared for five days on single- or mixed-plant diets. Significant differences in several cytochrome P450 activities and glutathione *S*-transferase were evident for nymphal grasshoppers feeding on different plant diets. However, the behavioral differences between the two species could not be explained by an underlying flexibility of detoxification response in *M. differentialis*, but lacking in *T. eques*. This is the first reported evidence that detoxification enzyme activities are affected by plant diet in polyphagous orthopterans.

Key Words—Cytochrome P450, glutathione, *S*-transferase, esterase, plant diet, *Melanoplus differentialis*, *Taeniopoda eques*.

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INTRODUCTION

Insect herbivores use a wide variety of foraging strategies in their search for nutrition. Some species are extreme specialists, feeding on a single tissue of a single plant species. Other insects are polyphagous, accepting plants from many different families. Even among such generalists, feeding patterns show variation. Some species initially feed on a variety of host plants, but tend to specialize on particular items as they gain experience with them, a process termed induced host plant preference (Jermy, 1987). In other cases insects appear actively to mix their diets, avoiding repeated meals on the same plant species (Bernays et al., 1992). The physiological mechanisms underlying these contrasting feeding behaviors are not yet known. Nevertheless, it is likely that the two behaviors will result in differing patterns of exposure to allelochemicals contained in the host plants. Insects ingesting a diverse diet will encounter relatively low amounts of a large array of compounds; indeed it has been suggested that dietary dilution of allelochemical intake any underlie selection for polyphagy (Freeland and Janzen, 1977). In contrast, behavioral specialization on specific host plants will narrow the range of allelochemicals encountered, but will increase the intake of secondary metabolites characteristic of the host plants that become preferred. These differences would seem to require different detoxification strategies. In particular, we would expect that the diverse diet would necessitate a relatively generalized resistance or detoxification mechanism, and the more restricted diet would correlate with a more specialized mechanism appropriate to the secondary metabolite profile of the host plant.

The most studied forms of herbivorous insect resistance to plant secondary metabolites are metabolic defenses (Berenbaum, 1991). The major classes of enzymes responsible for detoxifying plant compounds are glutathione *S*-transferases, various esterases, and cytochrome P450 monooxygenases. Glutathione *S*-transferase activities can be induced $\geq$30-fold (Egaas et al., 1992) and can metabolize plant chemicals such as allyl isothiocyanate (found in cruciferous plants) in some Lepidoptera (Wadleigh and Yu, 1988). Esterases have been implicated in the detoxification of phenolic glycosides in the papilionidae (Lindroth, 1989). Cytochrome P450s are known to metabolize a wide variety of plant compounds. The furanocoumarin xanthotoxin was shown to be detoxified by a specific cytochrome P450, CYP6B1, in *Papilio polyxenes* (Ma et al., 1994). Furthermore, nicotine metabolism, by an as yet characterized P450(s), was induced in tobacco hornworm larvae by feeding on nicotine in an artificial diet (Snyder et al., 1994).

Although there is an extensive literature on plant secondary metabolite detoxification in Lepidoptera (Berenbaum and Isman, 1989; Berenbaum, 1991), little is known about these processes in the Orthoptera. A few studies have demonstrated cytochrome P450 involvement in the detoxification of insecticides

and other xenobiotics (Benke and Wilkinson, 1971a,b; Singh and Thornhill, 1980; Yu, 1982; Kumi et al., 1991). The results of other studies have provided evidence that the toxicity of some plant compounds is reduced when the dose is via oral or topical routes rather than injection (Isman, 1985; Isman et al., 1987; Cottee et al., 1988; Champagne et al., 1989), implying that some types of detoxification mechanisms must be occurring after ingestion.

In this report, we describe the effects of varied plant diet on the detoxification activities of two grasshoppers with contrasting feeding behaviors. *Melanoplus differentialis* tends to show induced preferences on particular host plants (Howard, in preparation). In both field and laboratory studies *Taenipoda eques* actively mixes diet, frequently switching between host plants (Bernays et al., 1992). Last instars of both species were fed for five days with one of four single-plant diets or a mixed diet of all four plants and were subsequently assayed for 11 different detoxification enzyme activities. If *T. eques* is mixing to dilute its allelochemical intake, we would expect relatively low titers of many enzymes and little ability to induce activity in response to specific allelochemical challenges. In contrast, *M. differentialis* may be expected to tailor its detoxification enzymes to the secondary metabolite profile of its diet, which should result in high among-diet variation in enzyme activities.

METHODS AND MATERIALS

Insect Rearing Conditions. Laboratory cultures of *Taeniopoda eques* and *Melanoplus differentialis* used in this study were established from insects collected in the fall of 1989 from the Buenos Aires National Wildlife Refuge, 70 km southwest of Tucson, Arizona. Prior to our experiments, lab cultures were maintained on a standard diet of romaine lettuce and wheat bran. Insects were housed in groups in cubical metal cages with 30-cm wire screen sides. *T. eques* undergoes five instars prior to the adult molt, while *M. differentialis* undergoes six instars. We conditioned insects and analyzed enzyme activities in the last instar of each species.

In order to obtain adequate amounts of tissue for analysis, we conducted all experiments on groups of insects. Fifth instars of *T. eques* are significantly larger than sixth instars of *M. differentialis*, and to obtain similar amounts of tissue from both species we used groups of 8–12 *T. eques* and 15–20 *M. differentialis*. We conditioned insects for five days on one of five diets: either basil (*Ocimum basilicum*, Labiatae), kale (*Brassica oleracea* var. *acephala*, Cruciferae), mint (*Mentha piperita*, Labiatae), or tomato (*Lycospersicon esculentum*, Solanaceae), fed ad libitum, or a mixture of all four plants. We randomly selected groups of insects molting to the appropriate instar on a single day, and placed them in 8-liter transparent plastic buckets with the conditioning diet.

Plants were replaced and the buckets cleaned on a daily basis. Buckets were housed in a controlled temperature environmental chamber set on a 12L:12D cycle, with temperature cycling from 33°C (light phase) to 27°C (dark phase). Three replicate groups of insects were conditioned on each diet treatment.

Preparation of Tissues. Last instars were grouped into pools of three to four individuals. After removal of heads, legs, and wing pads, the midguts (MG) were dissected free of hindguts and Malpighian tubules (TUB). The hindgut and tubules were taken together as TUBs. The carcass pools were taken together as fat body (FB). The pooled tissues were homogenized in 0.1 M $NaPO_4$ buffer (pH 7.5) containing 10% glycerol, 1 mM EDTA, and 0.1 mM dithiothreitol. The homogenates [total volumes per tissue, from three to four individuals were 2 ml (TUB), 3 ml (MG), and 8 ml (FB)] were centrifuged at 10,000*g* for 15 min. The resulting supernatants were filtered through glass wool and centrifuged at 100,000*g* for 60 min to pellet microsomes. The final supernatant served as cytosol for glutathione *S*-transferase and esterase assays, while the microsomal pellets were individually resuspended in the homogenization buffer. Both tissue fractions were immediately frozen in liquid nitrogen and stored at −80°C until assayed for enzymatic activities. Total protein of cytosols and microsomes was assayed by the use of the bicinchoninic acid procedure (Sigma) for microtiter plates with bovine albumin as standard.

Enzyme Assays. All assays were performed on two to four different pools of tissues from both species of grasshopper. The linearity of enzyme activities was checked for each tissue of both species over the time course of the incubation by repeated sampling, and the data were analyzed by ANOVA.

Glutathione *S*-transferase (GST) and general esterase (EST) activities were assayed according to the procedures of Grant et al. (1989) with the following amounts of cytosol protein per assay: GST = 50 μg TUB, 50 μg FB, 100 μg MG, and EST = 100 μg TUB, 100 μg FB, 25 μg MG. Kinetic assays (5 min) were measured directly in 96-well microplates at 340 nm (GST) or 450 nm (EST) with a Thermomax microplate reader (Molecular Devices).

Aldrin (ALD) and heptachlor (HEP) epoxidation by microsomes were assayed according to Feyereisen and Vincent (1984) by using the following amounts of protein and incubation times: TUB, 0.25 mg, 45 min; FB, 0.25 mg, 15 min; and MG, 0.5 mg, 30 min. The products dieldrin and heptachlor epoxide were measured by electron capture on a Shimadzu GC-8A gas chromatograph.

The *N*-demethylation of *p*-chloro-*N*-methylaniline (PCN) was measured according to Snyder et al. (1993) on the following microsomal protein concentrations and incubation times: TUB, 0.25 mg, 30 min; FB, 0.25 mg, 20 min; and MG, 0.25 mg, 20 min. The production of *p*-chloroaniline was compared against a standard curve at 450 nm in the Thermomax plate reader.

O-Demethylation of 7-methoxycoumarin (7MC) was assayed according to Feyereisen and Vincent (1984) in a 30-min incubation with microsomal protein

concentrations of 0.125 mg TUB, 0.25 mg FB, and 0.5 mg MG. The product 7-hydroxycoumarin was measured against a standard curve at wavelengths of 370 nm excitation and 455 nm emission with a Perkin-Elmer 650-10S fluorescence spectrophotometer.

Biphenyl hydroxylation was assayed according to the procedure of Burke and Prough (1978) in a 30-min incubation with 0.125 mg TUB or 0.50 mg FB and MG of microsomal protein per assay. The products 2-hydroxybiphenyl (BP2) and 4-hydroxybiphenyl (BP4) were measured against standards at 290 nm excitation/420 nm emission and 270 nm excitation/330 nm emission, respectively, by fluorometric detection as indicated above.

The *S*-demethylation of 6-methylthiopurine (MTP) was measured according to Yu (1988) with the following protein concentrations and incubation times; TUB, 0.125 mg, 60 min; FB, 0.25 mg, 40 min; and MG, 0.25 mg, 40 min. The absorbance of produced formaldehyde was measured with the Nash reagent at 405 nm on the Thermomax microplate reader as outlined above.

Total P450 was measured according to the procedure of Omura and Sato (1964). The spectra were measured at 12°C on a Perkin-Elmer Lambda 19 spectrometer.

NADPH-cytochrome *c* reductase activity was assayed according to Snyder et al. (1993) with 10 μg of microsomal protein per 5 min assay for all tissue samples on the Thermomax plate reader.

RESULTS

Effects of Plant Diet. The midgut of *T. eques* fed on tomato or mint appeared thin, and 50% less total cytosolic protein was recovered from MG, FB, and TUB on these two diets. The average of the total cytosol protein per insect recovered for the three tissues of both species was as follows: MG = 28%, FB = 68%, and TUB = 4%. Diet had no effect on microsomal protein levels found in each tissue of both species. The average of the total microsomal protein per insect recovered for the three tissues of both species were: MG = 45%, FB = 45%, and TUB = 10%.

The midgut for both species exhibited only one significantly different enzyme activity in response to the different diet treatments: GST activity against CDNB was increased by 4- to 10-fold for the mixed diet in *M. differentialis*, and 6-MTP *S*-demethylation activity was significantly reduced by 2- to 3-fold in mint-fed *T. eques* (Table 1).

Fat body showed more significant enzyme activity responses to feeding on different plants in both grasshoppers (Table 2). For *M. differentialis*, feeding on tomato resulted in 2- to 7-fold decreases in epoxidation of aldrin and heptachlor, and 2- to 4-fold decreases in 4-hydroxylation activity of biphenyl. Feed-

TABLE 1. INFLUENCE OF DIET ON MIDGUT ACTIVITIES[a]

	GST	EST	PCN	CYC	ALD	HEP	BP4	BP2	7MC	MTP	TOP
Melanoplus											
Mixed	1900.0[b]	1689.1[a]	0.1[a]	20.9[a]	0.0[a]	8.3[a]	48.0[a]	1.2[a]	0.4[a]	21.7[a]	140.0[a]
	(70.0)	(248.5)	(0.1)	(1.4)	(0.0)	(1.5)	(11.5)	(0.2)	(0.1)	(2.6)	(11.0)
Basil	430.0[ab]	841.9[a]	1.8[a]	16.6[a]	11.7[a]	8.4[a]	109.0[a]	4.4[a]	1.1[a]	55.0[a]	120.5[a]
	(200.0)	(361.2)	(1.2)	(2.3)	(11.2)	(5.0)	(26.6)	(1.7)	(0.7)	(35.1)	(13.5)
Kale	230.0[ab]	736.9[a]	0.4[a]	22.7[a]	8.3[a]	15.2[a]	68.0[a]	1.8[a]	4.7[a]	35.0[a]	110.0[a]
	(60.0)	(202.6)	(0.3)	(0.4)	(2.3)	(2.1)	(16.0)	(0.3)	(2.1)	(9.7)	(7.0)
Tomato	200.0[a]	855.2[a]	0.5[a]	20.6[a]	2.7[a]	11.4[a]	52.3[a]	0.5[a]	1.3[a]	13.3[a]	126.5[a]
	(20.0)	(205.2)	(0.2)	(3.6)	(2.7)	(3.5)	(8.7)	(0.2)	(0.5)	(1.8)	(6.5)
Mint	340.0[ab]	204.6[a]	0.4[a]	21.3[a]	14.7[a]	30.5[a]	36.6[a]	1.5[a]	0.6[a]	35.3[a]	103.0[a]
	(140.0)	(41.2)	(0.2)	(2.0)	(14.7)	(8.8)	(18.3)	(0.7)	(0.4)	(4.9)	(8.0)
Taeniopoda											
Mixed	86.5[a]	741.3[a]	9.9[a]	11.4[a]	0.0[a]	6.5[a]	107.3[a]	5.5[a]	1.2[a]	36.0[a]	134.0[a]
	(9.7)	(241.5)	(1.9)	(1.7)	(0.0)	(1.3)	(14.3)	(0.6)	(0.4)	(4.6)	(7.0)
Basil	104.8[a]	506.1[a]	6.8[a]	14.9[a]	0.7[a]	10.2[a]	56.3[a]	1.5[a]	0.4[a]	24.3[ab]	130.0[a]
	(16.7)	(66.1)	(1.2)	(2.0)	(0.7)	(1.9)	(17.9)	(0.4)	(0.2)	(4.7)	(9.0)
Kale	171.9[a]	500.0[a]	5.9[a]	15.1[a]	0.0[a]	9.2[a]	111.7[a]	4.5[a]	0.7[a]	24.3[ab]	107.0[a]
	(51.9)	(76.0)	(1.7)	(0.7)	(0.0)	(6.7)	(49.4)	(2.1)	(0.2)	(2.6)	(2.0)
Tomato	87.5[a]	291.4[a]	2.4[a]	35.7[a]	0.8[a]	1.4[a]	113.0[a]	3.5[a]	0.8[a]	25.7[ab]	155.0[a]
	(18.1)	(62.6)	(0.5)	(21.1)	(0.8)	(1.4)	(61.0)	(1.3)	(0.2)	(4.7)	(14.0)
Mint	40.1[a]	181.4[a]	4.1[a]	14.0[a]	0.0[a]	1.5[a]	59.3[a]	2.9[a]	0.5[a]	14.7[b]	103.5[a]
	(1.2)	(26.7)	(0.4)	(1.9)	(0.0)	(0.8)	(9.4)	(1.5)	(0.2)	(2.3)	(10.5)

[a]Values are mean (SD), based on three to four different cytosolic or microsomal pools for each grasshopper species. Values for GST, EST, PCN., and CYC are expressed in nanomoles product formed per milligrams protein per minute, while ALD, HEP, BP4, BP2, 7MC, MTP, and TOP are expressed as picomoles product formed per milligrams protein per minute. Abbreviations are as follows: GST = glutathione s-transferase, EST = general esterase, PCN = *p*-chloro-*N*-methylaniline *N*-demethylation, CYC = cytochrome *c* reductase, ALD = aldrin epoxidation, HEP = heptachlor epoxidation, BP4 and BP2 = biphenyl 4- or 2-hydroxylation, 7MC = 7-methoxycoumarin *O*-demethylation, MTP = 6-methylthiopurine *S*-demethylation, and TOP = total cytochrome P450. Means within a column of the same species followed by the same superscript letters are not significantly different from each other.

TABLE 2. INFLUENCE OF DIET ON FAT BODY ENZYME ACTIVITIES[a]

	GST	EST	PCN	CYC	ALD	HEP	BP4	BP2	7MC	MTP	TOP
Melanoplus											
Mixed	2920.0[a]	98.6[a]	4.5[a]	51.0[a]	633.3[a]	374.5[ab]	1320.4[a]	7.0[b]	37.6[a]	298.3[a]	220.7[a]
	(940.0)	(20.2)	(1.2)	(7.5)	(103.4)	(95.6)	(301.2)	(3.6)	(21.1)	(141.0)	(78.7)
Basil	1040.0[a]	60.6[a]	2.7[a]	41.3[a]	657.0[a]	725.8[a]	657.0[ab]	11.0[ab]	53.0[a]	259.7[a]	81.4[a]
	(60.0)	(17.1)	(0.5)	(5.3)	(49.7)	(62.3)	(23.7)	(3.1)	(7.6)	(47.5)	(21.1)
Kale	1080.0[a]	82.6[a]	5.3[a]	35.5[a]	692.3[a]	798.8[a]	1443.3[a]	33.1[a]	355.2[b]	381.3[a]	120.5[a]
	(280.0)	(28.6)	(0.9)	(2.1)	(43.2)	(183.2)	(134.6)	(1.9)	(8.9)	(63.5)	(6.5)
Tomato	880.0[a]	46.3[a]	2.4[a]	36.2[a]	219.7[b]	111.1[b]	369.3[b]	15.4a[b]	49.9[a]	267.3[a]	100.8[a]
	(30.0)	(6.0)	(0.4)	(4.1)	(31.8)	(54.2)	(35.5)	(13.6)	(7.9)	(30.2)	(23.1)
Mint	1040.0[a]	61.7[a]	3.5[a]	37.2	453.5[ab]	4492[ab]	766.3[ab]	16.0[ab]	92.2[a]	267.7[a]	95.8[a]
	(90.0)	(7.6)	(0.7)	(3.3)	(23.5)	(69.0)	(169.5)	(4.5)	(12.8)	(39.4)	(9.2)
Taeniopoda											
Mixed	122.0[a]	22.6[a]	3.4[a]	26.8[a]	624.0[ab]	641.2[ab]	328.3[a]	15.1[a]	13.4[ab]	164.7[a]	39.5[a]
	(8.3)	(1.7)	(1.2)	(2.6)	(70.9)	(61.9)	(20.3)	(2.7)	(3.0)	(35.6)	(23.5)
Basil	264.8[a]	35.0[a]	2.9[a]	23.8[a]	552.3[a]	622.0[ab]	253.3[a]	14.9[a]	12.3[ab]	286.3[a]	34.0[a]
	(61.7)	(4.0)	(0.3)	(1.7)	(150.4)	(184.8)	(68.3)	(6.5)	(1.1)	(55.5)	(13.0)
Kale	143.1[a]	28.7[a]	2.2[a]	21.2	1204.0[b]	951.6[b]	403.7[a]	5.3[a]	26.2[b]	308.0[a]	49.0[a]
	(12.4)	(3.2)	(0.7)	(5.1)	(121.8)	(134.3)	(72.3)	(2.6)	(2.6)	(34.1)	(14.0)
Tomato	113.0[a]	40.3[a]	3.2[a]	19.4[a]	280.7[a]	158.3[a]	239.3[a]	10.3[a]	15.2[ab]	224.0[a]	59.0[a]
	(6.9)	(18.7)	(0.1)	(1.1)	(86.2)	(45.9)	(65.1)	(3.9)	(5.0)	(28.3)	(10.0)
Mint	123.8[a]	26.3[a]	2.1[a]	21.[a]	272.3[a]	295.3[a]	171.3[a]	22.7[a]	7.2[a]	378.7[a]	43.0[a]
	(33.1)	(3.1)	(1.1)	(3.0)	(134.4)	(31.7)	(27.3)	(2.9)	(2.3)	(62.7)	(6.0)

[a]Values are mean (SD), based on three to four different cytosolic or microsomal pools for each grasshopper species. Abbreviations and units of measurement are the same as in Table 1. Means within a column of the same species followed by the same superscript letters are not significantly different from each other.

ing on the mixed plant diet caused a 1.5- to 5-fold decrease in 2-hydroxylation of biphenyl, while a kale diet resulted in a 4- to 10-fold increase in 7MC *O*-demethylation activity. In *T. eques*, only the kale diet significantly affected enzyme activities, increasing ALD and HEP epoxidation by 4- to 6-fold, and 7MC 2- to 4-fold.

Malpighian tubule detoxification enzyme activities were also affected by feeding different plant diets to last instar nymphs of both species (Table 3). For *M. differentialis*, tomato resulted in 2- to 4-fold decreases in HEP activity, mixed plant diet significantly increased both hydroxylations—BP4 and BP2—by 9- to 20-fold and 7MC by 23- to 100-fold. Again in *T. eques*, only the kale diet resulted in significant changes in detoxification enzyme activities (ALD and HEP increased by 2- to 4-fold).

Tissue Enzyme Levels. The hierarchical distributions for specific tissue enzyme activities were similar for both grasshopper species as follows: GST (FB = TUB = MG), esterase (MG > TUB > FB), PCN *N*-demethylation (FB = TUB = MG), CYTC reductase (FB = TUB > MG), ALD and HEP epoxidations (TUB > FB > MG), BP4 and BP2 hydroxylation's (FB > TUB > MG), 7MC *O*-demethylation (FB > TUB > MG), MTP *S*-demethylation (FB > TUB = MG), and TOP (MG = TUB ≥ FB). The tissues were ranked according to the percentage of the amount of a particular enzyme activity from the whole insect (represented by three compartments: MG, FB, and TUB). The fat body was the most active tissue for five of 11 measures and Malpighian tubules for four of 11 measures. The midgut was the most active tissue for only two of 11 measures, while all tissues were equally active for two of 11 measures. Based on total protein recoveries, the fat body clearly has the most detoxification enzyme activities of the three grasshopper tissues examined.

Species Comparisons. Enzyme activities between species were not compared statistically, but some trends were apparent. Both GST and EST activities were higher in all three *M. differentialis* tissues than in *T. eques*. In the fat body, *M. differentialis* had higher TOP, 7MC, and CYTC activities. In midgut, *M. differentialis* had higher ALD while *T. eques* had higher PCN activities.

The variation in whole body enzyme responses (all three tissues combined) from diet to diet was significantly greater in *M. differentialis* (nine of 11 measures, $P = 0.0327$, sign test) implying that this species is more variable in its biochemical responses to the different diets than is *T. eques*. Comparing single plant diets to the mixed diet, *M. differentialis* has an average decrease in the 11 enzyme activities of 43%, while in *T. eques* the total activity increased by an average of 21%.

DISCUSSION

Diet Effects. This study is the first to demonstrate the induction of detoxification enzymes by variable plant diets in the Orthoptera. Almost all previous

TABLE 3. EFFECTS OF PLANT DIET ON GRASSHOPPER MALPIGHIAN TUBULE DETOXIFICATION ENZYME ACTIVITIES[a]

	GST	EST	PCN	CYC	ALD	HEP	BP4	BP2	7MC	MTP	TOP
Melanoplus											
Mixed	1400.0^{a}	439.2^{a}	13.7^{a}	54.6^{a}	1477.7^{a}	995.8^{a}	780.0^{b}	117.3^{b}	258.1^{b}	25.0^{a}	158.0^{a}
	(470.0)	(242.9)	(1.0)	(1.3)	(281.6)	(67.9)	(310.0)	(46.4)	(72.4)	(3.0)	(96.0)
Basil	410.0^{a}	212.4^{a}	6.8^{a}	38.8^{a}	1306.7^{a}	937.9^{a}	73.0^{a}	5.7^{a}	11.3^{a}	16.7^{a}	97.5^{a}
	(30.0)	(54.0)	(2.3)	(3.9)	(245.5)	(182.9)	(36.5)	(5.0)	(8.1)	(1.3)	(6.5)
Kale	520.0^{a}	308.8^{a}	7.1^{a}	43.8^{a}	1582.0^{a}	940.4^{ab}	56.7^{a}	7.7^{a}	6.8^{a}	11.0^{a}	73.5^{a}
	(170.0)	(90.7)	(3.3)	(12.4)	(130.0)	(46.4)	(6.7)	(0.7)	(0.3)	(4.5)	(19.5)
Tomato	330.0^{a}	117.3^{a}	3.7^{a}	24.6^{a}	538.0^{a}	237.9^{b}	86.0^{a}	12.3^{a}	2.7^{a}	14.3^{a}	100.0^{a}
	(10.0)	(27.6)	(0.5)	(8.9)	(213.7)	(43.6)	(86.0)	(12.3)	(0.4)	(3.9)	(7.0)
Mint	840.0^{a}	125.6^{a}	4.2^{a}	31.1^{a}	777.3^{a}	545.6^{ab}	50.0^{a}	6.8^{a}	3.3^{a}	14.3^{a}	154.5^{a}
	(450.0)	(65.4)	(2.4)	(0.7)	(255.4)	(93.0)	(25.2)	(3.5)	(0.4)	(2.3)	(72.5)
Taeniopoda											
Mixed	233.9^{a}	55.6^{a}	2.7^{a}	41.4^{a}	799.3^{a}	410.1^{a}	107.3^{a}	31.3^{a}	41.3^{a}	11.5^{a}	163.5^{c}
	(29.5)	(17.6)	(0.9)	(4.0)	(72.5)	(116.0)	(14.3)	(1.3)	(29.1)	(0.7)	(15.5)
Basil	264.1^{a}	86.4^{a}	3.1^{a}	31.4^{a}	792.0^{a}	796.3^{ab}	470.0^{a}	59.1^{a}	37.3^{a}	23.7^{a}	50.5^{a}
	(36.6)	(16.9)	(0.1)	(2.2)	(55.0)	(7.9)	(120.0)	(29.1)	(7.5)	(8.4)	(2.4)
Kale	281.6^{a}	55.5^{a}	3.7^{a}	28.3^{a}	1766.0^{b}	1498.8^{b}	555.0^{a}	89.0^{a}	111.8^{a}	22.7^{a}	99.6^{b}
	(25.9)	(6.2)	(1.9)	(6.1)	(19.0)	(91.0)	(5.0)	(5.5)	(48.8)	(7.5)	(3.2)
Tomato	205.7^{a}	61.9^{a}	2.4^{a}	27.0^{a}	797.3^{a}	398.1^{a}	215.0^{a}	32.2^{a}	49.1^{a}	17.5^{a}	66.0^{ab}
	(35.7)	(16.3)	(0.2)	(1.3)	(34.1)	(30.0)	(15.0)	(2.8)	(0.5)	(5.0)	(7.0)
Mint	201.6^{a}	45.2^{a}	3.4^{a}	29.9^{a}	1011.3^{ab}	697.1^{a}	290.0^{a}	47.9^{a}	91.7^{a}	28.7^{a}	61.4^{ab}
	(9.5)	(14.7)	(1.1)	(2.4)	(236.9)	(211.6)	(66.6)	(11.2)	(19.1)	(16.3)	(13.4)

[a] Data are reported as mean (CSD) for three to four different cytosolic or microsomal pools for each species. Means followed by the same superscript letters are not significantly different from each other. Abbreviations for enzyme activities are given in Table 1.

work on the detoxification enzymes of the Orthoptera has dealt with responses to synthetic pesticides. Further, as discussed by Berenbaum and Isman (1989), most of this work has concentrated on cockroaches. It has been shown that insecticides induced cytochrome P450 activity in cockroaches (Khan and Matsumura, 1972).

The presence of cytochrome P450s in orthopteran tissues was demonstrated in several studies. Benke et al. (1972) and Benke and Wilkinson (1971a,b) first demonstrated that cockroach fat body and cricket Malpighian tubules required NADPH as a cofactor for epoxidation activity. Carbon monoxide and piperonyl butoxide were found to inhibit the activities of Orthoptera subcellular preparations (Chakraborty and Smith, 1967; Benke and Wilkinson, 1971a,b; Feyereisen and Durst, 1978; Yu, 1982). Additionally, Kumi et al. (1991) demonstrated that coapplication of the cytochrome P450 inhibitor piperonyl butoxide significantly reduced the metabolism of benzo[a]pyrene in the cricket, *Acheta domesticus*, and Champagne et al. (1989) found that piperonyl butoxide synergized the toxicity of the plant allelochemical azadirachtin in the grasshopper *Melanoplus sanguinipes*.

The results of this study demonstrate significant activities of a variety of microsomal cytochrome P450s and several cytosolic detoxification enzyme activities in grasshopper tissues. Berenbaum and Isman (1989) used the data of Kulkarni et al. (1976) to argue that nonacridoid Orthoptera have low P450 activities. However, Yu (1982, 1983) reported similar epoxidation activities between orthopterans and lepidopterans. The activities of the two grasshoppers in this study can be compared with those from *Manduca sexta* in assays done by the same investigator (Snyder et al., 1993; Snyder and Glenndinning, 1996). The hydroxylation, *O*-demethylation, *S*-demethylation activities, and total cytochrome P450 were similar between the grasshoppers and *M. sexta*. Cytochrome *c* reductase and *N*-demethylation were lower in grasshoppers, while epoxidation activities ranged from much lower to five times higher than those found in *M. sexta*. The microsomal enzyme activities found in this study were also similar to values reported for locusts and crickets (Feyereisen and Durst, 1978; Yu, 1982). The activities of glutathione *S*-transferase were several hundred-fold less in grasshoppers than in *M. sexta*, although the significance of this is unclear.

The activity of Orthoptera cytochrome P450s appeared from many previous studies to occur along the following tissue hierarchy: Malpighian tubules > fat body > nerve cord > gut (Benke and Wilkinson, 1971a; Feyereisen and Durst, 1978; Singh and Thornhill, 1980). Yu (1982) has reported contrary data on mole crickets that show microsomal activities roughly equivalent in midgut and Malpighian tubules and significantly lower in fat body. Others have reported higher monooxygenase activities in the orthopteran midgut (Benke et al., 1972). In this study, the tissue distribution of grasshopper cytochrome P450 activities resem-

bled the results of Feyereisen and Fransworth (1985), who showed that cytochrome P450 activities could be higher or lower in midgut and fat body from the cockroach, *Diploptera punctata*, depending on the specific enzymatic substrate. At least several activities were highest in either midgut, fat body, or Malpighian tubules of both species of grasshoppers (Tables 1–3).

There is a small body of evidence to suggest that phytophagous orthopterans are able to detoxify plant chemicals and that this metabolism is mediated in part by cytochrome P450s. Several studies have shown that plant chemicals are much less toxic to grasshoppers when administered orally rather than by direct injection into the body cavity (Isman, 1985; Cottee et al., 1988; Champagne et al., 1989). Self et al. (1964) showed that *M. differentialis* and *M. femurrubrum* could efficiently metabolize nicotine. The major metabolite in grasshoppers was cotinine, a known metabolite of nicotine-metabolizing cytochrome P450s (Kyeramaten and Vesell, 1991). Another grasshopper, *M. sanguinipes*, metabolizes plant chromenes, furanocoumarins, and cardiac glycosides (Isman et al., 1987; Smirle and Isman, 1992). Smirle and Isman (1992) demonstrated metabolism of xanthotoxin to polar products, suggesting the involvement of cytochrome P450 oxidation in the metabolism of ingested plant chemicals.

The components of the various plant diets responsible for the changes in grasshopper detoxification enzyme titers in this study are unknown. Cruciferous plants such as kale can contain indoles such as indole-3-carbinol that can induce some CYP1A-mediated hydroxylations of steroid hormones in rats (Baldwin and Leblanc, 1992). Indole-3-carbinol is well known to induce both monooxygenase and glutathione *S*-transferase activities when fed to lepidopterous insects (Yu, 1984; Yu and Ing, 1984). Monoterpenes typical of the Laminaceae (basil and mint, in this case) induce cytochrome P450 levels in Lepidoptera (Brattsten et al., 1977; Brattsten, 1984). Tomato contains the steroidal alkaloid tomatine, various flavonoids, 2-undecanone, and 2-tridecanone but the effects of these on cytochrome P450s have yet to be determined. The methyl ketones, 2-undecanone and 2-tridecanone, are known to be toxic to Lepidoptera and Coleoptera (Dimock et al., 1982; Farrar and Kennedy, 1987; Lin et al., 1987). In addition, nonsecondary metabolite components of the diet may also profoundly affect P450 activities. In rats, P450 titers are influenced by the lipid and protein composition of the diet (Kaminsky and Fasco, 1992). Studies with defined artificial diets will be necessary to distinguish the effect of the various diet components.

Species Differences. Berenbaum and Isman (1989) have argued that both cuticle and gut peritrophic membranes of orthopterans are relatively less permeable to plant allelochemicals than those of lepidopterans. This lowered permeability would negate the need for an extensive microsomal detoxification system such as that found in the Lepidoptera. However, as reported here, grasshopper

detoxification activities are relatively high and probably play important roles in the adaptation to a wide range of host-plant secondary products.

Various hypotheses have been advanced to account for diet breadth. Freeland and Janzen (1974) suggested that polyphagy minimizes the potential toxic load of various allelochemicals in vertebrates. *T. eques* responds to enforced monophagy by elevating enzyme activities (relative to the mixed diet), but the cost of maintaining elevated P450 levels is not known. Although Neal (1987) found no energetic cost to induction of P450 in *Heliothis zea*, his measurements depended on indices of growth and nutritional utilization, which lack the sensitivity to measure subtle effects (Van Loon, 1991). Curiously, *M. differentialis* responded to the single plant diets by reducing most enzyme titers; only two fat body activities, BP2 and 7MC, increased, both in response to the kale diet. If we assume that there is a cost to maintaining elevated levels of detoxification enzyme activity, it is noteworthy that both grasshopper species tend to behave in a manner that reduces that cost.

Although *M. differentialis* is more variable in its response to the various diets, there is no evidence that it is able to tailor its detoxification enzymes to particular diets. Rather, several activities (PCN, CYC, ALD, HEP, BP4) are significantly positively correlated. In *T. eques* activities of ALD, HEP, BP4, BP2, and, to a lesser extent, 7MC are positively correlated. There are no negatively correlated activity levels in either species. Since most activities change in concert, the differences between the two species are due to the magnitude of the single-plant diet-related changes (greater for *M. differentialis*), and the direction of change (increased for *T. eques*, decreased for *M. differentialis*). The basis for the positive correlations is not known; the activities may be due to a small number of enzymes with very broad substrate affinities, or several enzymes may respond to the same regulatory signals. Nevertheless it is clear that behavioral differences between the species cannot be explained by an underlying flexibility of detoxification response present in *M. differentialis* but lacking in *T. eques*.

Both *T. eques* and the closely related *Romalea guttata* sequester noxious or toxic compounds from their food plants. This implies that the gut of grasshoppers that sequester secondary metabolites do not pose a major barrier to the uptake of allelochemicals. Midgut enzyme activities did not differ between *T. eques* and *M. differentialis*, so it is unlikely that the behavioral differences result from differential effectiveness of the gut as a barrier to allelochemicals, correlated with defensive strategies used by the two species. Jones et al. (1989) suggested that dietary mixing by *Romalea* is a mechanism for maximizing the diversity of compounds present in the defense secretion. This hypothesis cannot be excluded on the basis of the enzyme activities studied here. Behavioral differences between the two species may also relate to nutritional mixing or sensory effects (Bernays et al., 1992) or to ecological factors.

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IDENTIFICATION OF FLORAL VOLATILES FROM *Ligustrum japonicum* THAT STIMULATE FLOWER-VISITING BY CABBAGE BUTTERFLY, *Pieris rapae*

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Abstract—Floral scent compounds of *Ligustrum japonicum* that affect the foraging behavior of *Pieris rapae* adults were examined by means of chemical analyses, electroantennogram (EAG) responses, and behavioral bioassays; the behavioral biossays consisted of two tests: reflex extension of proboscis (REP) in response to odor, and attraction to scented and unscented artificial flowers. More than 30 compounds, including 2-phenylethanol, benzyl alcohol, and methyl phenylacetate as the major components were identified from *L. japonicum* flowers. Of these, 22 compounds were tested for their effect on foraging behavior. Phenylacetaldehyde (PA), 2-phenylethanol (PE), and 6-methylhept-5-en-2-one (MHO) elicited the highest REP responses, and benzaldehyde (BA) and methyl phenylacetate (MPA) evoked intermediate REP responses. EAG responses were not necessarily correlated with REP activities; the three high-REP compounds gave only moderate EAG responses, whereas two other compounds (ethyl phenylacetate and 2-phenylethyl acetate) that released high EAG responses showed low REP activities. In two-choice behavioral bioassays, flower models scented with any one of these high-REP compounds attracted significantly more adults, while compounds with low REP activities exhibited weak or no appreciable attractiveness. This suggests that the REP responsiveness closely reflects the attractiveness of a compound and could be an effective measure in elucidating which chemical attractants are involved in flower-visiting. A synthetic blend of five floral chemicals (PA, PE, MHO, BA, and MPA) displayed an attractiveness that was comparable to that of the floral extract and was more effective in attractiveness than the compounds tested singly. Consequently, it is highly likely that the flower-visiting by *P. rapae* to *L. japonicum* is mediated largely by floral scent chemicals and that a synergistic effect of the five floral components would be most respon-

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sible for attraction of the butterfly to this flower. The present results also strongly suggest that specific floral volatiles may facilitate close-range flower location by *P. rapae*, could serve in part as a cue for recognizing food sources, and also be profoundly implicated in flower preference.

Key Words—Flower-visiting, floral scent, *Pieris rapae*, Pieridae, *Ligustrum japonicum*, Oleaceae, proboscis extension, EAG, phenylacetaldehyde, 2-phenylethanol, 6-methylhept-5-en-2-one.

INTRODUCTION

Insect–flower associations have long been a pivotal subject for many entomologists because of their economic importance in agriculture and because of the coevolutionary history between flowers and pollinating insects. Adults of most butterfly species are effective pollinators and tend to visit a broad spectrum of plant species for nectar. Field observations, however, reveal that some flowers are frequently utilized by butterflies while others are not. In certain cases, flowers of particular plants are more preferred by particular butterfly species and flower constancy is sometimes prominent. Flower constancy, which varies with both the species of butterfly and the species of plant, appears to be an outcome of learning through the recognition of rewarding flowers (Lewis, 1989; Lewis and Lipani, 1990).

Insects are known to make use of a variety of sensory modalities in foraging, and the integration of visual, olfactory, and gustatory cues are usually involved in their orientation to and finding of food sources (Barth, 1991). Although sufficient information is not available at present on the mechanisms by which butterflies detect and recognize flowers, a few publications clearly demonstrate that the flower-visiting behavior of butterflies is affected to a large extent by the color of flowers and that colors are profoundly involved in flower recognition and preference (Ilse, 1928; Honda, 1976; Miyakawa, 1976; Scherer and Kolb, 1987a,b). Moreover, it is also established that butterflies can perceive airborne chemicals from food sources such as flowers, oak sap, rotten fruits, and so on (Ilse, 1928; Honda, 1973, 1976; Pellmyr, 1986) and show high levels of sensitivity to diverse volatile compounds (Topazzini et al., 1990). In several nocturnal moth species, attraction to flowers is guided mainly by floral sent (Weisenborn and Baker, 1990; Gabel et al., 1992; Zhu et al., 1993; Dobson, 1994), while feeding behavior of a diurnal *Zygaena* moth is triggered by simultaneous stimulation of vision and olfaction (Naumann et al., 1991). Likewise in butterflies, attraction to flowers is probably modulated olfactorily by floral odor. However, very little is known about how floral volatiles affect their behavior before and after alighting and which chemicals might play a critical role in butterfly–flower interactions.

The cabbage butterfly, *Pieris rapae crucivora* Boisd. (Pieridae), often visits flowers of *Ligustrum japonicum* Thunb. (Oleaceae). This shrub sprouts a panicle (5–15 cm) of many small flowers with a white funnel-shaped corolla (5–6 mm) in June in Japan. A brief photographic investigation showed the flowers have no visual and ultraviolet marks (nectar guides) on petals (unpublished), suggesting high reliance on cues other than visual ones in the foraging behavior of the butterfly to the flowers. We thus examined the chemical basis for attraction of *P. rapae* adults to *L. japonicum* flowers by means of chemical analyses, EAG, and behavioral bioassays.

METHODS AND MATERIALS

Collection of Volatile Floral Compounds. Fresh flowers of *L. japonicum* (192 g) collected in the Osaka prefecture were extracted with approximately 1 liter of isopentane for two days at 25°C. The extract was concentrated in vacuo at 15°C and the residue (1.77 g) was subjected to steam distillation.

Chemical Analysis of Floral Volatiles. The chemical composition of the floral volatiles was examined by GC and GC-MS. GC analyses were carried out with a Shimadzu GC-14A gas chromatograph on a FFAP fused-silica capillary column (0.25 mm ID × 50 m). The injection temperature was 250°C, and the oven temperature was programmed from 50°C to 230°C at a rate of 5°C/min. The flow rate of carrier gas (N_2) was 1 ml/min. Mass spectra were recorded at an ionization potential of 70 eV on a Shimadzu QP-2000 mass spectrometer interfaced to a Shimadzu GC-14A gas chromatograph, using the same capillary column and under similar operational conditions to those described above. Identification of the components was based on comparison of GC retention and mass spectral data with those of authentic chemicals.

Insects. Adults of *Pieris rapae crucivora* (2–7 days old) used for the experiments were obtained from stock cultures maintained in our laboratory. Larvae were reared on potted cabbage at 25°C with a 16-hr light, 8-hr dark regime. Adults were fed with 10% sucrose–water once daily and not allowed to contact real flowers throughout the experiments.

All behavioral and electrophysiological experiments were conducted at 25°C in an air-conditioned room with good ventilation.

Reflex Extension of Proboscis (REP). Some butterfly species have been reported to extend the proboscis spontaneously in response to certain colors or odors; they unroll the proboscis and perform probing and sucking movements immediately when such stimuli are given (Ilse and Vaidya, 1956; Myers and Walter, 1970; Honda, 1973, 1976). This behavior can be regarded as showing reflex reaction to specific stimuli and implying, without doubt, a feeding

response. Therefore, these stimuli seem likely to serve as cues by which insects recognize the presence of food sources. Since a pilot experiment revealed that *P. rapae* also responds to particular odors by exhibiting such behavior, we utilized REP responses as a measure for estimating the olfactory responsiveness of this species to floral components identified from *L. japonicum.*

Butterflies were starved for at least 12 hr prior to the experiment. Before starting the bioassay, they were permitted free flight for 2 hr in a plastic chamber illuminated externally with incandescent lamps (3500 lux) and were subsequently fed water only. Thereafter, one individual was introduced into a plastic mesh cage (15 × 13 × 8 cm) under similar illumination. Deodorized and humidified air, which was used as a carrier of odorants, was prepared by passing the air through a column packed with molecular sieves and a gas washing bottle filled with distilled water. Adults were pretested to determine whether they respond to the airstream alone (1400 ml/min); those that showed positive responses to the carrier gas were discarded.

The odor plume of a test chemical was delivered onto the antennae for 30 sec from a distance of 5 cm through a glass cartridge (5 mm ID × 25 mm) in which a piece of filter paper (5 × 30 mm) impregnated with a test chemical (5 μl) was placed. Each individual was assayed once a day, and the tests were replicated four times. The REP response of each trial was scored as 100% for food-seeking behavior with extension of the proboscis, 50% for half-unrolling the proboscis, and 0 for no response. The REP-eliciting activity of each compound was expressed as the mean percentage response of individual adults. REP responses to a total of 22 compounds were recorded from more than 10 individuals of each sex.

Tests for Attraction to Artificial Flowers. The effect of floral scent on a butterfly's alighting on flowers and on behavior after alighting (REP) was examined using scented and unscented flower models. The model was composed of a 50-ml Erlenmeyer flask (75 mm high) laterally covered with green paper, a doughnut-shaped yellow filter paper disc (22 mm ID, 50 mm OD) held horizontally on top of the flask, and a crumpled white paper towel inserted into the flask such that it protruded about 1 cm above the top. The disc was dyed with 0.1% aq. Tartrazine to promote attraction of the butterfly. In preliminary experiments, filter paper dyed with higher concentrations of Tartrazine solution was found to attract *P. rapae* stongly, whereas 0.1% solution only marginally attracted them.

Two treated and two control (or differently treated) flowers were diagonally placed at the corner of a square (50 × 50 cm^2) in the experimental arena (150 × 70 cm, 76 cm high), the floor of which was covered with green plastic plates to imitate green vegetation. Before the bioassay, each individual was conditioned in the same manner as in REP assays.

Samples tested were five selected floral components, a flower extract of *L. japonicum*, and its floral-scent mimic, which is a mixture of synthetic compounds (phenylacetaldehyde, 2-phenylethanol, 6-methylhept-5-en-2-one, benzaldehyde, and methyl phenylacetate) prepared to approximate the blend ratios of the compounds found in the floral extract. When individual floral components were assayed, the flask was filled with 50 ml of distilled water in which 50 mg of an authentic chemical was dissolved or dispersed. For the tests with the flower extract and its mimic, in which much smaller amounts of test chemicals were used, a given amount of sample dissolved in dichloromethane was uniformly applied to the paper towel of the model, while the unscented "flower" was treated with an equal amount of the solvent alone.

Five to eight adults of both sexes (sex ratio not controlled) were employed for each run and allowed free flight over the experimental arena under illumination with fluorescent lights (3500 lux). The total number of alightings and the number of alightings followed by proboscis extension by each individual during a 30-min period were recorded for the treated and the control. During the bioassay, the positions of flowers were rotated once every 10 min to minimize the influence of location and learning. Six replicates were performed for each trial using different individuals. Significance of differences between treatments and controls was analyzed by a Wilcoxon's signed-ranks test, while the effect of treatments was assessed by a χ^2 test.

Electroantennogram (EAG) Recording. Antennal responses to floral components were examined electroantennographically. An antenna was excised at its base and mounted vertically on a sponge that was placed in a Petri dish filled with Ringer's solution (Roelofs and Comeau, 1971). An AgCl-coated Ag plate was used as the indifferent electrode. The recording electrode consisted of a glass capillary filled with Ringer's solution (Pt wire inserted) and was brought into contact with the distal end of the excised antenna. This preparation was connected to a DPZ-115 input probe coupled with a DPA-100E amplifier (Dia Medical System), which was interfaced to a NEC PC-9801RA microcomputer. EAGs were visualized on a CRT and measured as the maximum amplitude of depolarization elicited by a stimulus.

A test compound dissolved in dichloromethane was deposited on a filter paper strip (5 × 30 mm). After evaporating the solvent at room temperature, the filter paper was placed in a sample cartridge (6 mm ID × 50 mm). An odor puff of 2 ml was mixed with a humidified airstream blowing continuously over the antenna at a rate of 350 ml/min through 6-mm-ID glass tubing, which terminated 1 cm from the antennal preparation.

A total of 22 floral components were tested individually at three doses (1, 10, and 100 μg) on five antennae of both sexes, each antenna of which originated from a different butterfly. All tests were repeated three times for each

antenna. Since the antennal responses decreased in amplitude over time and the interindividual variation in absolute response to a stimulus was considerable, standards (1 μg of hexan-1-ol) and blanks were included before each test chemical. The responses were averaged and expressed as percentages of the response to the standard.

Test Chemicals. Authentic chemicals used as olfactory stimuli were purchased from commercial sources (Tokyo Chemical Industry, Nakarai Chemicals, and Aldrich). Their purities as assessed by GC were above 97% except for β-myrcene (ca. 70%) and nerolidol [a mixture of *Z* (35%) and *E* (65%) isomers].

RESULTS

Chemical Composition of Floral Scent. More than 30 compounds were identified from the steam distillate of the extract of *L. japonicum* flowers (Table 1). The floral volatiles contained a variety of aromatic compounds, which seem to characterize the scent of this flower. 2-Phenylethanol, benzyl alcohol and methyl phenylacetate were the major constituents, comprising more than 20% of the volatiles detected.

REP Responses to Floral Components. In general, REP responses to individual components were not high; however, several compounds elicited conspicuous positive responses (Figure 1). Among the 22 compounds tested, phenylacetaldehyde, 2-phenylethanol, and 6-methylhept-5-en-2-one released relatively higher responses from both sexes, while intermediate responses were obtained from benzaldehyde and methyl phenylacetate. In contrast, elicitation of REP responses was weak for green leaf volatiles [(*Z*)-hex-3-en-1-ol and (*E*)-hex-2-enal] and ubiquitous terpenic alcohols such as linalool, geraniol, and nerolidol. Although sexual differences in intensities of REP responses were not remarkable in most cases, females showed considerably higher responses to (*Z*)-hex-3-en-1-ol, β-myrcene, benzaldehyde, and methyl benzoate, whereas males responded more potently to geraniol.

Effect of Floral Odor on Flower-Visiting Behavior. Flower-visiting is stimulated by some volatile compounds (Figure 2). The proportion of the number of alightings accompanied with proboscis extension on treated flowers to that on control flowers was calculated for samples shown in Figure 2; the ratio for each sample is given in parentheses. Phenylacetaldehyde (3.09), 2-phenylethanol (3.33), and 6-methylhept-5-en-2-one (5.59) significantly enhanced the frequency of alighting and subsequent REP on artificial flowers, whereas benzyl alcohol (2.89) exerted a weaker and (*Z*)-hex-3-en-1-ol (1.40) exerted no appreciable effect on foraging behavior, respectively. A synthetic blend (6.27) composed of five chemicals (phenylacetaldehyde, 2-phenylethanol, 6-methylhept-5-en-2-one, benzaldehyde, and methyl phenylacetate) was significantly more attractive than a single compound ($P < 0.05$).

TABLE 1. CHEMICAL COMPOSITION OF FLORAL VOLATILES FROM *Ligustrum japonicum*

Component	Area (%)
Hydrocarbon	
β-Myrcene	0.03
Limonene	0.08
(*E*)-β-Farnesene	0.14
Alkanes (C_{12}–C_{26})	28.20
Alcohol	
Hexan-1-ol	0.10
(*E*)-Hex-2-en-1-ol	0.29
(*Z*)-Hex-3-en-1-ol	0.90
Octan-1-ol	0.12
(*E*)-Oct-2-en-1-ol	0.16
Oct-1-en-3-ol	0.09
Linalool	0.08
Geraniol	0.14
(*E*)-Nerolidol	0.13
Benzyl alcohol	5.21
2-Phenylethanol	12.36
Aldehyde and ketone	
(*E*)-Hex-2-enal	0.02
Nonanal	0.19
Benzaldehyde	0.33
Phenylacetaldehyde	0.04
6-Methylhept-5-en-2-one	0.15
Ester	
2-Phenylethyl acetate	0.27
Methyl benzoate	1.16
Methyl salicylate	0.21
Methyl phenylacetate	4.50
Ethyl phenylacetate	0.36
Methyl esters of C_{16}–C_{18} acids	8.35
Acid	
Benzoic acid	0.24
Aliphatic acids (C_6–C_{18})	5.57
Phenol ether	
Estragole	0.60
Eugenol	1.78
Total	71.80

Also in another set of experiments with a much smaller dose of test chemicals, both the floral extract (5.17) and the same blend (3.62) significantly augmented the attractiveness (Figure 3, columns A and B). However, the blend showed far higher activity compared to the actual floral extract (Figure 3, column C). This may be due to the fixative effect brought about by coexisting waxy

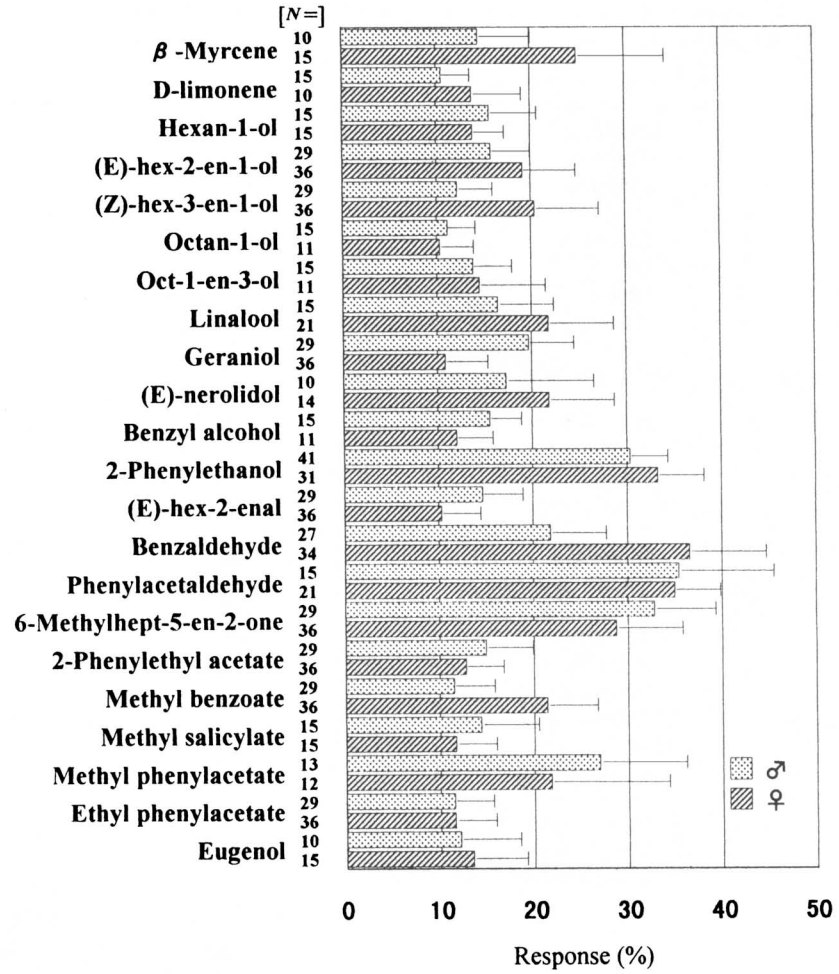

FIG. 1. REP responses (mean ± SEM) of male and female *P. rapae* to individual components of floral scent of *L. japonicum*. *N* = number of individuals tested.

components in the extract, which would considerably suppress the emanation of volatile components. At any rate, a combination of specific floral components seems to elicit flower-visiting from *P. rapae* synergistically. Moreover, in these tests the total number of alightings (including the case where proboscis extension was not observed) on each treated flower model was noticeably larger than that on the control except for the tests with benzyl alcohol and (*Z*)-hex-3-en-1-ol,

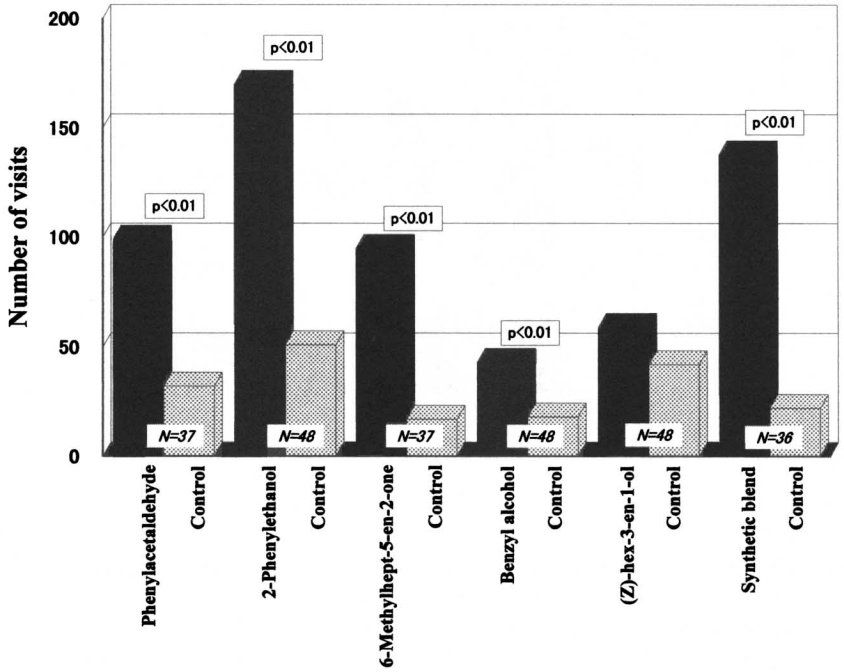

FIG. 2. Effect of floral scent compounds on the attraction of *P. rapae* adults to artificial flowers. Only the number of alightings accompanied by proboscis extension is shown. N = number of individuals tested.

in which almost the same number of alightings took place on the treatment and the control (data not shown).

EAG Responses to Floral Components. EAG responses (mean ± SEM) of both sexes to 21 floral components are shown in Figure 4. EAG responses of males to hexan-1-ol at doses of 1 (standard), 10, and 100 μg were 100, 276 ± 45.2, and 510 ± 83.97, respectively, and those of females were 100, 524 ± 105.2, 1067 ± 235.2, respectively. Female responses at higher doses were much greater than those of males.

At a dose of 1 μg, there were no significant differences in intensities among the compounds tested, although female responses to (*E*)-hex-2-enal (147 ± 24.4) and male responses to 2-phenylethanol (142 ± 17.3) were somewhat larger than to other compounds. At higher doses, however, both sexes responded more sensitively to ethyl phenylacetate (347 ± 41.8 for males, 492 ± 160.9 for females), and male response to 2-phenylethyl acetate (594 ± 182.6) was clearly higher. In contrast, two phenolic compounds—eugenol and methyl salicylate—

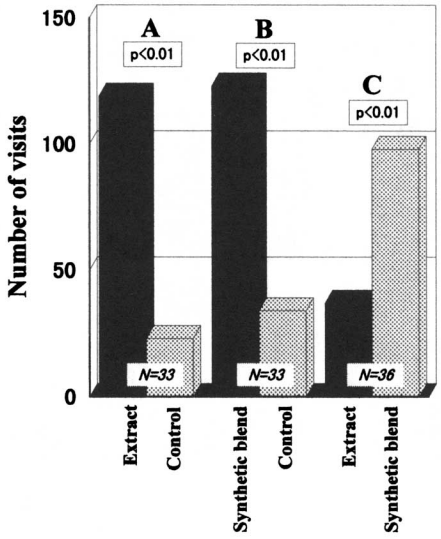

FIG. 3. Effect of floral extract and a blend of scent compounds from *L. japonicum* on the attraction of *P. rapae* adults to artificial flowers. Only the number of alightings accompanied by proboscis extension is shown. The quantity of the floral extract applied was 5 mg per flower model, which corresponds to that derived from 77 living flowers. For the synthetic blend 869 μg was applied per model; the blend consisted of five chemicals (see text) in the amounts and proportions found in 5 mg of the extract. N = number of individuals tested.

released weaker responses. Green leaf volatiles and other compounds characteristic of flowers evoked moderate to weak responses.

DISCUSSION

This study revealed the presence of many new components as well as those previously reported by Kikuchi et al. (1981). Adults of *P. rapae* showed definite REP responses to particular compounds emitted by flowers of *L. japonicum.* Several aromatic compounds, 2-phenylethanol (alcohol), phenylacetaldehyde, benzaldehyde (aldehydes), and methyl phenylacetate (ester), and an aliphatic ketone, 6-methylhept-5-en-2-one elicited relatively higher REP response. It is, therefore, highly likely that these floral chemicals serve as cues by which the butterfly recognizes food or food sources and that these are preferred by the butterfly. On the other hand, green leaf volatiles (C_6 compounds) exhibited low REP activities on the whole. This seems quite reasonable since green leaf com-

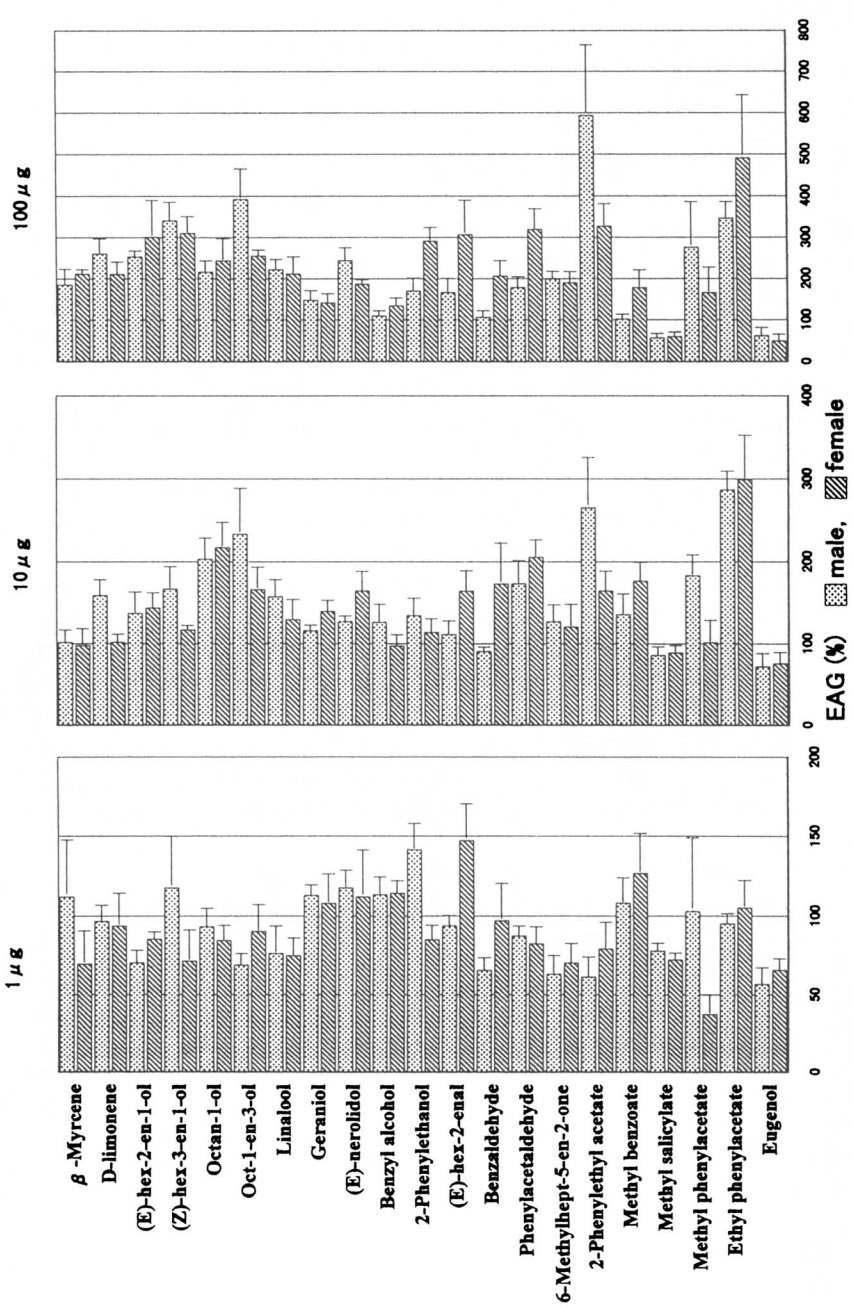

FIG. 4. Relative EAG response (mean ± SEM vs 1 μg of hexan-1-ol) of male and female *P. rapae* to three doses of individual scent components from *L. japonicum* flowers.

pounds are widespread in the natural environment and would not necessarily help insects in their accurate location of flowers. Although notable sexual differences in intensities of REP responses were observed with several compounds, their ecological and physiological meanings are not yet clear and remain to be investigated.

Compounds with high REP activities significantly stimulated flower-visiting, while those with low REP activities released weak attraction. Accordingly, the REP responsiveness, which seems to predict with considerable certainty the behavioral attractiveness of a compound, would be quite useful for elucidating the function of floral volatiles in the flower-visiting behavior of butterflies. Apparently, a blend of several components emanating from *L. japonicum* flowers was more effective than a single component, and was as effective as the whole floral extract in stimulating their location of food source. It is, therefore, evident that these chemicals synergistically interact to stimulate alighting on and REP responses to the flower.

Phenylacetaldehyde and/or 2-phenylethanol have already been reported to be attractive to cabbage looper moth and other moth species (Cantelo and Jacobson, 1979; Haynes et al., 1991; Heath et al., 1992). Taking account of the quantity of individual floral chemicals contained in *L. japonicum* and their respective REP activities, the chemical basis for the attraction of *P. rapae* to this flower may be attributed largely to the presence of 2-phenylethanol and methyl phenylacetate. Since *P. rapae* was not electrophysiologically sensitive to the five compounds that were considered to attract the butterfly, these chemicals are unlikely to elicit from foraging adults a klinotctic movement from a distance. The floral odor, in combination with visual stimuli, could play a significant role in short-range location of flowers and promote the chance of food finding.

At higher doses (>10 μg), 2-phenylethyl acetate and ethyl phenylacetate evoked relatively higher EAG responses, whereas the other tested compounds released moderate to weak responses. These results on the whole are consistent with the EAG data of *P. rapae* reported by Topazzini et al. (1990), although direct comparison is impossible, in that terpenic alcohols (linalool and geraniol) and benzaldehyde elicited moderate EAG responses.

It is to be noted that not all compounds eliciting moderate–high EAG responses showed high REP activities; phenylacetaldehyde, 2-phenylethanol, and 6-methylhept-5-en-2-one, which exerted moderate EAG activities, induced high REP responses, whereas 2-phenylethyl acetate and ethyl phenylacetate gave relatively high EAG responses despite their low REP activities. These results definitely demonstrate that EAG responsiveness does not necessarily correlate with REP activity. Apparently, the seeming discrepancy might have resulted from differential CNS processing of the individual compounds received. It is obvious that elicitation of the REP response, which is basically independent of

antennal sensitivity, is ultimately under the control of the CNS. In a recent report on EAG responses of the hawkmoth to floral scent compounds from *Clarkia breweri*, Raguso et al. (1996) inferred that diverse volatile compounds, to which the moth was olfactorily sensitive, could function as floral attractants for foraging adults. However, our results fail to lend support to their conclusion. It is, therefore, to be stressed that EAG data should be interpreted carefully when employed for elucidating the behavioral significance of bioactive compounds.

Earlier work has shown that in flower-visiting of *P. rapae*, the color of flowers displays a crucial function as a major determinant of flower selection (Miyakawa, 1976). In our experiment, the butterflies indeed visited scentless yellow models in considerable frequency. Nonetheless, the present findings clearly establish that the floral scent plays a crucial role in foraging by adults of *P. rapae* and is highly likely to mediate preference for flowers.

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IDENTIFICATION OF MAIN LIPID COMPONENTS OF MOLE RAT HARDERIAN GLAND

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Abstract—The Harderian gland of the mole rat has been demonstrated to have appeasement qualities on conspecifics. In this study total lipid content of the Harderian gland of the mole rat was evaluated and analyzed by means of thin-layer chromatography (TLC), densitometry, gas chromatography–mass spectrometry (GC-MS), FAB-MS, and NMR. More than 50% of the gland's fresh weight is composed of lipids, making it the highest Harderian gland lipid content ever found in rodents. Male and female lipid components differ considerably. Both have wax esters as the main lipid component, but in females the alcohol components were usually chains of 12 carbons, while in males they were mostly chains of 16 carbons. In addition, while in females the fatty acid residues were usually saturated, in males there was a double bond in the middle of the chain. As many as 25 different esters were found in females, and only seven in males. However, in the male an unusual volatile compound identified as 1-(3′)-hexenyl-benzene was found. This sexual dimorphism may show that the gland function is sex-specific, and gives support to our earlier reports that the mole rat Harderian gland may be a source of pheromones.

Key Words—Harderian gland, lipid, mole rat, pheromone, wax esters, appeasement, sexual dimorphism.

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INTRODUCTION

The Harderian gland is a tubuloalveolar lipid-secreting gland located at the posterior of the eyeball in animals possessing a third eyelid. Discovered by Harder in 1694, the gland was believed to function as a source of lubricant for the eye, but extensive studies have since suggested that it could have acquired other roles. Although a number of studies deal with various aspects such as hormonal control of the gland's activity, melatonin and porphyrin synthesis, thermoregulation, and pheromone production (for reviews see: Sakai, 1981; Olcese and Wesche, 1989; Payne, 1994), the gland's functional importance has yet to be clarified.

The mole rat (*Spalax ehrenbergi*) is a blind, solitary, highly aggressive subterranean rodent that shows striking behavioral and physiological adaptations to underground life (MacDonald, 1985; Nevo, 1991). It has no externally detectable eyes or external ears, and possesses a cylindrically shaped body, with no tail and very short fur. The rudimentary eye is atrophied, covered by a thick layer of skin and dark fur (Cei, 1946; Sanyal et al., 1990; De Jong et al., 1990) and rests upon a large Harderian gland. The Harderian gland of the mole rat has been recently shown to be sexually dimorphic (Shanas et al., 1996) and behavioral observations suggest that it could be implicated in pheromone production (Shanas and Terkel, 1995). Male Harderian glands have been demonstrated to have crucial roles in reducing the aggressiveness of both males and females (Shanas and Terkel, 1997). It would thus be interesting to find whether there are also chemical differences between the glands of the two sexes and to reveal the chemical nature of the putative pheromone.

Lipid is the main secretory material of the mammalian Harderian gland, but the quantity and components vary considerably among species. Phospholipids (found in rats and mice) and 1-alkyl-2,3-diacyl glycerol (found in mice, golden hamster, guinea pigs, and rabbits) were found to be the main constituents (Lin and Nadakavukaren, 1981; Murawski and Jost, 1974; Tvrzicka et al., 1988; Watanabe, 1980; Yamazaki et al., 1981; Harvey, 1991a; Kasama et al., 1973; Jost, 1974; Rock and Snyder, 1975), whereas wax esters and hydroxy fatty acid esters can be found only in rats and rabbits, respectively (Murawski and Jost, 1974; Tvrzicka et al., 1988; Rock et al., 1976).

In preliminary tests we found that the lipid component of the mole rat gland is considerably higher than in other rodents. However, the lipids have not yet been identified. In this study we first evaluated the total lipid content of the mole rat's Harderian gland, and then analyzed its contents by means of thin-layer chromatography (TLC), densitometry, gas chromatography–mass spectrometry (GC-MS), FAB-MS, and NMR.

METHODS AND MATERIALS

Animals. Since mole rats do not breed in captivity, all animals used in this study were adults trapped in the field in the Tel-Aviv area. After capture the animals were maintained in the laboratory at 24 ± 2°C under a 14L:10D lighting regime in individual plastic cages (30 × 20 × 13 cm) with wood shavings for bedding. They were fed rat pellets, carrots, and apples.

The animals were anesthetized with an intramuscular injection of a Ketalar and Rumpon mixture (Vetalar–Rompun 2%–saline; 3:0.3:7.7, v/v/v). A 1-cm incision of the skin covering the orbit was made on both sides of the head, exposing the Harderian glands. After ligating the blood vessels leading to the gland, the gland was quickly removed, weighed, and frozen at −70°C, until analysis. A total of 36 glands were used in this study as follows: 22 (5 females, 17 males) for determination of lipid content, TLC, and densitometry evaluations; 2 (one of each sex) for the NMR study; 8 (2 summer males, 2 winter males, 2 summer females, and 2 winter females) for GC-MS analysis; 2 (one of each sex) for saponification and subsequently GC-MS analysis; 2 (one of each sex) for FAB-MS.

Extraction of Lipids. Each Harderian gland was placed in liquid nitrogen according to Denton and Randle (1967) and pulverized. After transfer into a reaction tube the tissue was extracted 2 × 1 hr at 60°C with 5 ml $CHCl_3$–MeOH (8:5; v/v) under reflux (Folch et al., 1957). After centrifugation, the lipid extract was transferred into a second reaction tube. To 1 ml of the lipid solution 200 μl of water were added, shaken, and the upper phase (water-containing sugars and salts) was discarded. To the lower phase, Na_2SO_4 was added to remove water, filtered, and then brought to dryness by heating to 40–50°C under a stream of nitrogen (N_2).

Determination of Total Lipids. The dry lipid extract was dissolved in 5 ml $CHCl_3$–MeOH (8:5; v/v) and mixed under cooling; then 50 μl of the solution were transferred to a small tared aluminum bowl (14 mm diam.), which was heated to 120°C. After removal of the solvent, the weight was determined on a Cahn-Gram electrobalance (±0.1 mg). Multiplication by 100 yielded the weight of the total lipids per gland, which was normalized to mg/100 mg of glands fresh weight.

Thin-Layer Chromatography. The qualitative and quantitative distribution of lipid classes in Harderian glands was determined by TLC according to Egge et al. (1970). Slides measuring 2.5 × 7.5 cm were cleaned carefully, coated with silica gel (Camag D-O) by dipping two slides in well-stirred silica gel suspended in $CHCl_3$, and then dried in air. For separation, three solvent systems were used: (1) $CHCl_3$–MeOH–H_2O (75:25:4 v/v/v), (2) $CHCl_3$, and (3)

$CHCl_3$–*n*-hexane (1:3 v/v). The lipids were resolved by one-dimensional TLC with solvent 1 for the first centimeter, followed by solvent 2 for half the slide, and solvent 3 for the remainder of the slide. After each solvent the plate was dried in air. The separated lipids were visualized by 10% sulfuric acid in distilled water and a subsequent charring to 200°C for 5 min.

Densitometry. Quantitative evaluation of the relative distribution of lipids was analyzed by densitometry on a Joyce-Loebl Chromoscan. The peak integration was corrected by charring factors according to the carbon content and the relative distribution was calculated. The weight of each of the different lipids (mg/100 mg glands fresh weight) was determined from the relative distribution and the absolute total lipid weight.

Saponification. To the lipid samples in the reaction tubes, 500 μl of 0.5 N KOH–MeOH was added and left overnight at 4°C to produce fatty acid methyl esters (FAE) and free alcohols. Both components were separated on HPTLC, using the same solvent systems as described above.

After saponification, 500 μl of 1 N HCl were added, and the lipids were extracted twice by adding 2 and 5 ml *n*-hexane. The combined *n*-hexane phases were dried by Na_2SO_4. After removing the Na_2SO_4, freshly prepared diazomethane was added to the solution to convert the free fatty acids to corresponding FAE. The FAE and the alcohols were separated by preparative TLC, transferred into small tubes, and brought to dryness under N_2. The alcohols were converted to Alc-OTMS derivatives by adding 15 μl of *N*-methyl-*N*-trimethyl-silyl-trifluoracetamid (MSTFA). The separated FAE and alcohols were then analyzed by GC-MS.

GC-MS Analysis. GC-MS analysis was carried out for whole gland extracts and for the separated FAE and alcohols after saponification. The pulverized glands were ultrasonicated for 10 min in methylene chloride. The GC-MS measurement was performed using two methods: EI and CI. EI and CI GC-MS were performed with a HP model 5890/VG model Prospec GC-MS. Chromatography was performed on a capillary column (Restek: RTx-20, 0.18 mm × 20 m, 0.25-mm film thickness, and Restek: Stabilwax, 0.25 mm × 30 m, 0.25 mm film thickness). The carrier gas was helium at a flow rate of 1 ml/min. Column temperature was kept at 60°C for 1 min and then raised to 240°C at a rate of 10°C/min. The injector inlet was set to 250°C (split mode 1:50). Additional EI GC-MS measurement was performed with a HP model 5890/5971 GC-MS. Chromatography conditions including types of capillaries, carrier gas, and column temperatures were the same as above.

NMR. Samples were separated on 10-cm × 10-cm HPTLC-plates (silica gel 60 without indicator, Merck, No. 5633). After development according to Egge et al. (1970), lipids were visualized by iodine vapor and marked fractions were scraped off and isolated. The total lipids that were evaluated for the two

NMR samples were: 39.16 mg for the female gland and 74.29 mg for the male gland. After the wax esters were separated and cleaned twice, the actual weights of the samples for NMR evaluation were 12.1 mg for the female and 24.8 mg for the male.

For ^1H NMR, samples were dissolved in 0.5 ml $CDCl_3$ and NMR measurements were made on a Bruker AMX 500 by eight scans within a 1-min measuring time.

FAB-MS. The FAB mass spectra of two samples were recorded on a VG Analytical Zab-HF reverse geometry mass spectrometer (V.G. Analytical, Manchester, UK). The matrix was composed of 1-mercapto-2,3-propandiol (thioglycerol, TGL)–3 nitrobenzylalcohol (*m*-benzylalcohol, MNBL; 1:1, v/v), and ionization was achieved with Xe, FAB-gun 8.5 kV/1 mA. The accelerating voltage was 7 kV, and the spectra were evaluated on a SAM II/68K computer (KWS, Ettlingen, Germany).

RESULTS

Lipid Content. The lipid content of the Harderian gland was found to be 54 mg/100 mg tissue. Lipid content of male and female glands differed slightly. Male lipid content was 56.06 ± 8.83/100 mg gland weight. Female lipid content was 50.82 ± 14.49 mg/100 mg gland weight. The average gland weights in this study were 211.92 ± 69.88 mg for males, and 158.25 ± 64.46 for females. These are not significantly different [in a previous study (Shanas et al., 1996) we found that when compared to body weight, male glands weigh significantly more than female glands].

TLC separations showed a major spot—a nonpolar lipid—and several minor spots, which were identified as cholesterol, free fatty acids, and polar lipids. Saponification of the main component yielded fatty acids and alcohols, which verified that the main spots are mainly wax esters. No significant difference in lipid distribution was found between males and females.

Densitometry evaluations revealed that in females the main component accounted for about 74% of the total lipids, while in males it accounted for about 90% of the total lipids.

GC-MS Analsyis. From the GC-MS analysis of the methylene chloride (CH_2Cl_2) extracts of whole glands and of the separated FAE and alcohols, it was found that the combination of fatty acids and alcohols that form the wax esters differed between males and females and they also varied seasonally (Tables 1 and 2, Figures 1 and 2).

Females have a typical alcohol chain of 12 carbons, while the alcohol chain in the male is composed of 16 carbons. In addition, males usually have an

TABLE 1. COMBINATIONS OF FATTY ACIDS AND ALCOHOLS OF WAX ESTERS DERIVED FROM HARDERIAN GLANDS OF FEMALES DURING SUMMER AND WINTER[a]

$M+H^+$	Acid	Alcohol	Summer	Winter
311	8:1	12:0	+	+
313	8:0	12:0	+	+
339	10:1	12:0	+	+
341	10:0	12:0	+	+
365	10:1	14:1	+	+
367	12:1	12:0	+	+
367	12:0	12:1	+	−
369	12:0	12:0	+	+
393	14:1	12:1	+	±
395	14:1	12:0	+	+
397	14:0	12:0	+	+
397	12:0	14:0	+	−
421	14:1	14:1	+	+
423	16:1	12:0	+	+
423	14:0	14:1	+	−
425	16:0	12:0	+	+
449	16:1	14:1	+	+
451	18:1	12:0	+	+
453	18:0	12:0	+	+
477	18:1	14:1	+	+
479	18:1	14:0	+	±
481	20:0	12:0	−	±
507	18:1	16:0	+	−
507	20:0	14:1	+	−
507	20:1	14:0	+	−

[a]The glands were extracted in methylene chloride and the analysis was performed on a GC-MS, as described in the text.

unsaturated fatty acid component while that of the female is saturated. Seasonal differences were marked mainly by different ratios of the wax esters. In males, in addition to seasonal differences in ratios of wax esters, there was also a difference in constituents. A highly volatile compound was found only in summer male glands, and it was identified by GC-MS as 1-(3′)-hexenyl-benzene.

The ^1H NMR spectrum of male and female glands confirmed that the main lipid component of the gland is a wax ester (Figure 3). It also shows that contents of the male gland have about twice as many double bond compared with material from the female gland. It appears from the spectrum that in males there is only one type of double bond in the middle of the fatty acid residue. However, in females the position of the double bond in the chain is more varied.

TABLE 2. COMBINATIONS OF FATTY ACIDS AND ALCOHOLS OF WAX ESTERS DERIVED FROM HARDERIAN GLANDS OF MALES DURING SUMMER AND WINTER[a]

$M+H^+$	Acid	Alcohol	Summer	Winter
453	14:0	16:0	+	+
479	16:1	16:0	+	+
505	18:1	16:1	+	+
507	18:1	16:0	+	+
531	18:1	18:2?	+	+
533	18:1	18:1	+	+
535	18:1	18:0	+	+
537	18:0	18:0	−	+
481	16:0	16:0	+	−
563	18:1	20:0	+	±

[a]The glands were extracted in methylene chloride and the analysis was performed on a GC-MS, as described in the text.

The results from FAB-MS (Tables 3 and 4) support those of the GC-MS. The male gland shows molecular peak regions of 479, 505, and 533 ($M+H^+$), and the female gland shows molecular peak regions of 339, 367, 369, 393, 395, 397, 423 and 451 ($M+H^+$). Generally the fragmentation under the FAB-MS to fatty acid ions and alcohols led to fatty acid residues mass similar to those found in the GC-MS (Table 4).

DISCUSSION

The total lipid content found in the mole rat's Harderian gland is the highest ever found in rodents. More than 50% of the gland's fresh weight is composed of lipids. The composition of the main lipid content was found to be similar to adult rats (Murawski and Jost, 1974; Tvrzicka et al., 1988; Harvey, 1991a) but different from other rodents (Tvrzicka et al., 1988; Watanabe, 1980; Yamazaki et al., 1981; Harvey, 1991b). It is composed of wax-like esters of varied length. Fatty acid residues had a chain length of C_8–C_{20}, and alcohol residues of C_{12}–C_{18}. All were straight chains with no hydroxy residues, as confirmed by sililation.

Male and female lipid components differ considerably. In females the main alcohol component was a chain of 12 carbons, and the fatty acid was usually saturated, while in male it was of 16 carbons, and the fatty acid was usually unsaturated. In addition, the lipid composition in females is much more varied than in males. As many as 25 different esters were found in females, and only seven in males. However, in the male an unusual volatile compound was found

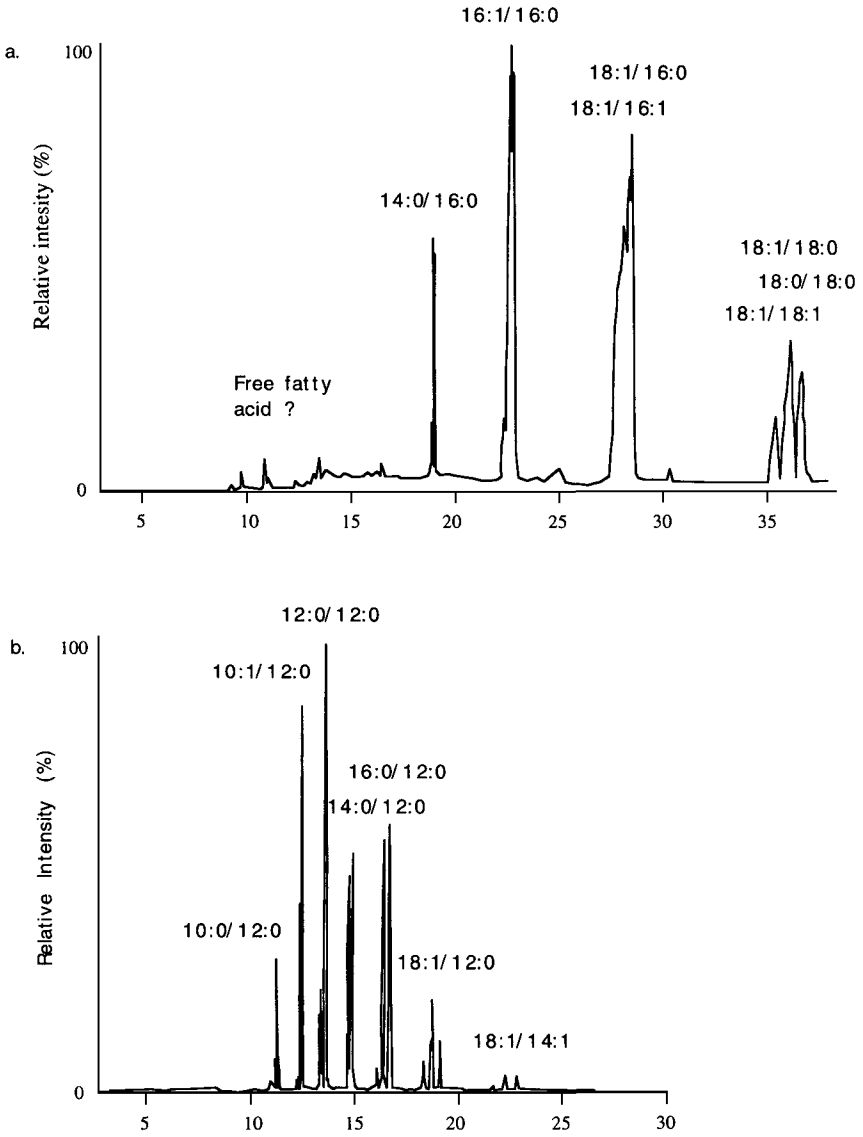

FIG. 1. GC spectrum and the major esters (combination of fatty acid/alcohol) of (a) male and (b) female Harderian glands during the winter. The glands were extracted in methylene chloride, and the analysis was performed on a GC-MS as described in the text.

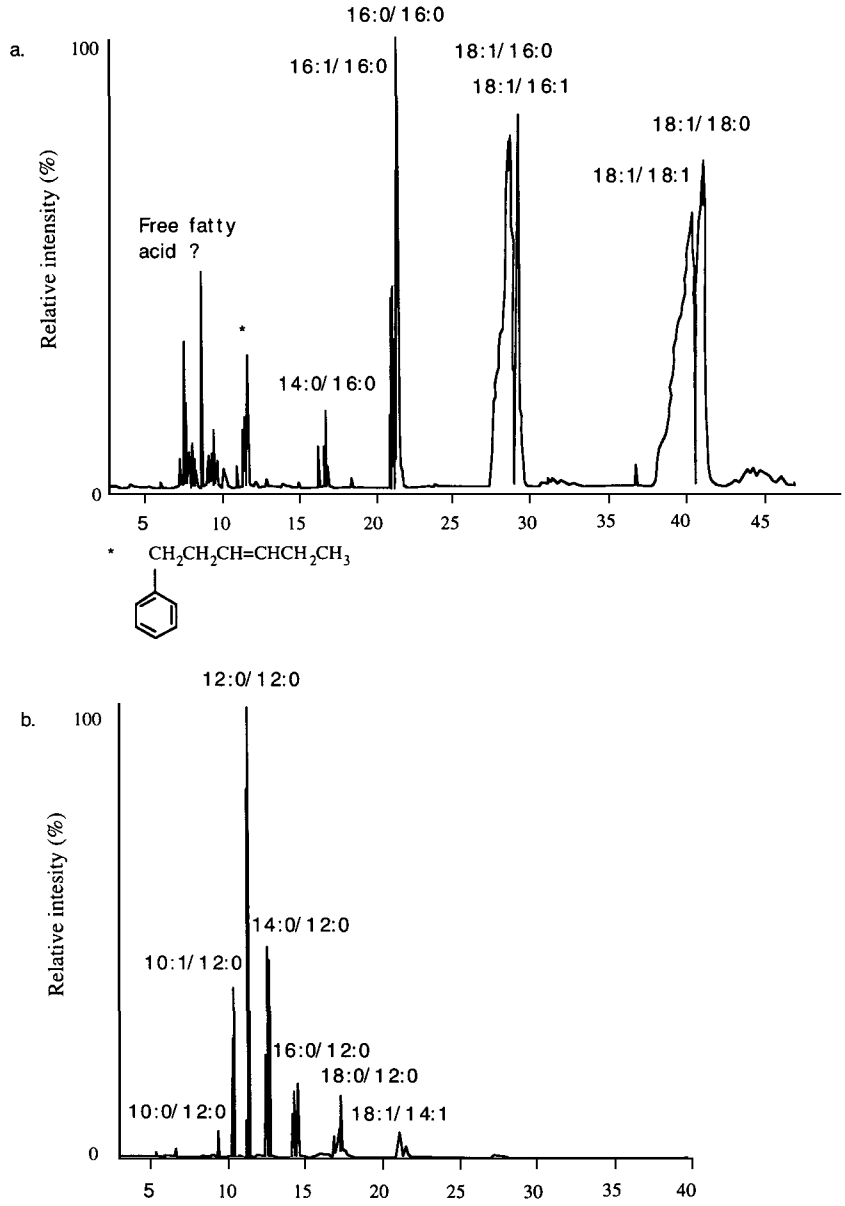

FIG. 2. GC spectrum and the major esters (combination of fatty acid/alcohol) of (a) male and (b) female Harderian glands during the summer. The glands were extracted in methylene chloride, and the analysis was performed on a GC-MS as described in the text.

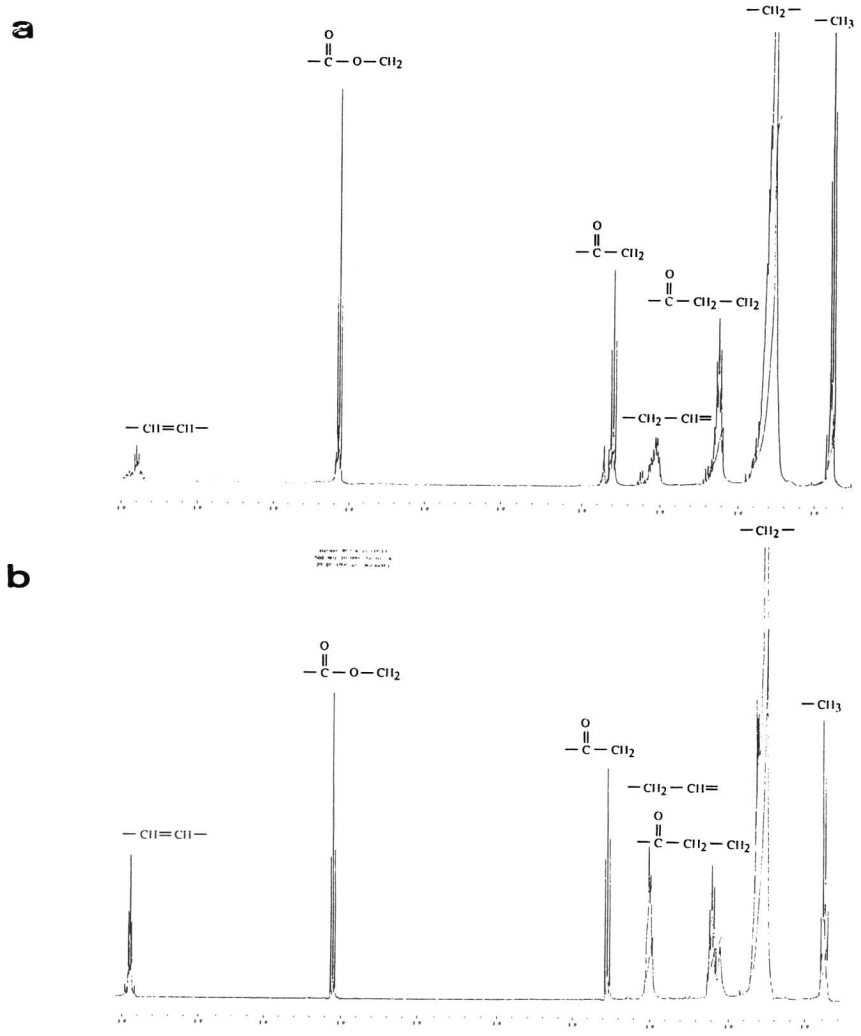

FIG. 3. The 500-MHz ^{1}H NMR spectrum of the Harderian gland of a (a) female (12.1 mg) and a (b) male (24.8 mg). Samples were dissolved in $CDCl_3$.

and identified as 1-(3′)-hexenyl-benzene. This sexual dimorphism may show that the gland function is sex-specific, in support of our earlier ideas (Shanas and Terkel, 1995) that the mole rat Harderian gland may be a source of pheromones. Another species for which pheromone production function was proposed for the Harderian gland is the golden hamster (Payne, 1977, 1979). This

TABLE 3. FAB-MS ANALYSIS OF MOLECULAR PEAK MASS OF MALE AND FEMALE HARDERIAN GLANDS IN WINTER

Peak mass	
$M+H^+$ male	$M+H^+$ female
477	337
479	339
505	367
507	369
533	393
	395
	397
	423
	451

species also has been found to have sexual dimorphism in the Harderian lipid content (Murawski et al., 1991; Seyama et al., 1995). Both sexes have 1-alykl-2,3-diacylglycerol and identical phospholipids, but the fatty acid and the alkyl compositions differ. Males had mostly straight-chain fatty acids, with C_{15} being the major one, while females had iso- and anteiso- branched chain acids and alkyl portions.

Seasonal studies did not show dramatic changes in gland composition. It is also hard to know at present how much of the seasonal variation is contributed by individual variation, since the seasonal study was carried out on only few glands. The breeding season of the mole rat is in the winter, but mole rats

TABLE 4. FAB-MS ANALYSIS OF FATTY ACID (FA) ION PEAK MASS OF MALE AND FEMALE HARDERIAN GLANDS IN WINTER

Male		Female	
FA ion mass	Fatty acid	FA ion mass	Fatty acid
201	12:0	173	10:0
229	14:0	201	12:0
237 ($M+H^+-H_2O$)	16:1	229	14:0
257	16:0	257	16:0
265 ($M+H^+-H_2O$)	18:1	285	18:0
285	18:0		
293 ($M+H^+-H_2O$)	20:1		

appear to encounter conspecifics in early and late summer too. The FAB-MS generally confirmed the results derived from the GC-MS, but it is possible that the slight differences between the two analyses reflect individual variations.

The mole rat Harderian gland is of great interest, since it may produce a pheromone that has appeasement qualities (Shanas and Terkel, 1995). Our behavioral studies (Shanas and Terkel, 1997) show that mole rats respond differently to female and male Harderian gland products and that males are capable of reducing conspecific aggression by expressing and spreading on the fur the Harderian gland contents. Thus the male's esters and the unique 1-(3′)-hexenyl-benzene found in this study may be candidates for an appeasement pheromone. Further studies will be needed to verify this hypothesis and to verify that the new substance found in this study, 1-(3′)-hexenyl-benzene, is indeed a physiologically active substance.

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