Volatile Components of Red Clover Leaves, Flowers, and Seed Pods: Possible Insect Attractants

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The volatile components associated with the main different parts of the red clover plant have been analyzed by using Tenax trapping and GLC–MS analysis. The major volatile components identified were, for the leaves, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenol, and (*E*)- and (*Z*)-β-ocimenes, for the flowers, acetophenone, methyl cinnamate, and 1-phenylethanol, and, for the seed pods, (*E*)- and (*Z*)-β-ocimenes, an unidentified sesquiterpene hydrocarbon, and longifolene.

Red clover (*Trifolium pratense* L.) is an important forage legume that is attacked by a complex of insect pests, many of which feed only on specific parts of the plant (Dickason and Every, 1955; Niemczyk and Guyer, 1963). For example, the clover seed chalcid [*Bruchophagous gibbus* (Boheman)] attacks only the seeds, and the nitidulid beetle [*Meligethes nigrescens* (Stephens)] feeds only on flowers. Lygus bugs (*Lygus* sp.), however, feed on buds, flowers, and seeds (Sorenson, 1939). A substantial part of the interaction between phytophagous insects and their host plants is based on chemical cues that emanate from the plant and evoke a specific behavioral response by the insect. Some insects differentiate plants based on cues perceived at a distance whereas others select hosts based on cues perceived after arrival on the plant (Kennedy, 1977). A knowledge of these mechanisms provides a better understanding of insect behavior and may lead to new innovative methods of pest control by manipulation of insect behavior.

We report here the identification of volatile constituents that emanate from fresh red clover leaves, flowers, and seed pods in search of compounds that influence the behavior of clover pests.

EXPERIMENTAL SECTION

Materials. Red clover (*T. pratense*, Florex variety) flowers, seed pods, and leaves were obtained from an experimental field in the grounds of the Western Regional Research Laboratory in Berkeley. Seed pods were also obtained from commercial seed fields near Corvallis, OR. The field-collected pods were estimated to be 10–14 days mature when picked (from the time of pollination). The Berkeley-grown plant material was generally picked and the isolation of volatiles begun within an hour or two. Plant material from Oregon was shipped by air (kept cool by ice in an insulated package) and received a few hours after picking. The isolation was generally begun a few hours after receiving the package.

Isolation of Volatiles Using Tenax Traps. The method used was essentially the same as that previously described by the authors for alfalfa volatiles (Buttery et al., 1982a). Batches of 400-g quantities of leaves, 200 g of flowers, and 200 g of seed pods were used for each isolation. Each batch of plant material was enclosed in a 5-L flask. Purified air (500 mL/min) was passed into each flask over the plant material and out through a Tenax trap (1.7 g of Tenax, 1.3 cm diameter × 7.0 cm long) by applying suction to the outlet of the trap. The inlet air, obtained from outside the building, was purified by passing through a 60 cm long × 15 cm diameter column of activated charcoal (Mallinckrodt, 8–12 mesh, periodically reactivated by heating under high vacuum). The isolation was continued for 24 h at room temperature. The trapped material was eluted from the Tenax trap with freshly distilled diethyl ether. The ether extracts from four to six traps were combined and concentrated by using a warm water bath and low holdup distillation columns to a small volume (ca. 5 μL). This was used in a single spitless injection onto the gas chromatography (GLC) capillary column for the GLC–MS analysis.

Capillary GLC–MS Analysis. The GLC column was a 150 m long by 0.64 mm i.d. Pyrex capillary coated with Carbowax 20M. The GLC temperature program conditions involved holding the oven temperature at 50 °C for 30 min, then increasing at 1 °C/min from 50 to 170 °C, and then holding isothermal at 170 °C. The column inlet pressure was 15 psi He. The mass spectrometer was a modified Consolidated 21-620 cycloidal-type instrument with 70-eV ionization voltage. The GLC–MS analyses were repeated several times on different batches of each clover part.

Authentic Chemical Compounds. Most authentic samples for comparison were obtained from reliable commercial sources or synthesized by established methods and repurified by GLC separation. Their identities were verified by spectral (MS and IR) means.

(*Z*)-β-Ocimene was obtained by isomerization of (*E*)-β-ocimene (4 g) by heating (100 °C) with benzenethiol (0.2 g) and 2,2′-azobis(isobutyronitrile) (0.02 g) for 3 h under argon. The Z form (25%) was separated from the (*E*)-β-ocimene (75%) by using a 3 m × 0.64 cm o.d. stainless steel GLC column packed with 80–100-mesh Chromosorb G-DMCS coated with 2% Carbowax 20M.

Authentic sesquiterpenes were obtained as previously described (Buttery et al., 1982a).

Preliminary Field Tests. The following compounds identified in this and other studies (Buttery et al., 1982a) were tested in a field of red clover grown for seed near Corvallis, OR. The compounds were dodecyl acetate, 2-phenylethanol, methyl salicylate, 1-octen-3-ol, hexanol, 1-phenylethanol, methyl cinnamate, decanyl acetate, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, hexyl acetate, (*E*)-β-ocimene, 1-penten-3-one, 2-methylbutanol, 2-hexanone, 2-heptanone, acetophenone, acetoin, hexanal, caryophyllene, and caryophyllene oxide. All compounds were impregnated into rubber stoppers (Arthur H. Thomas Co., Philadelphia, PA, catalog no. 8753-D42) in 1 mL of hexane at 0.1 and 1% concentrations. Each concentration was

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Table I. Volatile Compounds Identified in Different Parts of Red Clover

compound[a]	most intense ions[b] (one each 14 mass units)	Kovats' GLC index[c]	relative % leaves	flowers	pods
	Aliphatic Ketones and Aldehydes				
1-penten-3-one	55, *84*, 39	1000			4
2-hexanone	43, 58, *100*, 71, 81	1100		0.2	
2-heptanone	43, 58, 71, *114*, 99, 85	1190		0.7	
(*E*)-2-hexenal	41, 55, 69, 83, *98*	1200		6	3
	Aliphatic Alcohols				
2-methylbutanol	57, 41, 31, 70	1180			4
hexanol	56, 43, 31, 69, 84	1330	0.5	0.7	
(*Z*)-3-hexenol	41, 67, 55, 31, 82, *100*	1370	24	3	
(*E*)-2-hexenol	57, 41, 31, 82, 67, *100*	1380	4	0.4	
2-hydroxybutan-3-one (acetoin)	45, 73, *88*	1250		3	3
1-octen-3-ol	57, 43, 72, 55, 31, 85	1460	0.8	3	4
2,3-dihydroxybutane	45, 57, 31, 75, *90*	1590		2	
	Aliphatic Esters and Acids				
(*Z*)-3-hexenyl acetate	43, 67, 82, 54	1310	33	3	2
acetic acid	43, 60	1430			3
decyl acetate	43, 55, 70, 61, 83, 97	1670			3
dodecyl acetate	43, 55, 69, 83, 61, 97	1880			3
	Terpenoids				
(*Z*)-β-ocimene	93, 41, 79, 53, 67, 121	1230	5	3	6
(*E*)-β-ocimene	93, 41, 79, 53, 121, 67	1250	22	8	35
longifolene[d]	41, 91, 161, 55, 105, 79	1530			5
caryophyllene	41, 69, 93, 79, 133, 55	1570	0.1	0.4	3
(*E*)-β-farnesene	69, 41, 93, 79, 55, 133	1650	2		2
	Aromatic Compounds				
acetophenone	105, 77, *120*, 51, 43	1600		24	4
methyl salicylate	120, *152*, 92, 39, 65, 53	1730	0.4		
1-phenylethanol	79, 107, 43, 51, *122*, 63	1760		8	
2-phenylethanol	91, *122*, 65, 39, 31, 51	1890		1	
methyl cinnamate	131, 103, *162*, 77, 51, 39	2050		11	

[a] Complete mass spectrum and Kovats' GLC retention index are consistent with that of authentic samples except for longifolene. [b] The most intense ion every 14 mass units above *m/z* 34. Ions are listed in descending order of intensity with the molecular ion (if listed) in italic type. [c] Kovats' GLC index for the Carbowax 20M Pyrex capillary column. [d] No authentic sample available but the mass spectrum is consistent with published spectra (Stenhagen et al., 1974).

replicated 4 times and exposed in the field 4 days during Aug 17–30, 1983, in Pherocon 1C traps (Zoecon Corp., Palo Alto, CA). The field was infested with moderate populations of lygus bugs, nitidulid beetles, and the clover seed chalcid.

RESULTS AND DISCUSSION

The amount of volatile oil obtained by Tenax trapping was less than 0.1 parts per million (ppm) for the leaves and seed pods and of the order of 0.5 ppm for the flowers.

The compounds identified are listed in Table I. As for alfalfa [cf. Buttery et al. (1982a)] (*E*)-β-ocimene was a major component of the clover leaves and seed pods. A considerable proportion of (*Z*)-β-ocimene was also found in the clover although none had been detected in alfalfa. The sesquiterpene hydrocarbons caryophyllene and (*E*)-β-farnesene were found in both the leaves and pods. Longifolene was also found in the seed pods. However, no authentic sample of longifolene was available for direct comparison: the identification of longifolene was based on published information [cf. Stenhagen et al. (1974) and Anderson and Falcone (1969)] for both mass spectra and GLC retention data. The major sesquiterpene found in the pods (7% relative concentration of total volatiles) could not be identified. It gave the following mass spectrum (two major ions each 14 mass units, intensities in parentheses): 41 (100), 43 (35); 53 (25), 55 (54); 65 (20), 69 (23); 77 (38), 79 (34); 91 (92), 93 (45); 105 (87), 106 (69); 119 (74), 121 (18); 133 (43); 147 (26), 148 (38); 161 (23); 175 (51); 189 (49); 204 (40). A Kovats' GLC index off 1370 (Carbowax 20M) was obtained.

The terpene and sequiterpene hydrocarbons may be important olfactory cues to the insects after arrival on the plant. The alfalfa sesquiterpenes (*E*)-β-farnesene, γ-muurolene, and α-copaene were found to be quite attractive to the alfalfa seed chalcid in a laboratory olfactometer (Kamm and Buttery, 1983). The sesquiterpenes and terpenes may be located on the surface of the plant in the cuticular waxy material, which is well-known to contain other terpenoids usually in the form of triterpenoids or steroids [cf. Hadley (1981)]. The insects' first contact with the plant would be with this cuticular material.

The major component of the flowers was identified as acetophenone. The aroma of the flowers (to humans) in the authors' opinion seems to be mostly due to acetophenone. The reduced form of acetophenone, 1-phenylethanol, was also found in the flowers.

Comparison of the quantitative data in Table I shows some marked differences. The leaves contain by far the largest relative amounts of (*Z*)-3-hexenol (24%) and (*Z*)-3-hexenyl acetate (33%). (*Z*)-3-Hexenol has long been known as "leaf alcohol". (*Z*)-3-Hexenyl acetate is a less well known leaf component but had been found in potato plant leaves by Visser and Ave (1978) and by some of the authors in alfalfa (Buttery et al., 1982a) and barley leaves (Buttery et al., 1982b).

The clover flowers contain the largest relative concentration of the odorous aromatic compounds such as acetophenone (24%) and methyl cinnamate (11%) and the smallest relative amount of terpenoids.

The clover seed pods contain the highest relative concentration and the greatest variety of sesquiterpene hy-

drocarbons: these compounds may possibly play a role in selection of oviposition sites by the chalcid.

Comparison with Alfalfa Volatiles. Comparison of Table I with data obtained previously by the authors for alfalfa (Buttery et al., 1982a) shows many similarities and some marked differences. The nature of the volatiles in alfalfa flowers and clover flowers shows the major differences. Acetophenone, which is the major component of the clover flowers, does not occur in the alfalfa flowers. (*E*)-β-Ocimene, (*E*)-β-farnesene, and caryophyllene are common to both the alfalfa leaves (and pods) and the clover leaves (and pods). However, there are differences in the nature of the other terpene and sesquiterpene compounds between alfalfa and clover. With both alfalfa and clover the sesquiterpenes are at a higher relative concentration in the pods compared to the leaves (and flowers) of the same plant.

Preliminary Field Tests. Hexyl acetate, 2-hexanone, 2-heptanone, acetophenone, and acetoin appeared to be weak attractants for lygus bugs at the 1% concentration. None of the compounds tested was attractive to nitidulid beetles or seed chalcids. An unidentified humpbacked fly in the family Phoridae was strongly attracted to 1-phenylethanol at the 1% concentration. Unfortunately, this fly is not a pest and commonly feeds on decaying vegetation. We consider these data preliminary and not necessarily indicative of attractivity to the pest species. Some of the compounds tested are extremely volatile, and our baits may not have emitted an attractive concentration to the insect for more than several hours. Baits that control release of these compounds over a range of concentrations may give quite different results.

Registry No. 1-Penten-3-one, 1629-58-9; 2-hexanone, 591-78-6; 2-heptanone, 110-43-0; (*E*)-2-hexenal, 6728-26-3; 2-methylbutanol, 137-32-6; hexanol, 111-27-3; (*Z*)-3-hexenol, 928-96-1; (*E*)-2-hexenol, 928-95-0; acetoin, 513-86-0; 1-octen-3-ol, 3391-86-4; 2,3-dihydroxybutane, 513-85-9; (*Z*)-3-hexenyl acetate, 3681-71-8; acetic acid, 64-19-7; decyl acetate, 112-17-4; dodecyl acetate, 112-66-3; (*Z*)-β-ocimene, 3338-55-4; (*E*)-β-ocimene, 3779-61-1; longifolene, 475-20-7; caryophyllene, 87-44-5; (*E*)-β-farnesene, 18794-84-8; acetophenone, 98-86-2; methyl salicylate, 119-36-8; 1-phenylethanol, 98-85-1; 2-phenylethanol, 60-12-8; methyl cinnamate, 103-26-4.

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Fenvalerate Metabolism in Cotton Callus Tissue

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The metabolism of fenvalerate in cotton callus tissue proceeded in a manner similar to that of other pyrethroids in intact plants. Using chlorophenyl-^{14}C-labeled fenvalerate 97.0% and 94.7% of the label was recovered from the tissue in 4 and 8 days, respectively. The [*chlorophenyl*-^{14}C]fenvalerate was metabolized to conjugates of 2-(4-chlorophenyl)-3-methylbutyric acid while benzyl-^{14}C-labeled fenvalerate was metabolized to numerous conjugates, some of which were conjugates of 3-phenoxybenzoic acid and 3-(4-hydroxyphenoxy)benzoic acid. No similar conjugates of either chlorophenyl-^{14}C- or benzyl-^{14}C-labeled fenvalerate were found, indicating conjugation of the parent nuclear chain does not seem to exist.

Fenvalerate [α-cyano-3-phenoxybenzyl 2-(4-chlorophenyl)-3-methylbutyrate] is one of a number of synthetic pyrethroids that are used in controlling many insect pests of cotton and other crops. The metabolism of several pyrethroids has been examined in intact cotton plants under greenhouse and field conditions and in excised leaf disks (Ruzo and Casida, 1979). Under field conditions, about 30% of the ^{14}C-labeled permethrin was lost within 1 week (Gaughan and Casida, 1978). Permethrin, deltamethrin, and cypermethrin are degraded on or in cotton plants primarily by photoisomerization, ester cleavage, and conjugation reactions (Roberts, 1981; Ruzo and Casida, 1979; Wright et al., 1980). Plant tissue culture more efficiently utilizes the ^{14}C-labeled compounds and tends to optimize uptake and metabolism in pesticide studies compared to similar experiments under greenhouse or field conditions. In most tissue culture metabolism studies metabolites are qualitatively similar to intact plant studies. Plant tissue culture is advantageous in determining if the metabolites recovered are a result of true plant metabolism rather than that of microorganisms on the plant surface or photodegradation. This is important in evaluating short-term exposures of leaf disks to pesticides. Since fenvalerate is used on cotton to control lepidopterous pests (Ruscoe, 1980), we have examined the metabolic fate of

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