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IDENTIFICATION OF VOLATILES FROM FELLED *PINUS RADIATA* AND THE ELECTROANTENNOGRAMS THEY ELICIT FROM *SIREX NOCTILIO*

BY

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Volatiles emanating from *Pinus radiata* show only minor changes in composition with time after felling even though attractiveness of the felled timber to the wood wasp *Sirex noctilio* changes markedly over the first 3 weeks. Monoterpene hydrocarbons account for more than 95% of the volatiles and show little change in relative composition during this time. Oxygenated components (camphor, pinocamphone, isopinocamphone and *trans*-pinocarveol), which are absent or present in trace amounts immediately after felling, are produced in increasing amounts and account for 1% of the volatiles after 3 weeks. Their presence in the volatile fraction significantly increases the response from the antenna of *S. noctilio*. The major monoterpene hydrocarbons and oxygenated components were identified by chromatographic and spectral techniques.

Sirex noctilio F. has caused extensive damage to pine plantations in both New Zealand and Tasmania. Madden (1971) demonstrated that various kinds of damage to *Pinus radiata* D. Don rendered the trees more attractive to *S. noctilio*. Felled trees were highly attractive for 6—14 days with some attraction persisting for up to 3 weeks from felling; girdled trees were attractive over a much longer time (3—4 months). Treatments that placed the tree under stress for a limited time, such as induced attack by caged *S. noctilio*, also resulted in increased attractiveness to the wood wasp. The increased attraction could be due to one or more of the following factors: (1) increased permeability of the bark to host volatiles, (2) metabolic change producing greater amounts of some components, and (3) metabolic change producing components not present under normal conditions.

To investigate the cause of the variation in attractiveness of felled *P. radiata* to *S. noctilio* observed in the field, the change in composition with time of the volatiles emanating from logs was examined. The activity of these volatile substances on the antennae of *S. noctilio* was monitored using EAG methods. It had been demonstrated that the amplitude of the signals produced by female *S. noctilio* antennae in response to pure compounds was related to the amount of test

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material [amplitude of EAG signal α log (amount of test material)] and that different compounds provided different degrees of stimulation (Simpson, 1976).

METHODS AND MATERIALS

Trapping experiments

(a) To obtain sufficient material for chemical and bioassay studies 24 logs (10—15 cm diam, 1.5 m in length) were cut from freshly felled *P. radiata*, the ends were covered in aluminium foil to seal off resin, and the logs were then placed in a polyethylene envelope. The envelope was externally sealed with 5.1 cm Promura PVC tape. A closed system, with recirculation of the air in the envelope, was set up using glass tubing (1.5 cm o.d.) and minimal lengths of Nylex plastic tubing. Two sets of three large capacity glass cold traps were connected in parallel and led to a single glass spiral trap placed immediately before the compressor inlet. The large capacity glass traps were placed in line in the reverse manner to that normally used so as to avoid blockage of the central tube (see Blight, Grove & McCormick, 1969). The first trap in each set was packed in salt-ice and the remaining cold traps in powdered solid carbon dioxide. The air flow from the oil-free compressor (Compton type D-416-6) was controlled by a needle valve so that a volume equivalent to the enclosed space was circulated 20—30 times/hr. The polyethylene envelope and plastic tubing were placed in a vacuum drying cabinet before use and the glass tubing and traps were baked at 120°. A preliminary experiment in which logs were enclosed in aluminium foil instead of polyethylene showed that the polyethylene envelope did not affect the composition of the trapped materials.

Trapping was carried out for 24 hr each alternate day over a period of 21 days. When the compressor was not in operation the envelope was opened so that moisture did not condense on the logs or the envelope.

The traps were washed out between collections with redistilled ethyl ether (b.p. 35°, distilled through a 70-cm column packed with glass helices). The aqueous layer was saturated with AR NaCl before extraction with ether (two lots, 50 ml each); the solution was reduced to a small volume by distillation incorporating a Vigreux column. Materials collected from days 1—9 (4.41 g) and 11—21 (2.57 g) were combined after gas chromatographic (GC) analyses of the individual collections. Much of the monoterpene hydrocarbon fraction was removed under reduced pressure (20 mm Hg) using a Vigreux column and the remaining material was fractionated using micropreparative GC.

(b) To examine the volatiles diffusing only through the intact bark surface, a metal framework was constructed to support 16 logs 1.3 m in length and up to 20 cm in diameter in such a way that the cut ends of the logs were excluded from the enclosed airspace. The seal near the ends of the logs was formed using Utilex hose clamps placed over a strip of latex rubber around the polyethylene film, which formed the envelope and contained the framework. The arrangement of the cold traps and compressor was basically the same as in experiment (a). The cut ends of

the logs were treated with fungicide (ICI Captan A) and coated with mastic (Shell L Grafting Mastic) immediately after felling and cutting. The trapping was carried out at 20 ± 1° and the humidity within the enclosed space was monitored. The pumping rate was similar to that used in (a). After a similar work-up procedure the isolates were analysed by GC. Trapping was continued up to 33 days from felling.

Gas chromatography

A Philips PV 4000 gas chromatograph was modified in a similar manner to that described by Brownlee & Silverstein (1968) to permit the collection of material in thin glass capillary tubes. The splitting ratio was such that 2.5% of the effluent was directed to the FID.

Stainless steel columns (3 m × 3 mm) were used. The stationary phase loading was 10% on AW DMCS Chromosorb W, 60—80 mesh, and liquid phases used were Carbowax 20M, silicone oil (SF-96) / Igepal (20 : 1), Apiezon L, and Reoplex 400. Capillary columns (72 m × 0.76 mm i.d.) coated with Carbowax 20M and silicone oil (SF-96) / Igepal (20 : 1) by the method of Mon, Forrey & Teranishi (1967) were used with a model 104 Pye gas chromatograph. A splitter (with 9 : 1 vent to column) and a column flowrate of 6 ml/min nitrogen was used with the capillary columns. The inlet was heated to 180°. A Shimadzu R101 recorder equipped with series 200 Disc integrator was employed with both gas chromatographs.

Coupled gas chromatography-mass spectrometry

A silicone membrane separator similar in design to that described by Karasek (1969), but having a smaller contact area (5.1 cm × 2.8 mm) was located in the GC oven. The GC was coupled to an EAI Quad 300 mass spectrometer using Hoke diaphragm valves (type 4616 N4M) in a similar manner to the all-glass system described by Hawes, Mallaby & Williams (1969). The link was heated at 180°. Helium was used as the carrier gas; the mass-spectrometer gauge indicated a pressure of 2—4 × 10^{-6} torr with the GC oven at ambient to 150°. Mass spectra at 70 eV were recorded in the usual way.

Bioassay (EAG)

Equipment used was basically as described earlier (Simpson, 1976). Amplitude of EAG response to similar amounts of material collected on different days in experiment (a) was obtained. The EAG response to the total volatiles collected on day 19 was compared with the responses to the monoterpene hydrocarbon fraction isolated from this material, and with α- and β-pinene.

In the experiment in which the response of *S. noctilio* to fractions from the combined materials (days 11—21) was examined, the test material in redistilled diethyl ether was applied, using a microsyringe, to a 1 cm × 1 cm Whatman No. 1 filter paper placed in an aluminium cartridge. After the solvent had been allowed to evaporate, the cartridge was attached to a movable arm, connected to the air line and moved to a fixed position in front of the antennal preparation for a period of 2 sec. The new cartridges were positioned so that the distance from the

preparation was the same in each test. Duplicate runs with at least six antennae from different insects were made with each set of test fractions. The first material examined was re-run at the end of each set of fractions to check that antennal response had not altered significantly due the assay.

Where the combined materials (days 11—21; experiment a) were fractionated and the EAG response to *S. noctilio* was examined, different amounts of material were applied to the test papers proportional to the amounts of these materials emanating from felled *P. radiata*. Since the quantities used in the bioassay are contained in a fairly narrow range (ca. 50—250 and 1—70 $\times$ 10^{-8}g) and the major components have similar vapour pressure, the amount of material evaporating from the test paper will correlate fairly well with the amount applied (see Sower, Gaston & Shorey, 1971) and hence with concentration in the total volatiles.

The amount of test material was determined from GC analysis, on the basis of peak area, of a quantity equal to that used in the bioassay.

RESULTS

The major components of all collections were monoterpene hydrocarbons and constituted over 95% of the total volatiles, excluding water. The monoterpene hydrocarbons, identified from GC retention data and GC-MS, were shown to be the same as those found in *P. radiata* bark oil (Simpson & McQuilkin, unpubl.), but differed significantly in relative composition. Except for decreases in the amounts of myrcene and γ-terpinene the composition of the monoterpene hydrocarbons emanating from felled *P. radiata* remained fairly constant (Table I).

TABLE I

Monoterpene hydrocarbon composition of volatiles emanating from felled P. radiata *(experiment b; days 1, 23 and 33).*

Material	Amount (%)		
	day 1	day 23	day 33
α-Pinene	34.4	35.1	38.0
Camphene	0.6	0.6	0.7
β-Pinene / Sabinene	54.5	55.2	52.7
Myrcene	3.0	1.8	1.6
3-Carene	1.3	1.2	1.6
Limonene	2.2	2.7	2.4
β-Phellandrene	2.9	2.4	2.2
γ-Terpinene	0.4	0.2	0.1
p-Cymene	0.2	0.2	0.2
Terpinolene	0.6	0.5	0.5
	100.1	99.9	100.0

The EAG response to the volatiles collected at 15 and 19 days from felling was greater than the response to similar amounts of material collected at earlier times

TABLE II

EAG response of female S. noctilio *to volatiles from* P. radiata *collected at different times from felling (experiment a).*

Day from felling	Response[1] in μV	Amount[2] ($\times 10^{-8}$g)
3	716	75.6
7	788	60.9
11	792	61.6
15	968	56.8
19	927	57.0

L.S.D. = 149

[1] Average value of response from at least six antennae from different insects.
[2] Amount of material entrained into the airstream (flowrate 700 ml/min).

TABLE III

EAG response of female S. noctilio *to volatiles from felled* P. radiata *(day 19) and resulting fractions (experiment a).*

Material	Response[1] in μV	Amount[2] ($\times 10^{-8}$g)
Volatiles (day 19)	472	38.9
Monoterpene hydrocarbon fraction	382	38.0
α-Pinene	338	38.0
β-Pinene	382	36.6

L.S.D. = 52

[1] Average value of response from at least six antennae from different insects.
[2] Amount of material entrained into the airstream (flowrate 700 ml/min).

which indicated that the volatiles show increasing activity with time (Table II). Since the responses to the monoterpene hydrocarbon fraction isolated from material collected on day 19, α-pinene and β-pinene were similar but significantly less than the response to the total volatiles (Table III), the change in composition within the monoterpene hydrocarbon fraction, including the decrease in the relative amounts of myrcene and γ-terpinene, does not account for the increased EAG response.

Materials, less volatile than the monoterpene hydrocarbons, were present in only trace amounts immediately after felling but increased with time and by day 23 accounted for approximately 1% of the total volatiles from *P. radiata.* These materials were concentrated by fractional distillation. Camphor, pinocamphone, isopinocamphone and *trans*-pinocarveol were present in sufficient quantities to isolate using micropreparative GC, their identities being confirmed by comparison with authentic materials. Pinocamphone and isopinocamphone were prepared

TABLE IV

Amounts of camphor, pinocamphone, isopinocamphone and trans-*pinocarveol in volatiles emanating from felled* P. radiata *(as percent of total; experiment b).*

Day from felling	Camphor + Pinocamphone[1] (%)	Isopinocamphone (%)	*trans*-Pinocarveol (%)
1	0.01	0.01	0.01
14	0.02	0.02	0.01
23	0.09	0.11	0.02
27	0.24	0.26	0.12
33	0.29	0.29	0.15

[1] Relative proportion of camphor/pinocamphone was ca. 1:4.

TABLE V

EAG response of female S. noctilio *to volatiles from felled* P. radiata *(combined materials, days 11-21; experiment a) : first fractionation.*

Fraction No.	Response[1] in μV	Amount[2] ($\times 10^{-8}$ g)
1[3] (Monoterpene hydrocarbons)		
2	360	47.2
3	608	68.8
4	722	253.0
5	539	228.0
6	544	103.0

L.S.D. = 129

[1] Average value of response from at least six antennae from different insects.

[2] Amount of material in diethyl ether solution applied to the test paper.

[3] The monoterpene hydrocarbon fraction was not treated further in this study, although the EAG response by *S. noctilio* to the individual components present has been investigated (Simpson, 1976).

from(-)-α-pinene (Zweifel & Brown, 1964) and *trans*-pinocarveol from (-)-β-pinene (Coxon, Dansted & Hartshorn, 1970). The relative composition of these materials in the volatiles from felled *P. radiata* from day 1 to 33 is shown in Table IV.

The EAG responses by *S. noctilio* to fractions, excluding the monoterpene hydrocarbons, from the volatiles of felled *P. radiata* (combined materials days 11—21; experiment a) are shown in Tables V and VI. Under the test conditions, the amount of test materials used in the bioassay was directly dependent on the concentration of the components in the volatiles emanating from *P. radiata.* With the major oxygenated components, the vapour pressures and hence rates of evaporation from the test paper were very similar. Fraction 4 (Table V) showed highest activity and greatest amount of material (except for 1). After further fractionation, 4b, 3b, 4a, 5b and 5a (in order of decreasing activity) had high

TABLE VI

EAG response of female S. noctilio *to volatiles from felled* P. radiata *(combined materials, days 11-21; experiment a) : second fractionation.*

Fraction No.	Response[1] in μV	Amount[2] ($\times 10^{-8}$ g)	Composition or No. of components
2a	65	0.17	one components
b	77	0.90	one components
c	182	4.25	two components
d	85	1.63	one components
e	94	1.66	one components
f	112	8.33	one components
3a	178	1.07	one components
b	386	11.96	three components including fenchone
c	194	3.22	one components
d	166	0.69	mixture
4a	323	22.08	pinocamphone/camphor
b	537	57.24	isopinocamphone + minor amounts of terpinen-4-ol and thymol methyl ether
5a	220	70.80	*trans*-pinocarveol
b	273	12.30	*trans*-pinocarveol + minor component
6a	154	4.28	one components
b	163	5.88	one components
c	139	7.30	two components including borneol
d	142	1.63	mixture including citronellol

L.S.D. = 75

[1] Average value of response from at least six antennae from different insects.
[2] Amount of material in diethyl ether solution applied to the test paper.

activity (Table VI). Fractions containing more material did not necessarily show higher response. Although the EAG response of *S. noctilio* depends on concentration, the response also varies considerably from compound to compound (Simpson, 1976).

Using GC-MS and from GC retention data, 3b was shown to contain fenchone, 4a was identified as pinocamphone/camphor (ca. 4 : 1 using the SF-96 capillary column), 4b as isopinocamphone with minor amounts (i.e. <2%) of terpinen-4-ol and thymol methyl ether, 5a as *trans*-pinocarveol, and 5b as *trans*-pinocarveol with a minor unidentified component.

DISCUSSION

The EAG technique has proved useful in identification studies of lepidopterous pheromones (Roelofs *et al.*, 1971a, b) in conjunction with gas chromatography. Less use had been made of the EAG technique with food and ovipositional attractants although there have been preliminary studies with the pine cone moth *Dioryctria abietella* (Asher, 1970) and the Japanese beetle (Adler, Beroza & McGovern, 1972).

Madden (1971) has indicated that trees were most attractive in the field to *S. noctilio* 5—7 days after felling, although attraction could persist for up to 3 weeks. The present study has shown that the volatiles being released show increasing antennal response with similar amounts of test material over the same time (3 weeks). Changes taking place in the logs may have occurred more slowly under laboratory conditions and the quantitative aspects of release of materials require further examination.

The relative amounts of camphor, pinocamphone, isopinocamphone, and *trans*-pinocarveol in the volatiles emanating from felled *P. radiata* increased significantly (20 - 30 ×) over a period of 3—4 weeks. These components, which accounted for the major part (at least 80%) of the volatiles other than monoterpene hydrocarbons were shown to elicit high antennal response from *S. noctilio* compared to the hydrocarbons. The EAG responses to fractions from the volatiles from felled *P. radiata* suggest strongly that these oxygenated components were largely responsible for the increase in response with time after felling shown when antennae were stimulated with similar amounts of total volatiles.

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RÉSUMÉ

IDENTIFICATION DES COMPOSÉS VOLATILS DE PINUS RADIATA *APRÈS ABATTAGE ET LES ELECTRO-ANTENNOGRAMMES QU'ILS PROVOQUENT CHEZ* SIREX NOCTILIO.

Les composés volatils émanant de *Pinus radiata* ne montrent que de faibles changements dans leur composition après l'abattage, bien que l'attractivité des arbres abattus à l'égard de *Sirex noctilio* change nettement au-delà de 3 semaines. Pendant les premières 3 semaines suivant l'abattage les monoterpènes hydrocarbons représentent plus de 95% des composés volatils et leur composition varie peu durant cette période. Les composants oxygénés (camphor, pinocamphone, isopinocamphone et *trans*-pinocarveol) qui sont absents ou seulement présents à l'état de traces immédiatement après l'abattage, sont produits en quantités croissantes et représentent 1% des substances volatiles au bout de 3 semaines. Leur présence dans la fraction volatile accroît de façon significative la réponse antennaire de *Sirex noctilio*. Les principaux monoterpènes hydrocarbonés et les composants oxygénés ont été identifiés par les techniques chromatographiques et spectrographiques.

REFERENCES

ADLER, V. E., BEROZA, M. & MCGOVERN, T. P. (1972). Electrophysiological response of antennae of the Japanese beetle to attractants and related compounds. *J. econ. Ent.* **65** : 921-923.

ASHER, W. C. (1970). Olfactory response of *Dioryctria abietella* (Lepidoptera: Phycitidae) to slash pine cones. *Ann. ent. Soc. Am.* **63** : 474—476.

BLIGHT, M. M., GROVE, J. F. & MCCORMICK, A. (1969). Volatile neutral compounds emanating from laboratory-reared colonies of the desert locust *Schistocerca gregaria*. *J Insect Physiol.* **15** : 11—24.

BROWNLEE, R. G. & SILVERSTEIN, R. M. (1968). A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Analyt. Chem.* **40** : 2077—2079.

COXON, J. M., DANSTED, E. & HARTSHORN, M. P. (1970). *trans*-Pinocarveol from New Zealand turpentine. *J. chem. Engng. Data* **15** : 336.

HAWES, J. E., MALLABY, R. & WILLIAMS, V. P. (1969). An all-glass in line temperature-programmed interface for use in gas chromatograph-mass spectrometer combinations. *J chromat. Sci.* **7** : 690-693.

KARASEK, F. W. (1969). Techniques for flavour analysis. *Res. Dev.* **20** : 74, 76, 78.

MADDEN, J. L. (1971). Some treatments which render Monterey pine *(Pinus radiata)* attractive to the wood wasp *Sirex noctilio* F. *Bull. ent. Res.* **60** : 467-472.

MON, T. R., FORREY, R. R. & TERANISHI, R. (1967). Effects of addition of adsorption-reducing material with open tubular and packed column gas chromatography. *J. Gas Chromat.* **5** : 497-500.

ROELOFS, W. L., CARDÉ, R., BENZ, G. & VON SALIS, G. (1971a). Sex attractant of the larch bud moth found by electroantennogram method. *Experientia* **27** : 1438-1439.

ROELOFS, W. L., COMEAU, A., HILL, A. & MILICEVIC, G. (1971b). Sex attractant of the codling moth: characterization with electroantennogram technique. *Science, Wash.* **174** : 297-299.

SIMPSON, R. F. (1976). Bioassay of pine oil components as attractants for *Sirex noctilio* (Hymenoptera: Siricidae) using electroantennogram techniques. *Ent. exp. & appl.* **19** : 11-18.

SOWER, L. L., GASTON, L. K. & SHOREY, H. H. (1971). Sex pheromones of noctuid moth. XXVI. Female release rate, male response, threshold, and communication distance for *Trichoplusia ni*. *Ann. ent. Soc. Am.* **64** : 1448-1456.

ZWEIFEL, G. & BROWN, H. C. (1964). Hydroboration of terpenes. II. The hydroboration of α- and β-pinene - the absolute configuration of the dialkylborane from the hydroboration of α-pinene. *J. Am. chem. Soc.* **86** : 393-397.