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BIOASSAY OF PINE OIL COMPONENTS AS ATTRACTANTS FOR *SIREX NOCTILIO* (HYMENOPTERA: SIRICIDAE) USING ELECTROANTENNOGRAM TECHNIQUES

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A study was made of the response to components of pine oils and related compounds of antennae excised from female *Sirex noctilio* using electroantennogram (EAG) techniques. A response ratio of each compound was obtained after determining the response expected from the same amount of linalool, which was used as a reference material. Correlations were found between activity and chemical structure. Within each chemical class, compounds having a pinane skeleton and unsaturation showed the highest response. Activity differed between the *cis*- and *trans*-isomers of the same compound and varied according to the location of substituents with compounds possessing functional groups.

The wood wasp *Sirex noctilio* F. has been regarded as a potentially serious pest of conifers, especially *Pinus* spp. (Morgan & Stewart, 1966), since its introduction into New Zealand and Australia, and, in some plantations, it has caused extensive damage (Rawlings, 1955). Female *S. noctilio* are more likely to attack trees that are damaged or in suppressed or subdominant crown class (Coutts, 1965; Morgan & Stewart, 1966). Madden (1968, 1971) showed, further, that the attractiveness of trees to *S. noctilio* increased after felling or ringing, reaching a maximum after some days or weeks, e.g. 5-7 days after felling. Volatile components are important in the attraction of *S. noctilio* to host trees and attractants are present in the cambium-phloem (Madden, 1971; Madden, pers. comm.). However, it is not known if the attractiveness of felled or ringed *P. radiata* depends on (1) formation of materials resulting from tissue damage or altered metabolism, or (2) change in permeability of bark resulting in higher release of some (or all) normal components.

The electroantennogram (EAG) technique has been successfully applied to the study of pheromones both for screening and for establishing relationships between chemical structure and activity. Roelofs & Comeau (1971a) have shown that compounds corresponding to the natural pheromone produce the highest amplitude EAG signals of all compounds in the same chemical class, and that the more distant the homologue in a series the smaller is the amplitude of response.

As part of a programme to elucidate the chemical basis for the increased attractiveness of felled trees to *S. noctilio*, a study was made of relationships between

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structure and activity of pine oils components and related compounds. No satisfactory behavioural bioassay has yet been devised for the study of the responses of *S. noctilio* to host volatiles. In the present study, therefore, the electroantennogram (EAG) technique was used as a bioassay for the activity of the various test compounds, in order to determine which materials should be included in subsequent field studies.

MATERIALS AND METHODS

EAGs were recorded using a Beckman Type RS Dynograph with Type 9806A ac/dc Coupler. Chart speed was 1 mm/sec and maximum sensitivity $1\mu V/mm$. The antennal preparation was mounted on electrodes and isolated in an earthed Faraday cage. Electrical leads to the recorder were shielded up to attachment with the electrodes to minimize baseline noise level. Air (purified with activated charcoal) was passed through a glass injection system (tubing cross-sectional area 1 cm^2) equipped with a septum to permit introduction of the test material. Several different types of electrodes were evaluated; stainless steel electrodes were used during compilation of results in Table I, although platinum electrodes gave equivalent results. Glass-saline electrodes were used in some preliminary experiments, but were found to give less consistent results.

The antenna was cut from the insect (near the scape) and the distal two to three segments removed. Electrodes were inserted into each end of the antenna.

All test insects had emerged 1-2 days prior to assay from logs held in the Pittwater laboratory in Southern Tasmania. Older insects (7-12 days) gave less consistent responses. Antennae from both male and female *S. noctilio* responded similarly. Antennae from at least six insects were employed with each set of test materials, and each material was presented two to three times. With *S. noctilio* the responses to test materials were not affected by order of presentation.

Preliminary experiments showed that mixtures of compounds sometimes elicit abnormally large EAG responses. It was considered essential, therefore, that all test material should be of high purity.

The materials were carefully purified by micropreparative gas chromatography with polar (10% Carbowax 20M on AW DMCS Chromosorb G) and non-polar (10% SF-96/Igepal 20 : 1 on AW DMCS Chromosorb G) liquid phases and accessory equipment similar to that described by Brownlee & Silverstein (1968).

The relationship between amplitude of EAG signal and the amount of test material was examined for members of the various chemical classes. Various amounts of the test materials from 0.10 μl up to sufficient to give saturated vapour were placed in 120 ml conical flasks. After allowing sufficient time for equilibration (at least 2 hr), 0.5 ml of the test vapour was removed from the flask with a 1-ml glass syringe and injected into the airstream (flowrate 700 ml/min) passing over the antennal preparation, starting with the flask containing the smallest amount of material. At least 20 sec were allowed to elapse between successive presentations,

TABLE I

EAG response of S. noctilio *antennae relative to linalool*

Compound	Mean response* ratio	Mean of transformed responses†	SE of mean of transformed responses	Compound	Mean response* ratio	Mean of transformed responses†	SE of mean of transformed responses
β-Pinene	1.22	1.08	0.05	Pinocarvone	3.61	1.55	0.03
3-Carene	1.15	1.05	0.06	Pinocamphone	3.33	1.50	0.05
α-Pinene	0.94	0.96	0.05	Fenchone	2.93	1.44	0.07
Myrcene	0.82	0.91	0.01	Isopinocamphone	2.61	1.41	0.03
α-Thujene	0.82	0.90	0.04	Camphor	2.39	1.36	0.06
β-Phellandrene	0.81	0.90	0.03	Verbenone	2.30	1.35	0.04
Sabinene	0.76	0.88	0.03	Piperitone	1.91	1.28	0.02
Camphene	0.76	0.85	0.09	Carvone	1.45	1.16	0.02
1-Limonene	0.68	0.83	0.02	Pulegone	1.33	1.12	0.02
Terpinolene	0.66	0.81	0.05	β-Ionone	0.68	0.82	0.04
α-Terpinene	0.64	0.80	0.04				
Tricyclene	0.63	0.78	0.05	*trans*-Verbenyl acetate	1.98	1.29	0.03
d-Limonene	0.60	0.77	0.04	Geranyl acetate	1.26	1.07	0.07
p-Cymene	0.55	0.72	0.06	Linalyl acetate	1.18	1.05	0.06
α-Phellandrene	0.53	0.71	0.05	α-Terpinyl acetate	0.94	0.96	0.04
γ-Terpinene	0.44	0.63	0.05	Neryl acetate	0.73	0.85	0.05
				α-Fenchyl acetate	0.58	0.76	0.03
cis-Verbenol	2.65	1.41	0.04	Isobornyl acetate	0.56	0.73	0.05
trans-Verbenol	2.37	1.37	0.02	Bornyl acetate	0.55	0.72	0.04
α-Fenchol	1.71	1.22	0.05	Citronellyl acetate	0.51	0.69	0.05
Geraniol	1.60	1.16	0.08				
Myrtenol	1.55	1.19	0.02	Thymol	0.64	0.80	0.04
α-Terpineol	1.53	1.18	0.03	Carvacrol	0.31	0.46	0.06
Isopulegol	1.43	1.15	0.04				
trans-Pinocarveol	1.28	1.10	0.04	1,8-Cineole	1.62	1.20	0.05
Terpinen-4-ol	1.25	1.09	0.03	Carvacrol methyl			
Nerol	1.04	1.01	0.04	ether	0.84	0.91	0.04
Isopinocampheol	1.01	0.99	0.04	Thymol methyl ether	0.37	0.56	0.03
Linalool	*1.00*	—	—				
Borneol	0.95	0.97	0.03				
Menthol	0.74	0.85	0.06				
Citronellol	0.74	0.85	0.06				
Myrtenal	2.60	1.41	0.02				
Geranial	1.42	1.13	0.06				
Neral	1.26	1.09	0.04				
Citronellal	0.83	0.91	0.04				

* Mean of ratio of the response (Y) to compound and the expected response (X) to an equal amount of linalool, using antennae from at least six insects.

† Mean of transformed ratios $\log_{10}$ (10 Y/X).

although preliminary experiments had established that there was no significant reduction in the EAG response due to adaptation even where presentations were as frequent as 1/sec.

The response to materials relative to the response to linalool was obtained using a similar procedure. Approximately 2 mg of each test compound was placed in a 120-ml conical flask. The order in which the test vapours were presented was rotated and the first used was presented again at the end of the trial to check that response of the antenna had not altered significantly during the assay. Linalool (at saturated vapour pressure) and eight to ten test compounds and the antennae from at least six insects were included in each assay.

The long-chained alcohols and esters to which many of the lepidopterous pheromones belong (Evans & Green, 1973), have vapour pressures so similar that the amount of material reaching the antennae preparation need not be monitored.

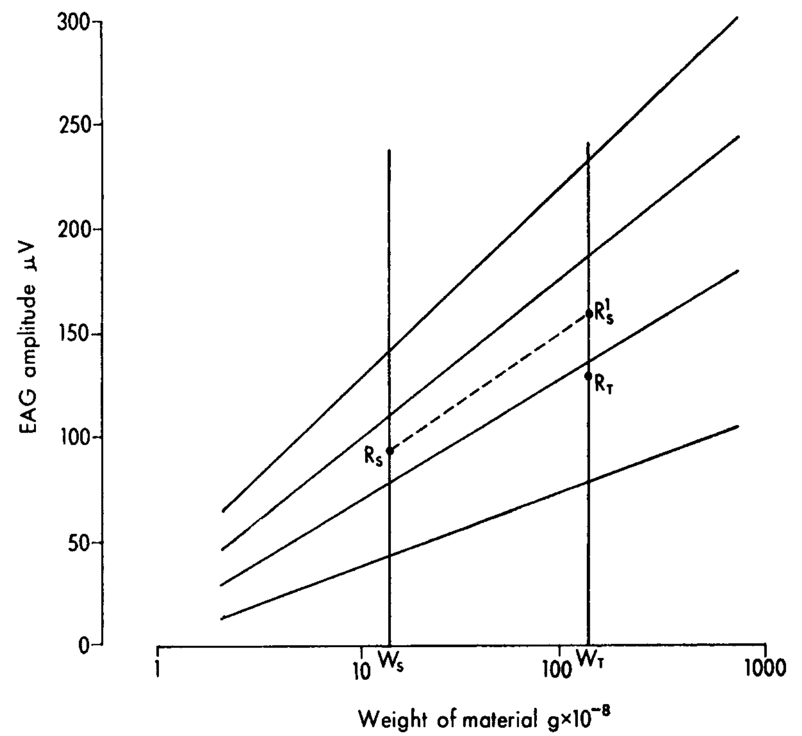

Fig. 1. Method of calculating the response of individual antennae to test compound relative to the response to linalool (response ratio). The four sloping lines represent the relationship between the weight of linalool and the EAG amplitude for four different antennae giving different amplitudes of response. R_T is the amplitude of the EAG elicited by a weight W_T of the test compound and R_S that elicited by a weight W_S of linalool. R'_S is the response which could be expected to be elicited by a weight W_T of linalool, the slope of the line R_S R'_S being similar to that of weight-response lines for antennae giving responses similar in amplitude to those given by the test antenna. The response ratio for the test compound for the particular test antenna is R_T/R'_S

However, when the effects of materials with markedly different vapour pressures are being investigated, as in the present study, it is necessary to do so. An amount of vapour equal to that used in the bioassay was therefore removed with a 1-ml Hamilton Gas Tight syringe from each flask and injected into a Pye model 104 gas chromatograph equipped with a flame ionization detector (FID), Shimadzu R101 recorder and Series 200 Disc integrator. The integration count was converted to weight of test material on the basis of calibration with known amounts of linalool.

A measure of the responsiveness of individual antennae to each test material relative to the response to linalool was obtained by the method detailed in Fig. 1. The mean response ratios shown in Table I are the means of the individual response ratios obtained from at least six different antennae.

RESULTS

Relationship between amplitude of EAG signal and amount of test material

There was a direct relationship between amplitude of the EAG signal and the logarithm of the amount of material, for all compounds examined, except at the high concentrations which were obtained with very volatile materials. At these

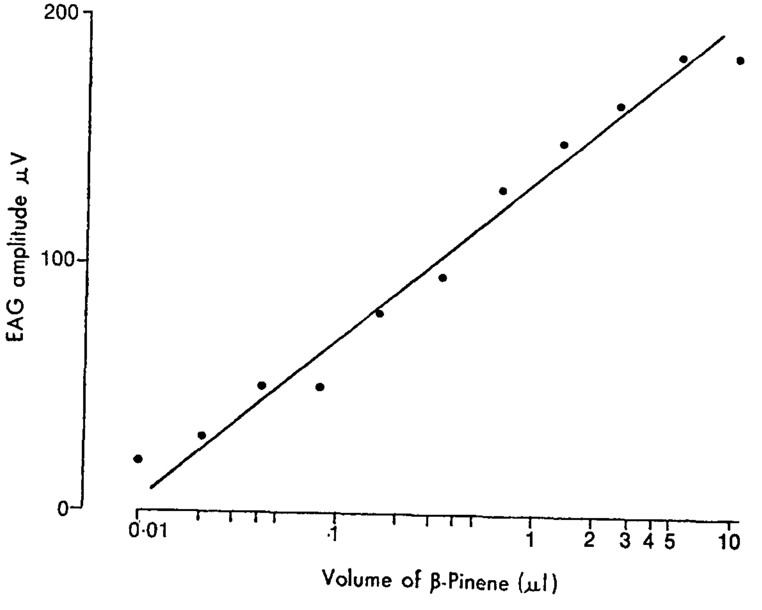

Fig. 2. Typical EAG response of single female *S. noctilio* antenna to increasing amounts of β-pinene. Volume refers to quantity of β-pinene placed in test flasks (120 ml). 0.5 ml of equilibrated vapour was injected into the airstream (700 ml/min).

high concentrations the EAG signal had more than one peak. The response of a female *S. noctilio* antenna to β-pinene is shown in Fig. 2. Similar relationships between concentration and EAG amplitude were demonstrated by Roelofs & Comeau (1971b) and Payne, Shorey & Gaston (1970). This type of relationship has been shown in studies of the responses of many organisms to chemical and physical stimuli and is traditionally known as the Weber-Fechner Law (see Moncreiff, 1967).

Relationship between structure and EAG response

To determine if differences between mean responses in relation to those of linalool were significant the standard error of the transformed responses (log $10 \times R_T/R'_S$) was calculated. The mean response, mean transformed response and standard error are shown in Table 1.

(1) Monoterpene hydrocarbons. The compounds of this group which have a bicyclic skeleton and unsaturation showed high activity, in particular, β-pinene (mean response ratio 1.22), α-pinene (0.94) and 3-carene (1.15). The only other materials to show moderate to high activity were β-phellandrene (0.81, p-menthane, skeleton, two double bonds) and myrcene (0.82, acylic, three double bonds).

Tricyclene (0.63) which has three ring systems shows low activity and p-cymene (0.55), which has the only aromatic structure, has similar activity to α-phellandrene (0.53) and γ-terpinene (0.44).

(2) Alcohols. Highest activity in this group was shown by *cis*-verbenol (2.65), *trans*-verbenol (2.37) and to a lesser extent myrtenol (1.55). All these compounds have a pinane skeleton and one double bond. The remaining compounds having a pinane skeleton, *trans*-pinocarveol (1.28, one double bond) with the hydroxyl in the 2-position and isopinocampheol (1.01) with no unsaturation showed lower responses. The geometric isomers geraniol (1.60) and nerol (1.04) showed significantly different activity, while the related compound containing one less double bond, citronellol (0.74, one double bond) showed lower activity. Other examples of compounds having related structure but differing in the number of double bonds were the menthanes isopulegol (1.43) and menthol (0.74). In all instances the material having the smaller number of double bonds has correspondly less activity.

(3) Aldehydes. Few aldehydes were included because of the correspondingly small numbers of primary alcohols. However, myrtenal (2.60, pinane skeleton, one double bond) showed high activity.

(4) Ketones. The ketones exhibited the highest response values of any compounds examined. Pinocarvone (3.61) and pinocamphone (3.33) both of which have a pinane skeleton, showed exceptional activity. Verbenone (2.30) with the keto-group in the 3- rather than 2- position had less activity. The high response values

of these materials are of interest since camphor, pinocamphone and isopinocamphone have been identified in the volatiles from felled *P. radiata* which are highly attractive to *S. noctilio* (Simpson & McQuilkin, unpubl.).

The only ketone which showed comparatively low activity was β-ionone (0.68), which is biochemically related to the sesquiterpenes rather than the monoterpenes.

(5) Acetates. The highest response in this group was shown by *trans*-verbenyl acetate (1.98, pinane skeleton, one double bond). The bicyclic unsaturated acetates showed fairly weak response: α-fenchyl (0.58), bornyl (0.55) and isobornyl acetate (0.56). The order of response shown by the acyclic acetates geranyl (1.26), neryl (0.73) and citronellyl acetate (0.51) was the same as that found for the corresponding alcohols and aldehydes.

(6) Phenols and ethers. Thymol and carvacrol and the corresponding methyl ethers showed fairly weak responses. Only 1,8-cineole (1.62) with two-ring systems had moderately strong activity.

DISCUSSION

In each chemical class, compounds having a pinane skeleton and unsaturation showed high activity. This is evident from the responses given by α- and β-pinene (hydrocarbons), *cis*-verbenol, *trans*-verbenol and myrtenol (alcohols), pinocarvone, pinocamphone and isopinocamphone (ketones), myrtenal and *trans*-verbenyl acetate.

There are numerous examples [isopulegol/menthol, (geraniol, nerol)/citronellol, *trans*-pinocarveol/isopinocampheol, (geranial, neral)/citronellal, pinocarvone/(pinocamphone, isopinocamphone) and (geranyl, neryl)/citronellyl acetate] of compounds having similar structures but differing in the number of double bonds where the material having the greater number of double bonds shows higher activity.

The location of a functional group (α-terpineol/terpinen-4-ol) or position of a double bond (β-phellandrene/α-phellandrene) clearly affects activity and the *trans*-isomer geraniol has higher activity than nerol (*cis*-isomer) — a relationship which also holds for the corresponding aldehydes and acetates.

It is generally accepted that EAG amplitude is an indication of the total level of receptor activity on the antennae. The relationship between EAG amplitude and behaviour is much less certain, since receptor activity would be elicited by both attractants and repellents. For *S. noctilio* the relation between EAG amplitude and behavioural response to host volatiles could be elucidated only by extensive field experiments, but it is interesting to note that α- and β-pinene, β-phellandrene, myrcene and 3-carene, which are major components of the volatiles emanating from felled *P. radiata* (Simpson & McQuilkin, unpubl.) have relatively high activity within the group of monoterpene hydrocarbons examined. With the Japanese beetle some correlation was found between EAG amplitude and trap captures in the field in tests of attractants and related compounds (Adler, Beroza & McGovern, 1972).

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RÉSUMÉ

TESTS BIOLOGIQUES DU CARACTÈRE ATTRACTIF DES COMPOSANTS DE L'HUILE DE PIN À L'ÉGARD DE SIREX NOCTILIO *(HYMENOPTERA: SIRICIDAE) EN UTILISANT LES TECHNIQUES DE L'ÉLECTRO-ANTENNOGRAMME*

Cette étude est basée sur les réponses antennaires de *Sirex noctilio* aux composants des huiles de pin et à quelques composés chimiques apparentés, en utilisant la technique de l'électro-antennogramme appliquée à une antenne isolée prélevée sur une femelle de cet insecte.

Un taux de réponse à chaque composé a été établi par comparaison à la réaction enregistrée à l'égard d'une égale quantité de linalool réaction utilisée comme unité de référence.

On a mis en évidence certaines corrélations entre la structure chimique et l'activité biologique des composés. A l'intérieur de chaque classe de composés, ceux qui sont insaturés et qui possèdent un squelette pinane ont la plus grande activité biologique. Cette activité biologique diffère entre les formes *cis-* et *trans-* d'un même composé et varie avec la position des groupes fonctionnels, avec également la position et le nombre des doubles-liaisons.

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