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Article

Electrophysiological Responses of Eucalyptus Brown Looper *Thyrinteina arnobia* to Essential Oils of Seven *Eucalyptus* Species

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A lagarta-parda, *Thyrinteina arnobia*, causa grandes prejuízos à cultura do eucalipto, destacando-se como o principal lepidóptero desfolhador; portanto, medidas alternativas de controle são necessárias. Neste trabalho foi avaliada, pela técnica de eletroantenografia (EAG), a interação dos voláteis dos óleos essenciais de sete espécies de *Eucalyptus,* frente às antenas de fêmeas e de machos de *T. arnobia*. Foram também identificados 28 compostos voláteis bioativos contidos no óleo essencial de *E. grandis,* utilizando a detecção eletroantenográfica acoplada à cromatografia a gás (CG-EAD). Estes resultados indicam que CG-EAD é uma ferramenta muito útil na triagem de compostos bioativos presentes em extratos de plantas e sugerem que *T. arnobia* utiliza vários destes terpenos como sinais para encontrar seu hospedeiro.

Eucalyptus is frequently attacked by the Brazilian eucalyptus brown looper, *Thyrinteina arnobia*. This caterpillar is regarded as the main lepidopterous pest of *Eucalyptus* and yet no practical and environmentally acceptable method of control currently exists. Electroantennographic techniques (EAG) have never before been used to detect semiochemicals that affect the behavior of *T. arnobia*. Thus, in this work, the ability of *T. arnobia* males and females to detect volatile essential oils of seven *Eucalyptus* species was investigated by EAG. We demonstrated that *T. arnobia* antennal olfactory system clearly showed differential sensitivity to several compounds, by coupled gas chromatography-electroantennographic detection (GC-EAD). Twenty-eight compounds were identified that elicited responses in *T. arnobia*, indicating that GC-EAD analysis may well be a useful means of screening active plant extracts for compounds that contribute to the observed behavior of this defoliator. The results also suggest that this species uses several volatile cues to find its host.

Keywords: *Thyrinteina arnobia*, Eucalyptus brown looper, *Eucalyptus* spp., essential oil, electroantennography

Introduction

The genus *Eucalyptus*, native to Australia, is widely cultivated in Brazil, covering an area of more than three million hectares. This country is the world's leading producer of eucalyptus-based fiber and of the 6.3 million tons of cellulose produced here annually, the majority is extracted from this exotic species. *Eucalyptus* plantations also supply wood to the furniture industry and help to reduce the pressure on native forests.[1]

The Brazilian eucalyptus brown looper, *Thyrinteina arnobia* (Stoll, 1782) (Lepidoptera: Geometridae), is the most harmful of the *Eucalyptus* pests in Brazil, causing severe losses in wood production through defoliation. This moth attacks several genera of the Myrtaceae family, including six *Psidium* species, eight *Campomanesia* species, nine *Eugenia* species and twenty *Eucalyptus* species. Biological data on *T. arnobia,* reared on leaves of *Eucalyptus* spp, have been recorded by a number of groups.[2-6]

Several strategies have been tried and considerable effort spent on the development of methods to control this pest, yet no practical and environmentally acceptable one currently exists. Control is made very difficult by the huge area of plantations and by the height of the trees.[4]

Herbivorous insects often interact with plants by means of volatile semiochemicals, particularly to locate hosts upon which to feed or oviposit.[7-9] Essential oil is typically a blend of compounds, principally terpenoids, which

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represent the dominant class in *Eucalyptus* leaves. Because the essential oils of different species often share the same monoterpenes,[10] a particular species' odor may have components typical of one or more species. Thus, knowledge of these volatiles could be used as the basis for a control strategy.

Some species of the genus *Eucalyptus* exhibit high essential oil contents in their leaves and others have medium or low contents. Some *Eucalyptus* species are characterized by their sclerophyllous foliage,[11] *i.e.*, high specific leaf weight or leaf toughness,[12] while other species present softer leaves, these characteristics being considered adaptive responses to abiotic factors. The seven species of *Eucalyptus* used in the present study were chosen on the basis of these characteristics. In preliminary experiments, we selected *Eucalyptus* species that vary from those with sclerophyllous foliage and high oil content (*E. camaldulensis* and *E. citriodora*) to those with softer leaves and intermediate oil content (*E. grandis*, *E. saligna*, *E. urophylla* and *E. cloesiana*), while another has sclerophyllous foliage and low essential oil content (*E. maculata*).[2,3,10] Besides, choice assays applied to *T. arnobia* larvae,[4] showed that *E. urophylla*, *E. saligna*, *E. grandis* and *E. cloesiana* species are preferred hosts of eucalyptus brown looper. They provide propitious conditions for their development and reproduction, whereas *E. camaldulensis* and *E. citriodora* are less suitable hosts because chemical and/or morphological factors on the leaves of these species could have adverse effects on the biology of *T. arnobia* larvae. *E. maculata* has never been mentioned as a *T. arnobia* host.

To understand the interactions mediated by semiochemicals, it is necessary to study the factors involved in the olfactory perception of these compounds in order to identify those that could attract *T. arnobia*. Up to now, neither potentially attractive semiochemicals, nor electroantennographic techniques have been applied to *T. arnobia.* Thus, we compared the detection sensitivity of *T. arnobia* males and females to volatile components of essential oils of seven *Eucalyptus* species, using the electroantennographic technique (EAG). Moreover, gas-chromatography linked on-line to electroantennography (GC-EAD) study was performed with volatiles sampled from *E. grandis* essential oil.

Experimental

Mass Spectra were recorded on a Shimadzu QP5000 (EIMS) at 70 eV. Gas chromatography was performed in a Shimadzu GC-17A with H_2 as carrier and using a DB-5 (30 m x 0.25 μm ID, 0.25 mm film thickness) capillary column with helium as carrier gas at a flow-rate of 1.6 mL min^{-1}. The temperature was held initially at 60 °C for 2 min and then increased at a rate of 3 °C min^{-1} to 240 °C. The injector was in the split mode at 225 °C. The interface temperature was 250 °C.

Eucalyptus essential oil extraction and analysis

Fresh leaves of *E. grandis* Hill ex Maiden, *E. citriodora* Hook, *E. camaldulensis* Dehnh., *E. saligna* Sm., *E. urophylla* S. T. Blake, *E. cloesiana* were collected in the Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil and *E. maculate* was collected in Universidade Federal de Viçosa, MG, Brazil. The collects were done in forest species arboretum, located in the experimental plantation of these universities and identified by the Dr. C. F. Wilcken. The leaves were randomly collected from approximately six-years-old *Eucalyptus* trees.

The fresh leaves (400 g) of each species were submitted to steam distillation for 4 h, using a Clevenger apparatus. The essential oils in the distillate were dried over anhydrous Na_2SO_4 and kept in the freezer. The essential oil that provoked the highest depolarization in *T. arnobia* female antennae was analyzed by GC-MS. Components were identified by determination of their retention indices relative to those of a homologous series of *n*-alkanes[13] by co-injection with authentic samples, and by comparing fragmentation patterns in mass spectra with those stored on the spectrometer database and bibliography.[14]

Insects

T. arnobia specimens were obtained from a laboratory where they were grown on artificial diets,[4] established in the Insect Bioassay Laboratory of the Universidade Federal de São Carlos, SP, Brazil. Pupae were sexed and placed individually in plastic vials (6 cm x 6 cm ID) for emergence of adults. Male and female pupae were maintained in a growth chamber at 25 ± 1 °C and 60 ± 5 % r.h., in a 12:12 L:D photoperiod.

Electroantennography and coupled Gas Chromatography-Electroantennographic Detection (GC-EAD)

The electroantennographic and the coupling of Gas Chromatography-Electroantennographic Detection (GC-EAD) were done as described in the literature.[7,13-15]

Antennae of 1-2 day old male and female moths were used for electroantennographic experiments (EAG) and electroantennographic detection (EAD).[16] Each antenna was pulled from the head with forceps and a few segments were cut off at the base and the tip.[17] The antenna was

then fixed between two stainless steel electrodes by pushing the base and tip into droplets of an electrically conductive gel (Spectra 360® electrode gel) applied to the metal electrodes.

EAG experiments were performed in order to elucidate the sensitivity and selectivity of the antennal receptors of *T. arnobia*. The EAG response was evaluated as follows: the volatile compounds or control were released from Pasteur pipettes containing a piece of filter paper (*ca.* 0.8 cm^2) previously impregnated with 10 µL of a freshly prepared solution of each test essential oil in hexane, after the solvent had evaporated. The puff containing the test essential oil (from seven *Eucalyptus* species) was delivered into a continuously humidified and purified air stream (1.2 L min^{-1}) passing for 0.3 seconds through the impregnated filter paper in the pipettes. Control stimulation was made at the beginning and the end of each series of EAG experiments. The test essential oils were then applied randomly at intervals of 60 seconds. EAG amplitudes in response to the essential oils were expressed in relation to the responses to the control (hexane), because of the large differences in overall sensitivity between individual antennae, and to compensate the decline in antennal sensitivity during a measuring session. In this normalization procedure, the responses to the control were defined as 100 %. The values obtained between two calibration references (controls) were calculated by linear interpolation between those references values. The Syntech EAG software (Syntech®, Hilversum, The Netherlands) calculated the normalized values automatically. The essential oils were tested on 10 antennae each of female and male *T. arnobia*. The mean normalized responses of the different compounds were submitted to ANOVA for statistical analysis and compared by the Tukey test ($P < 0.05$).

The GC-EAD coupling consisted of a Shimadzu 17-A chromatograph equipped with a flame ionization detector and a Syntech electroantennography system – EAG (Syntech®, Hilversum, The Netherlands). The column effluent (carrier gas hydrogen) was mixed with a nitrogen make-up gas flow (12 mL min^{-1}) before it was split to the FID and the heated transfer capillary leading to the antennal preparation of the EAD. The separated compounds were eluted from the transfer capillary into a humidified and purified air stream (1.2 L min^{-1}), which led them directly to the antennal preparation. To synchronise the retention times monitored by the FID and the EAD, the lengths of the capillaries after the splitting were exactly equal. The same chromatographic conditions as for the GC-MS analyses were used for the chromatographic separation. The FID was kept at 280 °C, whereas the temperature of the transfer capillary was maintained at 290 °C to avoid condensation.

Results and Discussion

Secondary metabolites in plants of Myrtaceae are dominated by the presence of mixtures of C_{10} and C_{15} terpenoids that are generally called essential oils and can be found in many plant families. The essential oil constituents of the genus *Eucalyptus* (Myrtaceae) have been well characterized.[10] *Eucalyptus* species produce numerous volatile compounds in large amounts, especially isoprenoids (here referred to as terpenes), which are accumulated in glands abundantly distributed throughout the leaf parenchyma and bark.[18] Quantitative data on the essential oils of seven eucalypts are shown in Table 1. *E. citriodora* furnished the

Table 1. Amounts of the essential oil in leaves from *Eucalyptus* spp

Eucalyptus Species	Weight (g)[a] of oil	(%)
E. cloesiana	0.70	0.17
E. saligna	0.76	0.19
E. citriodora	2.64	0.66
E. camaldulensis	2.54	0.63
E. grandis	1.26	0.31
E. urophylla	1.18	0.29
E. maculata	0.30	0.07

[a] from 400 g of leaves.

Table 2. Relative percentages of the most frequent compounds in essential oils from *Eucalyptus* leaves

Compound	KI	*E. cloesiana*	*E. saligna*	*E. citriodora*	*E. camaldulensis*	*E. grandis*	*E. urophylla*	*E. maculata*
α-Pinene	933	76.08	25.91	0.28	6.12	40.55	8.03	39.4
β-Pinene	975	2.21		0.58	2.37	0.23	4.59	6.87
p-Cymene	1022	0.16	24.38		2.13	13.13	2.5	
Limonene	1025	2.66	1.57		14.22	2.66	7.13	2.68
p-Mentha-2,4(8)-diene	1081	0.43	0.7		1.22	0.78	0.65	
Terpin-4-ol	1174	0.38	2.29		2.64	1.27	0.89	
α-Terpineol	1189	3.81	2.19		2.83	2.90	2.97	0.82
α-Gurjunene	1408		0.24		0.24	0.29	0.33	0.2
β-Caryophyllene	1418	2.33	0.25	0.52		1.19		10.34
Spathulenol	1576	1.9	0.32			0.95		0.55
Nerolidol	1583	0.98	0.37		0.88	0.57	0.29	3.78

KI = KI observed.

largest quantity of oil (2.64 g, 0.66 %) followed by *E. camaldulensis* (2.54 g, 0.63 %). *E. maculata* furnished the smallest quantity (0.30 g, 0.07 %).

The relative percentages of the most frequent compounds in essential oils are shown in Table 2. α-Pinene showed 100 % frequency, since it was present in all seven *Eucalyptus* oils analyzed. β-Pinene, limonene and nerolidol were present in six *Eucalyptus* species. α-Pinene is the monoterpene most frequently quantified by investigators in *Eucalyptus* species,[19-21] but it was not always the dominant component in the *Eucalyptus* species studied in this work (Table 3). Four species (*E. cloesiana*, *E. saligna*, *E. grandis* and *E. maculata*) showed α-pinene as the dominant component. The other species could be classified as having dominant citronelal (*E. citriodora*) or 1,8-cineole (*E. camaldulensis* and *E. urophylla*).

Mean depolarizations achieved by *T. arnobia* male and female antennae in response to the essential oils of *E. grandis, E. citriodora, E. camaldulensis, E. saligna, E. urophylla, E. cloesiana* and *E. maculata*, are shown in Figures 1 and 2. Electroantennographic responses in both sexes were significantly higher than that for the control (hexane). These results indicate that *T. arnobia* adults recognize some volatile compound in the *Eucalyptus* essential oils; that is, in the antennae of both sexes there are neuron receptors (chemoreceptors), present in sensillas, which detected compounds in the essential oils. Usually, receptors for plant volatiles are basiconic sensillas that are generalist neuron receptors.[22]

The mean responses of the males to the seven oils ranged from 1.21 ± 0.28 mV (*E. saligna*) to 0.82 ± 0.25 mV (*E. maculata*), the control giving 0.42 ± 0.11 mV, while in the females response ranged from 0.73 ± 0.082 mV (*E. urophylla*) to 0.46 ± 0.09 mV (*E. maculata*) and 0.26 ± 0.04 mV (control). The normalized peak responses of males and females to the essential oils are in Figures 1 and 2, respectively.

The number, location and also size and form of olfactory sensillas present in the male antenna could explain the higher sensitivity of the *T. arnobia* male antennae. The male antenna flagellum, which has a bipinnate structure, is more specialized than the female antenna, which is filiform (threadlike). Further studies have to be conducted to prove whether this hypothesis is true.

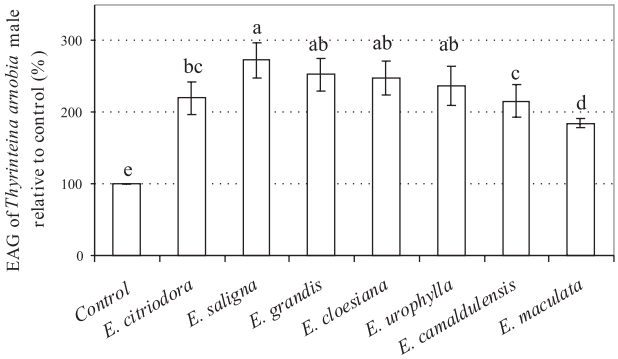

Figure 1. Mean EAG (± SD) elicited from *T. arnobia* male antennae, at concentration of 10 mg mL^{-1}, of *Eucalyptus* essential oils. Mean values marked with the same letter are not significantly different at $P < 0.05$, on the basis of the Tukey test (N = 10 antennae).

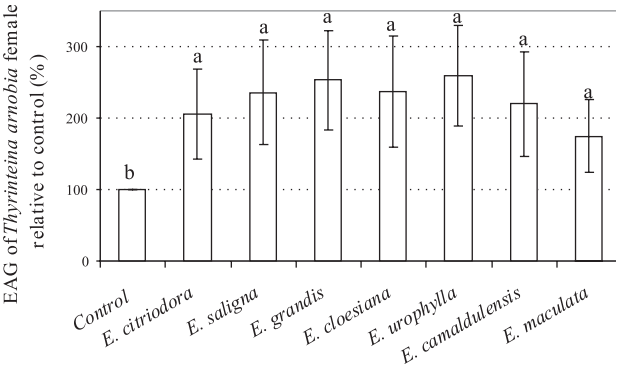

Figure 2. Mean EAG (± SD) elicited from *T. arnobia* female antennae, at concentration of 10 mg mL^{-1}, of *Eucalyptus* essential oil. Mean values marked with the same letter are not significantly different at $P < 0.05$, on the basis of the Tukey test (N = 10 antennae).

E. grandis essential oil was selected for the coupled gas chromatography-electroantennographic detection (GC-EAD) experiment because it caused the largest depolarization signal in both the male and female antennae in the EAG. Furthermore, this is the species most prevalent in commercial forestation in Brazil.

The composition of *E. grandis* essential oil shown by our results is given in Table 4. Component relative concentrations were calculated from GC peak areas and

Table 3. Relative percentages of dominant compounds in the essential oils of leaves of 7 *Eucalyptus* species

Compound	KI	*E. cloesiana*	*E. saligna*	*E. citriodora*	*E. camaldulensis*	*E. grandis*	*E. urophylla*	*E. maculata*
α-Pinene	932	76.08	25.91	0.28	6.12	40.55	8.03	39.4
p-Cymene	1021	0.16	24.38		2.13	13.13	2.5	
1,8-Cineole	1028		6.86		52.82	0.45	53.11	
γ-Terpinene	1054		24.63		6.79	16.25	0.69	
neo-iso-3-Thujanol	1139			11.84				
Citronellal	1150			75.99	1.09			
β-Caryophyllene	1417	2.33	0.25	0.52		1.19		10.34

KI = KI observed.

Table 4. Composition of essential oil (%) from *Eucalyptus grandis* leaves

Compound	KI Adams[12]	KI observed	%
N.I.	N.I.	913	0.31
α-Pinene	939	933	40.55
Camphene	954	946	0.30
β-Pinene	979	975	0.23
α-Phellandrene	1003	1004	1.83
n-Pentyl isobutyrate	1057	1012	0.55
N.I.	N.I.	1015	0.27
p-Cymene	1022	1022	13.13
Limonene	1029	1025	2.66
1,8-Cineole	1031	1028	0.45
(*Z*)-β-Ocimene	1037	1033	0.20
(*E*)-β-Ocimene	1050	1043	0.34
γ-Terpinene	1060	1053	16.25
N.I.	N.I.	1066	0.38
p-Mentha-2,4(8)-diene	1086	1081	0.78
N.I.	N.I.	1083	0.18
Linalool	1097	1093	0.84
endo-Fenchol	1117	1106	0.42
α-Campholenol	1126	1119	2.29
trans-Pinocarveol	1139	1132	0.27
trans-Pinocamphone	1163	1156	0.17
Isoborneol	1162	1161	0.76
Terpin-4-ol	1177	1174	1.27
α-Terpineol	1189	1189	2.90
trans-Carveol	1217	1218	0.45
α-Gurjunene	1410	1408	0.29
β-Caryophyllene	1419	1418	1.19
Aromadendrene	1441	1438	0.60
N.I.	N.I.	1442	0.30
α-Humulene	1455	1453	0.17
Seychellene	1460	1460	0.88
N.I.	N.I.	1462	1.60
Bicyclogermacrene	1500	1497	2.20
(*E*,*E*)-α-Farnesene	1506	1511	0.19
Flavesone	1547	1546	0.43
Spathulenol	1578	1576	0.95
Nerolidol	1563	1583	0.57
N.I.	N.I.	1590	0.50
N.I.	N.I.	1592	0.30
N.I.	N.I.	1600	0.23
Leptospermone	1623	1620	1.82

N.I.= compound not identified.

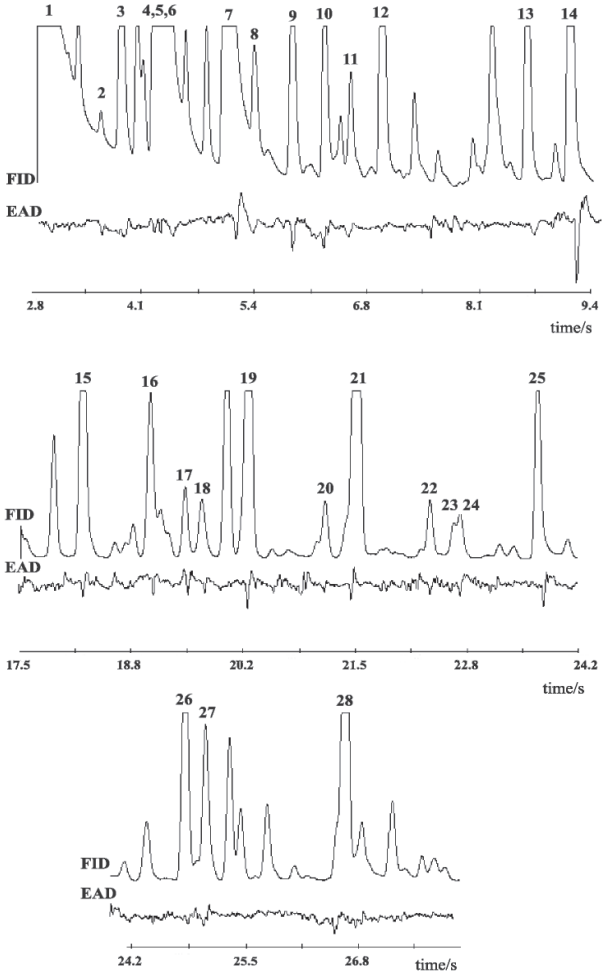

Figure 3. CG-EAD response of *T. arnobia* male antennae to *E. grandis* essential oil (0.1 mg mL^{-1}). The numbers indicate the peaks that elicited electrophysiological responses: α-pinene (1), β-pinene (2), α-phellandrene (3), *p*-cymene (4), limonene (5), 1,8-cineole (6), γ-terpinene (7), *p*-mentha-2,4,(8)-diene (9), linalool (11), α-campholenol (12), terpin-4-ol (13), α-terpineol (14), β-caryophyllene (15), aromadendrene (16), bicyclogermacrene (21), (E,E)- α-farnesene (22), flavesone (25), spathulenol (26), nerolidol (27) and leptospermone (28). (N = 5). (RT, Retention times = min).

they were arranged in order of GC elution. Forty compounds were found in this essential oil, thirty-two of which were identified, these corresponding to 95.9 % of the total eluted peaks in the chromatogram of the essential oil (Table 4). It was notable that the chromatographic peaks from the oil analysis consisted largely of terpenes. The major component was α-pinene (40.5 %), and the other two monoterpenes present in substantial amounts were γ-terpinene (16.2 %) and *p*-cimene (13.1 %).

The high olfactory sensitivity of *T. arnobia* to "green leaf volatiles" has never been demonstrated before. The present study shows that *T. arnobia* can detect many terpenes that are emitted by the *E. grandis* essential oil. The antennal olfactory system clearly showed differential sensitivity to different compounds, by GC-EAD. With this technique it was possible to identify twenty-eight compounds in the *E. grandis* oil that elicited responses in *T. arnobia* female antennae as well as in male antennae. Considerable effort was made to identify all stimulatory compounds among the volatile fractions eluted from the eucalyptus oil (Figure 3); however, only twenty could be identified: α-pinene (1), β-pinene (2), α-phellandrene (3), *p*-cymene (4), limonene (5), 1,8-cineole (6), γ-terpinene (7), *para*-mentha-2,4,(8)-diene (9), linalool (11), α-campholenol (12), terpin-4-ol (13), α-terpineol (14),β-caryophyllene (15), aromadendrene (16), bicyclogermacrene (21), (*E,E*)-α-farnesene (22), flavesone (25), spathulenol (26), nerolidol (27) and leptospermone (28). In the chromatogram (Figure 3), compounds 4, 5 and 6

coeluted, but elicited three electrophysiological responses (peaks). Further studies will be conducted in order to identify the other active compounds.

Many of these terpenoids have biological activity as herbivore attractants, repellents, or feeding stimulants.[23-25] The essential oil components α-pinene and β-pinene are well known mediators of insect behavior.[23] Additionally, 1,8-cineole is electrophysiologically active and acts as an attractant for the banana weevil *Cosmopolites sordidus*.[26] This terpenoid is also a feeding deterrent and oviposition repellent to mosquitoes[27] and repellent and toxic against stored-grain beetles.[28] In *Oxyops vitiosa*, 1,8-cineole, limonene and α-terpineol give rise to antennal (EAG and GC-EAD) and y-tube olfactometer signals, suggesting that these components elicit an attraction response in the adults of this weevil.[29] In our study, α-terpineol was the compound that elicited the greatest response in *T. arnobia* female and male antennae. Another active terpenoid, *trans*-nerolidol, functions either as an insect attractant[30-32] or as an antifeedant.[33] (*E*,*E*)-α-Farnesene, the main constituent of apple odor, stimulates oviposition and attracts neonate larvae and adult females of *Cydia pomonella* at short range.[34] This compound is also found in many other plants. Linalool acted electrophysiologically on *C. pomonella* female antennae.[35] α-Pinene, β-pinene, *p*-cymene, limonene, 1,8-cineole, linalool, from the oils of *Eucalyptus globulus, E. tereticornis* and *E. camaldulensis*, induced electrophysiological activity in *Phoracanta semipunctata* antennae.[7] β-Caryophyllene from the potato plant, *Solanum tuberosum* and the mahogany trees, *Swietenia macrophylla*, was electrophysiologically active on antennae of *Perillus bioculatus*[36] and *Hypsipyla grandella*,[37] respectively.

The fact that twenty-eight *Eucalyptus* essential oil components elicited responses from *T. arnobia* female and male antennae in this study is strongly indicative that GC-EAD analysis may be a useful method of screening active plant extracts for compounds that contribute to the observed behavior of this moth.[38] The results obtained by EAG and EAD also suggest that this species uses several volatile cues to find a host. Given the promising results of this study, it remains to be determined what behavioral roles is played by these allelochemicals, and whether compounds based on these active compounds will be sufficiently attractive and specific in the field to form the basis of population monitoring or control of the Brazilian Eucalyptus brown looper.

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