Electrophysiological Responses of *Atta sexdens rubropilosa* Workers to Essential Oils of *Eucalyptus* and its Chemical Composition

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The leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908 is the most harmful of the *Eucalyptus* pests, causing severe losses in wood production through defoliation. Various strategies have been tried and effort spent on the development of methods to control this pest, however no practical and environmentally acceptable one currently exists. In this work the chemical composition of the essential oil of seven *Eucalyptus* species was identified and the selectivity and sensitivity of antennal receptors of *A. sexdens rubropilosa* workers to the volatile compounds were determined using the electroantennographic technique (EAG and GC-EAD). Analysis by GC-EAD showed in *E. cloesiana* and *E. maculata*, respectively, seventeen and sixteen terpenes that elicited responses in ant workers' antennae, indicating the potential role of the essential oils as allelochemicals that determine the choice of the foraging material.

Key words: Leaf-Cutting Ant, Essential Oil, Electroantennography

Introduction

The leaf-cutting ants are the major insect problem to agriculture and forestry in Brazil and *Atta sexdens rubropilosa* Forel, 1908 is one of the principal species of this group. It is the most harmful of the *Eucalyptus* pests, causing severe losses in wood production through defoliation (Cherret, 1986; Fowler *et al*., 1989). Various strategies have been tried and substantial effort spent on the development of methods to control this pest, yet no practical and environmentally acceptable one currently exists.

Several plant secondary metabolites exhibit biological activities that have the potential to exert an effect on the physiology and/or behaviour of insects, and it is usually considered that such compounds are involved at some point in plant-insect relationships (Herrera and Pellmyr, 2002). The knowledge of the volatiles of essential oil of the genus *Eucalyptus* could be used as the basis for a control strategy of the leaf-cutting ants, given that they have a well-developed olfactory system (Hölldobler and Wilson, 1990).

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 *A. sexdens*. To date, the study with electroantennography (EAG) measurements applied to *A. sexdens rubropilosa* is restrict to two common trail pheromone components, 4-methylpyrrol-2-carboxylate and 2-ethyl-3,6-dimethylpyrazine (Kleineidam *et al*., 2005).

In this work, we identified the chemical composition of the essential oils of seven *Eucalyptus* species, which were selected on the basis of foliage characteristics, oil content and resistance by not preference (Anjos *et al*., 1987; Andrade *et al*., 1989; Berti-Filho *et al*., 1991; Boland *et al*., 1991; Anjos and Santana, 1994; Vendramin *et al*., 1995). Besides, the selectivity and sensitivity of antennal receptors of *A. sexdens rubropilosa* workers to volatile compounds of these essential oils were determined, using the electroantennographic technique (EAG). Gas chromatography linked on-line to electroantennography detection (GC-EAD) studies were also performed with volatiles sampled from *E. cloesiana* and *E. maculata* essential oils. This is the first report of the use of GC-EAD technique for *A. sexdens rubropilosa*.

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Materials and Methods

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. F. Blake and *E. cloesiana* were collected in the forest species arboretum, located in the experimental plantation of the Universidade Estadual Paulista, Botucatu, SP, Brazil. *E. maculata* leaves were obtained from Universidade Federal de Viçosa, MG, Brazil. The leaves were randomly collected from approx. six-year-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 at 0 °C. Essential oils were analyzed using a Shimadzu GC-17A gas chromatograph fitted with a fused silica DB-5 capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness; J and W Scientific) and 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. The chromatograph was coupled to a Shimadzu QP5000 mass selective detector (EIMS) at 70 eV. Components were identified by determination of their retention indices relative to those of a homologous series of *n*-alkanes (Dool and Kratz, 1963), by co-injection with authentic samples, and by comparing fragmentation patterns of mass spectra with those stored in the spectrometer database and bibliography (Adams, 1995).

Collection of the insects

A. sexdens rubropilosa workers were collected from six colonies located at the campus of the Universidade Federal de São Carlos, Central Region of São Paulo State, Brazil. Only larger foragers were collected, *i.e.*, leaf-carrying workers walking along trails.

Electrophysiology

Antennae of *A. sexdens rubropilosa* workers were used for electroantennographic experiments (EAG) and electroantennographic detection (EAD). Each antenna was pulled from the head with forceps and two segments were cut off at the base (Bjostad, 1998). 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, Parker, Orange, New Jersey) applied to the metal electrodes. The antennal responses were amplified and recorded with a data acquisition controller and software EAG (Syntech, Hilversum, The Netherlands).

EAG experiments were performed in order to elucidate the selectivity of the antennal receptors of *A. sexdens rubropilosa* workers. 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 s 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 essential oils were then applied randomly at intervals of 90 s. 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 calculated the normalized values automatically. The essential oils were tested on 20 antennae of *A. sexdens rubropilosa* workers. 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 equipment was used to identify individual compounds in the *E. cloesiana* and *E. maculata* oils capable of eliciting an electroantennographic response. Ten replicates were recorded for *A. sexdens rubropilosa* workers antennae, using essential oils from the two species. The GC-EAD instrument consisted of a Shimadzu 17-A gas chromatograph equipped with a flame ionization detector (FID) coupled to an electroantennography system and a DB-5 column (30 m ×

0.25 mm ID, 0.25 μm film thickness), using the following temperature program: 60 °C for 1 min, rising at 3 °C min^{-1} to 150 °C, held for 10 min, and finally rising at 1 °C min^{-1} to 280 °C and held for 16 min. The column effluent (carrier gas hydrogen) was mixed with a flow of nitrogen make-up gas (12 ml min^{-1}) before it was split to the FID and to a heated transfer capillary leading to the antennal preparation. From the transfer capillary the compounds entered a humidified and purified air stream (1.2 l min^{-1}), which carried them directly over the antennal preparation on the steel electrodes. 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

By the electroantennographic technique a high selectivity of the antennae of *A. sexdens rubropilosa* to the species *E. cloesiana*, *E. camaldulensis*, *E. urophylla*, *E. saligna*, *E. grandis*, *E. citriodora* and *E. maculata* was evident (Fig. 1). These results indicate the potential role of the essential oils as allelochemicals that determine the choice of the foraging material. When we compared the results obtained with the literature data we can infer that *E. cloesiana*, followed by the species *E. camaldulensis*, *E. urophylla* and *E. saligna* are the species preferred for the forage in relation to *E. grandis* and *E. citriodora*. These two last species are more preferred than *E. maculata*. These results are in agreement with the results of behavioral assays obtained by Andrade *et al.* (1989) and Vendramin *et al.* (1995), where the species *E. camaldulensis*, *E. urophylla* and *E. saligna* were preferred in comparison to *E. grandis* and *E. citriodora*. These two species were considered resistant by not preference. Anjos *et al.* (1986) verified that among the 20 species of eucalyptus more planted in Brazil, *E. maculata* was considered highly resistant to *A. sexdens rubropilosa*. Anjos and Santana (1994) demonstrated the negative effect in the behavior and in the survival of *A. sexdens* caused by *E. maculata* leaves; this effect was not evidenced for the species *E. grandis* and *E. citriodora*.

Composition of each essential oil of *Eucalyptus* is given in Tables I and II. Component relative concentrations were calculated from GC peak areas and arranged in order of GC elution.

E. cloesiana and *E. maculata* essential oils were chosen for the coupled gas chromatography-electroantennography detection (GC-EAD) experiment because they caused, respectively, the greater and the smaller depolarization in the *A. sexdens* antennae by the EAG technique.

It was verified that *E. cloesiana* essential oil presented 32 compounds 15 of which were identified, corresponding to 93.84% of the total oil mass (Tables I and II). The major component in the oil was α-pinene corresponding to 76.08% of the total mass. It was observed that the oil is constituted basically by terpenes.

Thirty-seven compounds were found in the *E. maculata* essential oil, 18 of which were identified, equivalent to 74.2% of the total mass (Tables I and II). Again, the major component was α-pinene (39.4%) followed by β-caryophyllene (10.34%), β-pinene (6.87%) and limonene (2.68%).

This research is pioneering in the employment of the GC-EAD technique to verify the olfactory sensitivity of *A. sexdens rubropilosa* workers. With

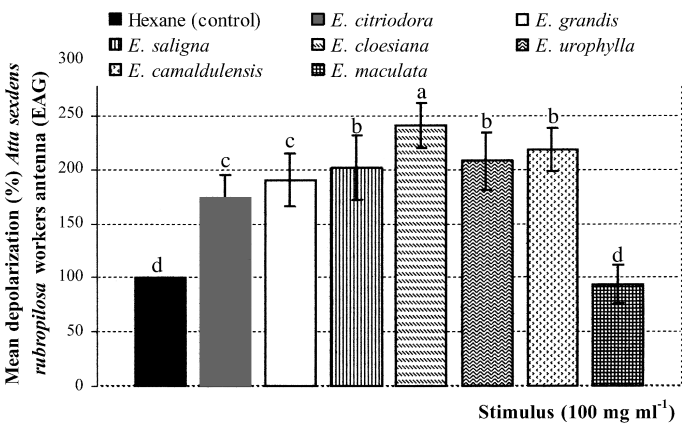

Fig. 1. Mean EAG (± SD) elicited from *Atta sexdens rubropilosa* worker antennae at the concentration of 100 mg ml^{-1} of *Eucalyptus* essential oils. Mean values marked with the same letter are not significantly different at $P < 0.05$ on the basis of the Tukey test ($N = 20$ antennae).

Table I. Essential oil composition (%) from *E. cloesiana* (ECL), *E. saligna* (ES), *E. citriodora* (EC), *E. camaldulensis* (ECA), *E. grandis* (EG), *E. urophylla* (EU) and *E. maculata* (EM) compared to Adams (1995).

Compound identified	Adams KI	ECL (%) KI obs	ES (%) KI obs	EC (%) KI obs	ECA (%) KI obs	EG (%) KI obs	EU (%) KI obs	EM (%) KI obs
Tricyclene	926	0.42			0.09		0.16	
α-Pinene	932	76.08	25.91	0.28	6.12	40.55	8.03	39.4
α-Fenchene	946	0.17	0.09			0.3		
β-Pinene	975	2.21		0.58	2.37	0.23	4.59	6.87
Myrcene	991				0.61		0.76	
α-Phellandrene	1004		0.53		0.19	1.83	4.02	
n-Pentyl isobutyrate	1012		0.42			0.55		
Isosylvestrene	1014				0.15		0.16	
o-Cymene	1021	0.16	24.38		2.13	13.13	2.5	
Limonene	1024	2.66	1.57		14.22	2.66	7.13	2.68
1,8-Cineole	1028		6.86		52.82	0.45	53.11	
(*Z*)-β-Ocimene	1033					0.2	2.92	0.93
(*E*)-β-Ocimene	1043					0.34	0.68	
γ-Terpinene	1054		24.63		6.79	16.25	0.69	
2,4(8)-*p*-Menthadiene	1080	0.43	0.7		1.22	0.78	0.65	
Linalool	1093		0.28		0.14	0.84	0.19	
exo-Fenchol	1106		0.13			0.42		
α-Canpholenol	1119		1.27			2.29		
trans-Pinocarveol	1132		0.27			0.27		
neo-iso-3-Thujanol	1139			11.84				
Citronellal	1150			75.99	1.09			
trans-Pinocamphone	1156		0.11			0.17		
Isoborneol	1161		0.21			0.76		
Terpin-4-ol	1174	0.38	2.29		2.64	1.27	0.89	
p-Cimen-8-ol	1183		0.09			2.9		
α-Terpineol	1188	3.81	2.19		2.83	2.90	2.97	0.82
trans-Carveol	1218		0.46					
Citronellol	1332			5.42				
p-Cimen-7-ol	1287		0.15					
Carvocrol	1302		0.12					
α-Terpenyl acetate	1350		0.37				4.38	
α-Gurjunene	1408		0.24		0.24	0.29	0.33	0.2
β-Caryophyllene	1417	2.33	0.25	0.52		1.19		10.34
Aromadendrene	1437	0.85			0.94	0.6		2.81
Seychellene	1460		0.17		0.38	0.88		0.73
Bicyclogermacrene	1497		0.98		0.55	2.2	0.2	
(*E*,*E*)-α-Farnesene	1511					0.19		
Geranyl isobutyrate	1517		0.28					
α-Cadinene	1524						0.23	0.40
Elemol	1550							0.53
α-Cadinene	1559							0.30
Spathulenol	1576	1.9	0.32			0.95		0.55
Nerolidol	1583	0.98	0.37		0.88	0.57	0.29	3.78
γ-Eudesmol	1631				0.1			0.38
β-Eudesmol	1649	0.48			0.36			0.66
α-Eudesmol	1652	0.67			0.28			1.02
Guaiol epimer	1658							0.45

the GC-EAD technique it was possible to detect 17 bioactive compounds present in *E. cloesiana* essential oil that stimulated the antennae of *A. sexdens* workers (Fig. 2). Of these bioactive compounds 12 were identified as: α-pinene (peak 1), β-pinene (2), limonene (3), 2,4(8)-*p*-menthadiene (4), terpin-4-ol (7), α-terpineol (8), β-caryophyllene (9), aromadendrene (10), spathulenol (13), nerolidol (14), β-eudesmol (16), and α-eudesmol (17).

In the *E. maculata* essential oil (Fig. 3), by GC-EAD, it was possible to detect 16 bioactive compounds. Efforts were made to identify all 16 of the stimulatory compounds among the volatile frac-

Table II. Not identified compounds observed in the essential oil from *E. cloesiana* (ECL), *E. saligna* (ES), *E. citriodora* (EC), *E. camaldulensis* (ECA), *E. grandis* (EG), *E. urophylla* (EU) and *E. maculata* (EM).

Compound not identified KI observed	ECL (%)	ES (%)	EC (%)	ECA (%)	EG (%)	EU (%)	EM (%)
906	0.23			0.08			6.23
913	0.27	0.86			0.31		
921	0.19			0.13			4.1
931							
963							0.4
965				0.06			
1015		0.35			0.27		
1033				0.16			
1066		0.36			0.38		
1083		0.1			0.18		
1092			0.47				
1099				0.1			
1106	0.2					0.15	
1140				0.21			
1143			0.51				
1164	0.22		0.88				
1196		1.32					
1255				0.22			
1267		0.16					
1332			0.7				
1336							0.62
1342						0.17	
1358	0.56						
1366	0.8						
1374							0.5
1386							0.25
1395		0.17					
1413		0.27					
1442					0.3		0.44
1453	0.32				0.17		1.12
1476			0.56				
1479			0.84				
1482				0.86			
1488							0.19
1491				0.21			
1495							1.37
1498	0.71		1.13				1.51
1504	0.16						
1522	1.16						
1545	0.39						
1566		0.11					0.31
1590	0.21	0.34	0.27	0.24	0.5		0.96
1592		0.24			0.3		0.99
1597							3.99
1600		0.14			0.23		0.81
1620					1.82		
1622		0.14		0.13			0.82
1627	0.34						0.67
1649	0.51						
1652	0.48						
1668	0.67						0.58
1972						0.42	

tions eluted from the oil of eucalyptus; however, only 11 could be identified: α-pinene (peak 1), β-pinene (2), limonene (3), α-terpineol (5), β-caryophyllene (8), aromadendrene (9), elemol (11), spathulenol (12), nerolidol (13), β-eudesmol (15), and α-eudesmol (16).

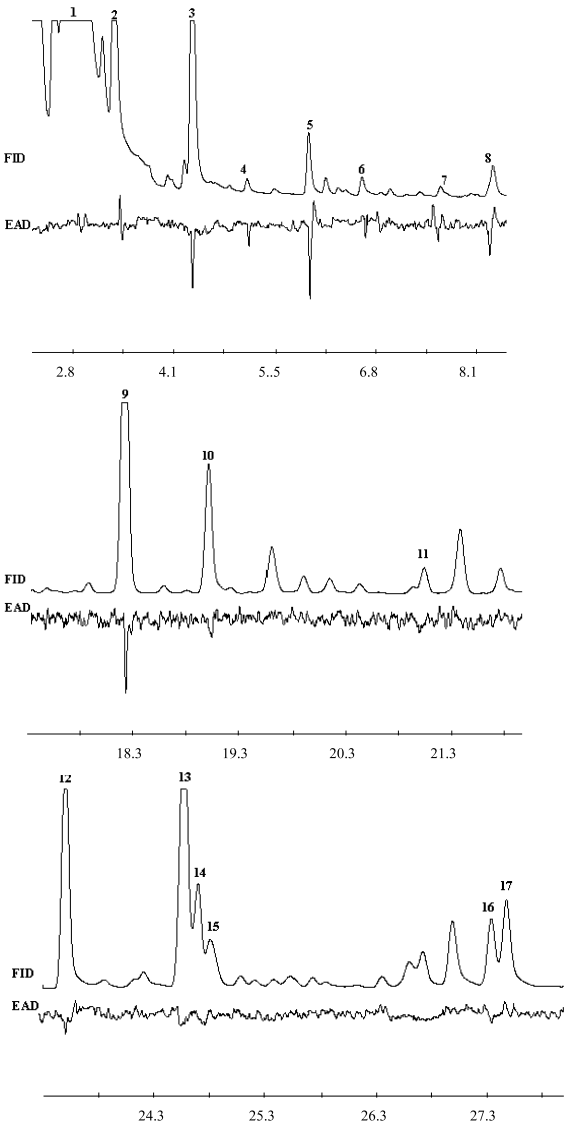

Fig. 2. GC-EAD response of *Atta sexdens rubropilosa* worker antennae to *E. cloesiana* essential oil (0.1 mg ml^{-1}). The numbers indicate the peaks that elicited electrophysiological responses ($N = 10$).

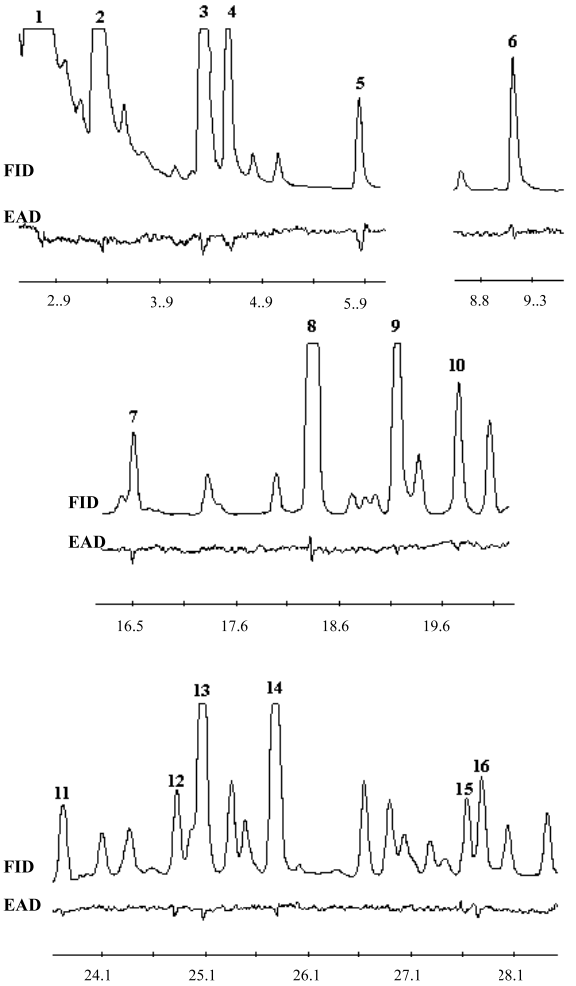

Fig. 3. GC-EAD response of *Atta sexdens rubropilosa* worker antennae to *E. maculata* essential oil (0.1 mg ml^{-1}). The numbers indicate the peaks that elicited electrophysiological responses ($N = 10$).

Ten bioactive compounds were almost chemically identical in *E. cloesiana* and *E. maculata* species. The compounds 2,4(8)-*p*-menthadiene (4) and terpin-4-ol (7) were present only in *E. cloesiana* essential oil while elemol (11) only in *E. maculata* essential oil. Of these results it can be inferred that the difference in the depolarizations, obtained for EAG and EAD, could be related to these compounds and mainly to the ratio of each compound, since in each oil they presented a different percentage. Besides, the differences in depolarizations could also be attributed to bioactive compounds not identified in each essential oil. According to Barnola *et al.* (1997) and Barnola and Cedeño (2000), the concentration of some terpenes, mainly β-pinene and β-caryophyllene, would be responsible for the difference in the attack of the *A. laevigata* leaf-cutting ant observed in the forestry species *Pinus caribaea*. In accordance with the same authors, β-caryophyllene, with occurrence in high percentage, would have a repellent

effect to *A. laevigata*. Of this form, the difference in the antennal response noticed for *A. sexdens* by EAG in *E. maculata* can be related with the presence of blend in high percentages of β-pinene (6.87%) with β-caryophyllene (10.34%); hence in *E. cloesiana* (2.21% β-pinene and 2.33% β-caryophyllene) and in other *Eucalyptus* species these compounds were present in low concentrations. Moreover, Marsaro *et al*. (2004) demonstrated, by behavioral assays, that hexane extracts of *E. maculata* interfered in recognition mechanism among workers. The main active compounds identified from this plant were elemol and β-eudesmol. These compounds may be responsible for the resistance of this species to ant attack.

In this study, it was verified that some terpenes, found in essential oils of *E. cloesiana* and *E. maculata*, are bioactive in the antennae of *A. sexdens rubropilosa* workers. These results indicate that the GC-EAD analysis is a useful technique for the selection of compounds present in plant extracts that have importance in the behavior of insects. The results obtained from the electroantennographic technique also suggest that *A. sexdens rubropilosa* workers use some volatile compounds as signals to find the host. But the relevance of these allelochemicals remains to be determined, *i.e*, if the bioactive compounds are satisfactorily attractive and specific in the field.

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