Cuticular Hydrocarbons of *Heterotermes tenuis* (Isoptera: Rhinotermitidae): Analyses and Electrophysiological Studies

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Termites have become an important pest of *Eucalyptus* and *Pinus* reforestations, sugarcane and other cultures. An alternative for the control of this pest would be the use of attractive traps that take in account the social behavior of these insects. Diverse factors are important for the insects in the localization of the habitat and the choice of the food and specific odors can facilitate this. Studies referring to *Heterotermes tenuis* (Isoptera: Rhinotermitidae) are scarce. The objective of this work was to analyze the tergal cuticular extract of *H. tenuis* and determine the selectivity and sensitivity of its antennae to the components of this extract by electroantennography (EAG). The composition of the cuticular extract was determined by GC-MS analysis. The hydrocarbons found were restricted to linear alkanes, being most abundant C_{24} to C_{27} that comprises *ca.* 65% of the total. Olefins were not detected. EAG and behavioral test responses to the cuticular hydrocarbons were greater and significantly different from the control and the high selectivity of the antennae to the extract indicates its potential as chemical messenger. Cuticular hydrocarbons mixture is species-specific and can be used to identify a given taxon without the diagnostic castes, soldiers or imagoes. Difference in the composition appears to relate with the type of habitat of specie.

Key words: Heterotermes tenuis, Cuticular Hydrocarbons, Electroantennography

Introduction

Termites are abundant and diverse in most parts of South America, particularly in tropical lowland forests, savannas (Cerrado) and grasslands. Several native termite species have been reported as agricultural and forest pests. The main pest species belong to Rhinotermitidae (*Heterotermes* and *Coptotermes*) and Termitidae (*Cornitermes*, *Procornitermes*, *Syntermes* and *Nasutitermes*). The most affected crops by *H. tenuis* in South America are sugarcane, upland rice, maize, cotton, soybean, coffee, cassava, *Eucalyptus* and *Pinus* reforestations (Berti Filho, 1995).

The problems with termites attacking commercial forests as *Eucalyptus* and *Pinus* have increased in Brazil and *H. tenuis* is reported attacking trunks of adult trees (Berti Filho, 1995). In São Paulo State, in the sugarcane crops, *H. tenuis* constitutes one of the most frequent and distributed pests being able to cause damages up to 10 t/ha/year (Arrigoni *et al.*, 1989).

Studies referring to *H. tenuis* are scarce; therefore its occurrence as pest in Brazil is relatively recent. Species of the Rhinotermitidae family live in diffuse (spread) nests in the soil. When active in foraging behavior, they make "shelter tubes" of soil material mixed with their saliva and fecal matter. They feed of the wood in decomposition and often attack vital parts of the plants like trunks and roots (Macedo, 1995).

Placing persistent insecticidal barriers in the soil around the roots has conventionally prevented damage to plants by subterranean termites. In the past, organochlorines (lindane, aldrin, dieldrin, chlordane and heptachlor) were applied almost exclusively as seed dressing or to the soil in planting holes or as furrows treatments. With the prohibition of these insecticides, alternatives for control of subterranean termites more ecologically acceptable that traditional chemical control measures become necessary. Researches with new chemicals of low environmental impact are being carried out, as well as alternative methods like biological con-

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trol with entomopathogenic fungi and physical barriers as plastic cylinders. Another alternative is the use of attractive bait for termites monitoring. It is generally performed by means of coil-corrugated cardboard, inside of a plastic bottle (Almeida and Alves, 1995; Wilcken and Raetano, 1995). Insects use several factors for localization of the habitat and choice of the food. In some cases, specific odors facilitate this localization and the identification of the alimentary source (Hubbell and Wiemer, 1983). For the best understanding of the interactions mediated by odors, *i.e.*, semiochemical, it is necessary to study the aspects involved in the olfactory perception of these compounds through electroantennographic experiments (EAG). Some authors suggested that cuticular hydrocarbons might act as semiochemicals (Howard and Blomquist, 1982). At the present, the use of semiochemical as a possible attractant for *H. tenuis* is unknown.

The electroantennographic technique which measures electrical activities of the insect antennae in response to olfactory stimulus has been used for decades to evaluate the sensitivity of the chemical receptors to semiochemicals. To date, the use of this technique for *H. tenuis* antennae has not been published. Thus, the objective of this research work was to analyze the tergal cuticular extract of *H. tenuis* and determine the selectivity and sensitivity of its antennae to components of this extract by electroantennography (EAG).

Materials and Methods

Collection of the insects

H. tenuis workers were collected in the agricultural zone of Indiaporã, located in the northwest of São Paulo State, Brazil, from January to February 2000. The collection was carried out with cylindrical corrugated cardboard baits (10 cm diameter, 15 cm height) buried under soil, 20 cm deep, next to poles infested with this termite species.

Preparation of H. tenuis extracts for the chemical analysis

The *H. tenuis* extract was prepared in the Laboratory of Termites, Biotério do Instituto de Biologia, Universidade Estadual Paulista, Rio Claro, SP. For the preparation of the extract for EAG bioassays and chemical analyses 250 workers of *H. tenuis* were used. The abdomens were sectioned, under a stereomicroscope and the guts removed, remaining only the tergites, which were extracted for 24 h in 3.0 ml purified hexane. The extract was concentrated to about 500 μl and stored at – 18 °C for further analysis.

Chemical analysis

The extracts were analyzed by gas chromatography in a Shimadzu 17-A chromatograph equipped with DB-1 column (30 m × 0.25 mm *i.d.* 0.25 μm film thickness; J & W Scientific, Folsom, California) coupled to a Shimadzu QP 5000 mass spectrometer, using helium as carrier gas. Injections of 1 μl of splitless tergal cuticular extracts were made at 250 °C, using a temperature program of 60 °C/1 min, 2 °C/min to 240 °C, then 5 °C/min to 280 °C held for 10 min. Electron impact mass spectra (70 eV) were obtained in the *m/z* range of 33–250.

Electroantennographic (EAG) bioassays

H. tenuis antenna was excised (pulled out of the head) by means of a forceps and a few segments were cut off at the base and the tip (Bjostad, 1998). The antenna was then fixed between two stainless steel electrodes by placing the base and tip into droplets of an electrically conductive gel (Spectra 360® electrode gel; Parker, Orange, New Jersey) applied onto the metal electrodes. The specimen with the antennal preparation was placed in such a way that the humidified air directed the compounds eluted from the stimulus delivery over the antenna. The antennal responses were amplified and recorded with a data acquisition controller and software EAG (Syntech, Hilversum, The Netherlands).

The EAG experiments were performed in order to elucidate the selectivity of the antennal receptors of *H. tenuis*. To evaluate the EAG response the compounds were released from Pasteur pipettes containing a piece of filter paper (circa 0.8 cm^2) impregnated with 5 μl of the *H. tenuis* cuticular extract and passed over the antennae. One Pasteur pipette containing a filter paper impregnated with 5 μl of the hexane solvent was used as control. The puff containing the tergal cuticular extract of *H. tenuis* was delivered into a continuously humidified and purified air stream of 1.2 l/min, passing for 0.3 s through the impregnated filter paper in the pipettes. Control stimulation was made at the beginning and at the end of every series of EAG experiments. The *H. tenuis* cuticular

extract was then applied at intervals of 90 s and tested using 10 antennae of *H. tenuis* workers.

Behavioral bioassay

The activity of tergal cuticular extracts in termites was performed by a Y-choice bioassay. *H. tenuis* workers of the last instar were immobilized under low temperature and by means of a forceps, under a stereomicroscope; all the tergites were removed without any remains of fat body tissue or digestive tract. The tergites were extracted for 24 h in 2.0 ml purified hexane and stored at – 18 °C until analysis. The extract was concentrated to about 10^{-2} worker equivalents/μl (WE/μl), which in preliminary bioassays showed high activity in termites. For the control purified hexane was used.

The extract of tergites was assayed using a Y-choice bioassay on filter paper discs (15 cm in diameter). On the Y stem (3 cm) and on one of the Y branch (7 cm), the tergite extract was drawn with an adjustable micropipette (Boeco, Hamburg, Germany) containing 1 μl of extract per 1 cm of trail. Another extract of pure solvent was placed on the other Y branch. One individual was gently deposited from a small perspex vial onto the Y base. The bioassay was repeated 30 times for both workers and soldiers. For an activity test, the distance covered by each individual was observed. The response was considered positive if the individual covered the Y since its base up to tip of each branch. For each test a new individual and a new paper were used to avoid trail deposition.

Statistics analysis

The mean values of the electroantennographic (EAG) responses were calculated automatically by software EAG for Windows. The mean responses of the different compounds were submitted to ANOVA for statistical analysis and compared using the Tukey test ($P < 0.05$). For the choice test, the number of termites that chose one trail was recorded and the data were compared by a Monte-Carlo test (Vaillant and Derridj, 1992), based upon chi-square at level of $P < 0.05$.

Results and Discussion

The hexane extract of tergal cuticles of *H. tenuis* workers was analyzed by GC-MS. Eleven components were identified as linear aliphatic hydrocarbons. They are the main constituents of the cuticular extract, and correspond to a homologous series initiating in eicosane, and finishing in triacontane. The corresponding percentage of each component in the extract (%) is respectively: 0.8, 2.2, 2.5, 3.1, 3.1, 3.3, 3.5, 3.4, 2.5, 1.6 and 1.0.

The identification of these substances was made on the basis of the mass spectra analysis, as well as co-injection of the extract with standards, in this case that commercially available hydrocarbons [triacontane ($C_{30}H_{62}$), pentacosane ($C_{25}H_{52}$) and heneicosane ($C_{21}H_{44}$)], besides confirming its presence, had corroborated to an interpolation that confirmed the existence of other hydrocarbons that complete the homologous series. For this, the Kovats retention index was determined (Adams, 1995).

According to Woodrow *et al.* (2000) and Haverty *et al.* (2000), the cuticular hydrocarbons are divided in two different groups. The first group is comprised of normal alkanes, monomethylalkanes and olefins in the range of 23 to 27 carbons. The other group is comprised of higher molecular weight olefins or methyl branched compounds with carbon numbers in the range of 37 to 45. The *n*-alkanes probably contribute to the largest degree of waterproofing of all cuticular hydrocarbons due to their closely-packed nature. Alkenes with low molecular weight probably are used to moderate lipid viscosity. Because insects cannot synthesize *n*-alkanes longer than 34 carbons (Haverty *et al.*, 2000), long chain alkenes might exert a dual role of regulation viscosity and retaining water at higher temperatures, given the lack of superior, high-molecular-weight alkanes. Page *et al.* (2002) reported that *Heterotermes* sp. specimens produce a continuous 9,17-dimethylalkanes series from C_{27} to C_{30}, and a 9,19-dimethylalkanes series from C_{28} to C_{30}. They also produce 9,21-dimeC_{29} and 9,21-dimeC_{31}. These dimethylalkanes characterized in *Heterotermes* sp. have not been identified in *H. tenuis*. This specie presents the profile of the first group of hydrocarbons, therefore the alkanes are linear and the carbon number varied from 20 to 30, and there are no olefins. The olefins are characteristic of the relative species to the families Kalotermitidade and Termopsidae, but other species of subterranean termites of the Rhinotermitidae often contain a significant fraction of olefins in the cuticular hydrocarbons mixture as *Reticulitermes virginicus*, *R. flavipes* and *R. hageni* (Jenkins *et al.*, 2000). However, in *Coptotermes vastator* and *C. formosanus* species was not evidenced the presence of olefins (Haverty *et al.*,

Table I. Types and relative percentage (%) of cuticular hydrocarbons of three species of Rhinotermitidae termites.

Hydrocarbons	Species		
	C. formosanus[a]	*C. vastator*[a]	*H. tenuis*[b]
Olefins[c]	0	0	0
n-Alkanes[d]	2.5	15.5	95.0
Methyl-branched alkanes[e]	97.0	83.0	0

[a] *Coptotermes formosanus* Shiraki and *Coptotermes vastator* Light, endemic and introduced species in Hawaii, respectively (Haverty *et. al.*, 2000).
[b] *Heterotermes tenuis* endemic species from Brazil.
[c] Olefins: comprising monoenes, dienes and trienes.
[d] *n*-Alkanes: homologue series (C_{20} to C_{30}).
[e] Very diversified mixture of compounds with chain ranged from C_{25} to C_{39}.

2000), corroborating with the results found for *H. tenuis*. *C. formosanus* and *C. vastator* present aliphatic hydrocarbons with different structures of *H. tenuis*, since the linear alkanes are minority while the most abundant are the branched ones (Table I).

The electroantennography (EAG) techniques have been extensively used for moths (Lepidoptera), mosquitoes (Diptera), aphids (Homoptera), beetles (Coleoptera) and other insect orders (Bjostad, 1998), however it is unknown for Isoptera mainly *H. tenuis* species. In this work, we have carried out electroantennographic experiments with *H. tenuis* antennae, and they were effective because the depolarization of the antenna for different stimulation and the response was selective. Then, it was demonstrated that the *H. tenuis* antennae possess chemoreceptors for the tergal cuticular extract (Fig. 1). The mean action potential recorded was 5099.2 mV for tergal cuticular extract of workers and 2597.6 mV and 2288.6 mV for the hexane and air controls, respectively. The mean responses obtained for tergal cuticular extract of *H. tenuis* workers were significantly higher than mean response for controls (Fig. 2).

In the behavioral test of choice the total preference (Monte Carlo test, $p < 0.05$) for cuticular extract in the concentration of bigger activity (10^{-2} tergites/μl) was verified for workers and soldiers (Table II), confirming the results obtained by the electroantennographic study. The high selectivity of the antennae of *H. tenuis* workers to the extract indicates its potential as a chemical messenger.

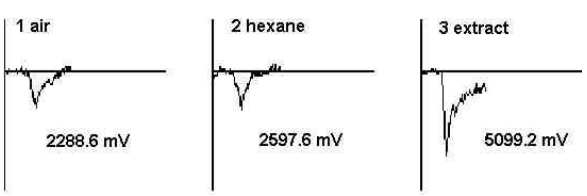

Fig. 1. Eletroantennograms (mV) of *H. tenuis* workers as a response to tergal cuticular extract, hexane and air controls (N = 10 antennae).

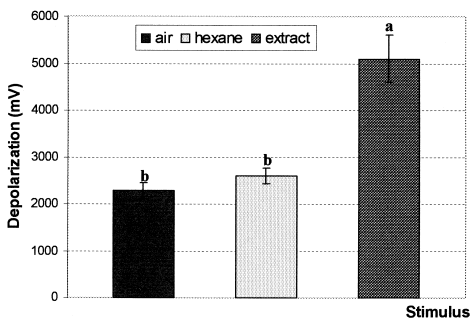

Fig. 2. Electroantennographic (EAG) response (mV) of *H. tenuis* workers to different stimuli: air, hexane, and tergites extract (N = 10 antennae). The stimulus was delivered from impregnated filter paper by using a continuously humidified and purified air stream of 1.2 l/min. Means followed by the same letters are not different by Tukey test ($p \leq 0.05$).

Table II: Choice bioassay for *H. tenuis* between extracts of tergites and control.

Caste	Extract	
	Tergites	Control
Workers[a]	30*	0
Soldiers	30*	0

[a] Last instar worker.
* Significant difference (Monte Carlo test, $p < 0.05$). N = 30 repetitions for each caste.

The composition of the hydrocarbons mixture is genetically controlled (Coyne *et al.* 1994). Cuticular hydrocarbons mixture is species-specific and can provide an alternative method to identify a given taxon without the diagnostic castes, soldiers or imagoes (Haverty *et al.*, 1991). Hydrocarbons might also serve as an important semiochemical cues for caste and species recognition (Howard and Blomquist, 1982). Thus, the results obtained by the electroantennographic analysis indicate that *H. tenuis* workers recognize the cuticular hydrocarbons of its proper species, or even of the proper colony. In accordance with Haverty *et al.* (2000), the difference in composition of cuticular hydrocarbons mixture is also related to the type of habitat of each species. The chemical analysis of the extract demonstrated a profile of termite cuticular hydrocarbons similar to termites that inhabit subterranean and humid places, which is the same for *H. tenuis.*

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