Lethal and Behavioral Effects of Amazonian Plant Extracts on Leaf-Cutting Ant (Hymenoptera: Formicidae) Workers

by

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ABSTRACT

Leaf-cutting ants are economically important pests in neotropical agricultural and forestry ecosystems. The present study aimed to assess the effect of extracts of six amazonian plants (*Banara guianensis*, *Clavija weberbaueri*, *Mayna parvifolia*, *Ryania speciosa*, *Spilanthes oleracea* and *Siparuna amazonica*) on survival of workers of the leaf-cutting ant species *Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans*, and also their effect on the mobility of these species. All of these extracts had some insecticidal effect on the ants and *S. oleracea* extract was the most toxic to all ant species studied. For *A. laevigata* the extract of *M. parvifolia* also was highly toxic. Only the extract of *R. speciosa* had an effect on walking behavior of ants, reducing the total distance moved and the walking velocity.

Keywords: leaf-cutting ants, plant extracts, plant toxicity, walking behavior.

INTRODUCTION

Ants are dominant social insects in most terrestrial ecosystems. True leaf-cutting ants, *Atta* spp. and *Acromyrmex* spp. (Hymenoptera: Formicidae), are dominant herbivores in the Neotropical region. Ants of these genera cause severe damage to agriculture and forests because they cut fresh plants, including flowers, fruits, leaves and twigs to cultivate their symbiont fungus. Moreover, in addition to direct crop damage, their impacts on agriculture include loss of land surface and destruction of farm roads because of their large colonies, leading to accidents involving machinery and livestock (Hölldobler & Wilson 1990, Zanetti *et al.* 2003).

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The main control method used against these pests are insecticides applied in different formulations such as dry powders, granulated baits, thermo-fogging and liquefied gases. Historically, leaf-cutting ants were successfully controlled by the application of synthetic organic insecticides formulated as granulated baits (Boaretto & Forti 1997, Della Lucia & Araújo 2000). Although many insecticides have been tested as baits against leaf cutting ants, the market was dominated by those with dodecachlor. In recent years it was shown that this compound can seriously pollute the environment so it has been banned and the new baits for these pests have either sulfluramid or chlorpirifos (Zanuncio 1999). However, these compounds also may cause damage to the environment, reaching not only target pests but also non-target species including man. Thus, other control strategies with higher specificity and less aggressiveness toward the environment should be investigated (Morini *et al.* 2005).

Among potentially cost-effective and environmental friendly alternatives to synthetic insecticides are the botanical extracts, which can be toxic to workers of leaf-cutting ants, to their fungi, or to both. Plants such as neem (*Azadirachta indica*), sesame (*Sesasum indicum*), castor bean (*Ricinus communis*), common rue (*Ruta graveolens*) jack bean (*Cannavalia ensiformis*), black sage (*Cordia verbenacea*) billygoat weed (*Ageratum conyzoides*), peppermint (*Mentha piperita*) and spotted gum (*Eucalyptus maculata*) have been tested for their control, but their efficiency is relatively low compared to synthetic organic insecticides (Bueno *et al.* 2004; Hebling *et al.* 1996, Takahashi-Del-Bianco 2002, Marinho *et al.* 2006, Ribeiro *et al.* 2008). For this reason, more research should be conducted to test plant extracts and demonstrate possible applications for future use in leaf-cutting ant control.

The singular focus of most toxicological studies is survival/mortality estimates. But there are other factors that warrant closer attention. Insecticides used to control ants as baits must be lethal at low concentrations and have slow action. Furthermore, to be effective in control, the compound must not modify the walking behavior of ants, which could reduce the effectiveness of control by preventing the ants' return to the nest (Boaretto & Forti 1997). Thus, the study of the effect of these products in the behavior of ants is as important as the study of their toxicity.

Therefore, our study had the objective of evaluating the effect of extracts of six amazonian plants (*Banara guianensis*, *Clavija weberbaueri*, *Mayna* *parvifolia*, *Ryania speciosa*, *Spilanthes oleracea* and *Siparuna amazonica*) on survival of workers of the leaf-cutting ant species *Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans*, and also their effect on the walking behavior of these species.

MATERIALS AND METHODS

Insect species and plant material

The bioassays were performed with workers of *A. sexdens rubropilosa*, *A. laevigata* and *A. subterraneus molestans*. The workers were caught in colonies located around the Campus of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais State, Brazil.

The following plants were subject to extraction and bioassays: *Banara guianensis* Aubl., *Clavija weberbaueri* Mez., *Mayna parvifolia* Sleumer, *Ryania speciosa* Vahl., *Spilanthes oleracea* L. and *Siparuna amazonica* Mart. ex A. DC. These plants were chosen based on popular knowledge of natives from the Amazon.

Samples of 500 g of the canopy of each plant species were collected from the Amazon rain forest, state of Acre, Brazil. Each sample was placed in a 1L erlenmeyer flask, with enough hexane to submerge the plant material. The solvent was removed under filtration after 48 hours. The hexane extracts were concentrated under low pressure and reduced temperature (<50°C). The plant extracts were stored at low temperature for subsequent bioassays.

Toxicity bioassays

The stored extracts were diluted with acetone as solvent to a concentration of 5 mg mL^{-1}. The experimental design was completely randomized with five replications. Each experimental unit consisted of a glass petri dish (9.5 cm x 2.0 cm) containing ten insects.

Bioassays were conducted by topical application. For each individual insect, 1.0 µL of the test extract was applied on the thoracic tergite, via a 10 µl Hamilton micro syringe. To achieve this, each ant was immobilized by using a tweezer while they received the solution on their pronotum. In a control experiment, carried out under the same conditions, 1.0 µL of hexane was applied on each insect.

After application, the insects were kept in individual Petri dishes containing honey + water at proportions of 1:1 and pure water. The honey and water were supplied in plastic containers of 1.5 cm diameter and 1.0 cm high. The Petri dishes were placed in an incubator at 25 ± 0.5 °C, 75 ± 5% relative humidity and 12 hours photophase. The mortality counts were made after 1, 3, 6, 12, 24, 48 and 72 hours of treatment. Mortality calculations included dead individuals and those without movements.

Behavioral bioassays

Behavioral bioassays were carried out in glass arenas (3.0 cm high × 15 cm inner diameter) containing filter paper fully sprayed with extracts diluted in hexane (control treatments were sprayed with hexane only).

The inner walls of each arena were covered with Teflon® PTFE (DuPont) to prevent insects from escaping. A single insect was placed in each arena (always at the center of the arena). Twenty arenas (i.e. independent replicates) with individual insects were used for each treatment in the behavioral bioassay, and no insect mortality was observed within the 10 min exposure (trial duration) used for the bioassays.

The movement of each insect within the arena during 10 min was recorded using a Canon® NTSC video camcorder (XL1 3CCD; Canon USA, Lake Success, NY) equipped with a 16x video lens (zoom XL 5.5–88mm) and digitally transferred to a computer for subsequent analysis using the software Studio version 9 (Pinnacle Systems, Mountain View, CA). The movement of the insects was recorded for each arena using the software EthoVision Pro 3.0 (Noldus Information Technology, Sterling, VA). EthoVision detected the insect's position using the subtraction method after applying an erosion and dilation filter.

Average movement parameters were calculated for the treatments to determine differences in ant response to extract-sprayed surfaces. The parameters calculated were total distance moved (cm) and velocity (cm s^{-1}).

Data analysis

Mortality data and the results of behavioral bioassays were submitted to analysis of variance and their averages were compared using the Scott-Knott test at $p<0.05$ (Scott & Knott 1974). Regression analyses were also used to determine time-mortality curves for the concentrations and extracts used.

The confidence intervals were calculated at 95% of probability to verify the differences among the curves over the time.

RESULTS AND DISCUSSION

Insecticide toxicity: time–mortality responses

The plant extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, *R. speciosa*, *S. oleracea* and *S. amazonica* had an insecticidal effect against *A. sexdens rubropilosa*, *A. laevigata* and *A. subterraneus molestans* workers, since significantly higher mortality of treated ants occurred when compared to that of control ants (Fig. 1).

Among the plant extracts tested, the extract of *S. oleracea* at the concentration of 5 mg mL^{-1} exhibited the highest toxicity to all leaf-cutter species, causing 100% mortality at 70, 56 and 60 hours after application on *A. laevigata*, *A. sexdens rubropilosa* and *A. subterraneus molestans* workers, respectively (Fig. 1, Table 1). Ribeiro e*t al.* (2008), evaluating the toxic effect of four hexane extracts (*Ruta graveolens, Cordia verbenaceae, Mentha piperita* and *Ageratum conyzoides*) when applied on workers of *A. sexdens rubropilosa* and *A. subterraneus molestans* obtained lower mortalities (> 40%) at the same concentration and with similar methodology. In this study, mortality reached 100% only for concentrations of 50 and 100 mg mL^{-1}, depending on the plant extract. This indicates that extract of *S. aleracea* had significantly higher mortality and can be further used to control leaf-cutting ants.

For *A. sexdens rubropilosa* only *S. oleracea* extract showed a considerable insecticidal effect. The other extracts showed significantly higher mortality than control, but mortality caused by them did not reach 50%. The mortality caused by *C. weberbaueri* and *M. parvifolia* was the same and both differed from the control immediately after application (Fig 1, Table 1).

As far as *A. laevigata* is concerned we can say that in addition to the extract of *S. oleracea*, the extract of *M. parvifolia* also showed high insecticidal effects. For this extract, the mortality reached 82% 72 hours after treatment. The extracts of *B. guianensis*, *C. weberbaueri* and *R. speciosa* led to the same mortality in *A. laevigata* (68% at 72 hours after application) and differed from control immediately after application. The mortality caused by *S. amazonica* was 58% at 72 hours after application. Unlike the other extracts, *S. amazônica* extract caused worker mortality in a short amount of time (47% mortality at

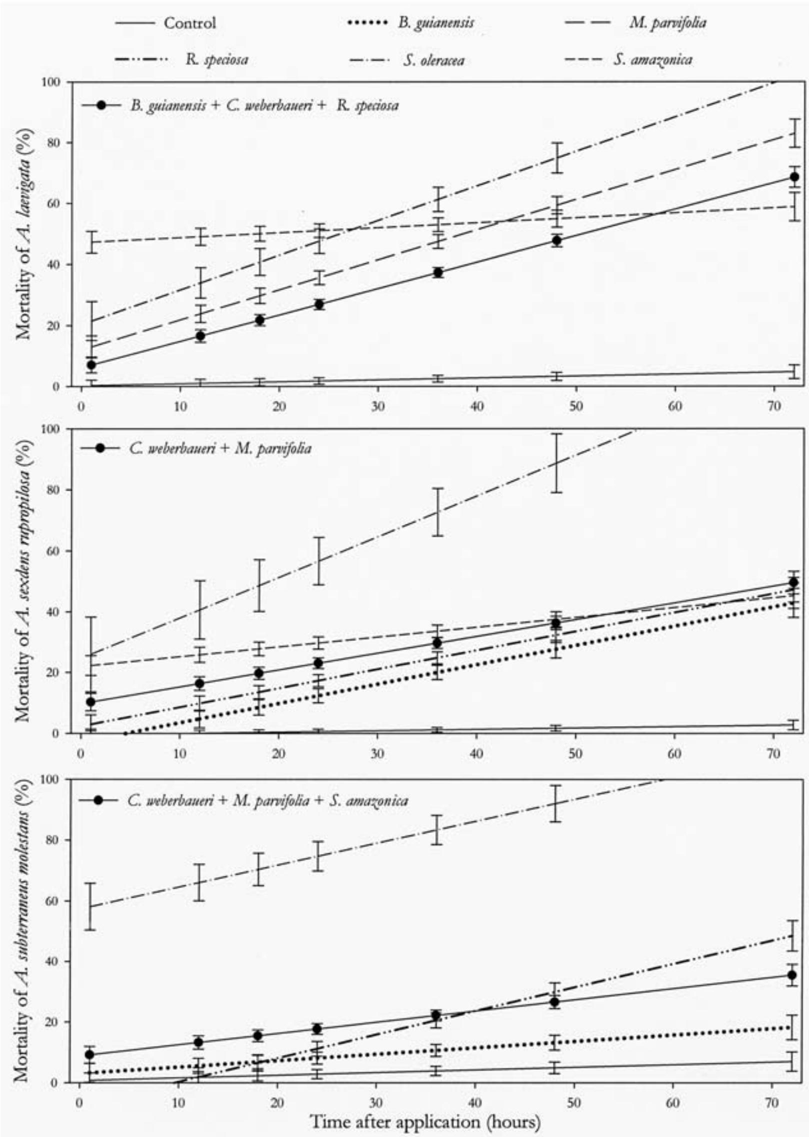

Fig. 1. Mortality (%) of workers of *Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans* caused by extracts of six plant species and control, applied topically at the concentration of 5 mg mL^{-1}. Vertical bars indicate confidence interval (CI) at 95%. Regressions represented by a continuous line with black circles indicate that the curves do not differ among themselves at a 95% CI and were therefore grouped together. Information related to the regressions is found in Table 1.

Table 1. Data of regression analysis for plant extracts applied topically on workers of *Atta laevigata*, *Atta sexdens rubropilosa* and *Acromyrmex subterraneus molestans*.

Extracts	Regression equations*	R^2	F-values	Probability
Atta sexdens rupropilosa				
B. guianensis	y=-2.88+0.64x	0.83	$F_{1,34}$=164.77	<0.0001
C. weberbaueri				
	y=9.75+0.55x	0.75	$F_{1,69}$=201.50	<0.0001
M. parvifolia				
R. speciosa	y=2.35+0.62x	0.87	$F_{1,34}$=219.98	<0.0001
S. amazonica	y=21.94+0.32x	0.69	$F_{1,34}$=55.92	<0.0001
S. oleracea	y=24.65+1.33x	0.66	$F_{1,34}$=65.23	<0.0001
Control	y=-0.44+0.04x	0.12	$F_{1,55}$=7.24	0.01
Atta laevigata				
B. guianensis				
R. speciosa	y=6.24+0.86x	0.85	$F_{1,104}$=572.35	<0.0001
C. weberbaueri				
M. parvifolia	y=12.08+0.98x	0.93	$F_{1,34}$=414.81	<0.0001
S. amazonica	y=47.15+0.16x	0.26	$F_{1,34}$=11.30	0.002
S. oleracea	y=20.39+1.13x	0.84	$F_{1,34}$=176.59	<0.0001
Control	y=0.27+0.06x	0.11	$F_{1,55}$=6.91	0.01
Acromyrmex subterraneus molestans				
B. guianensis	y=3.05+0.21x	0.43	$F_{1,34}$=25.39	<0.0001
C. weberbaueri				
M. parvifolia	y=8.83+0.37x	0.48	$F_{1,104}$=93.66	<0.0001
S. amazonica				
R. speciosa	y=-7.43+0.78x	0.87	$F_{1,34}$=221.15	<0.0001
S. oleracea	y=57.41+0.72x	0.60	$F_{1,34}$=49.10	<0.0001
Control	y=0.75+0.09x	0.12	$F_{1,55}$=7.01	0.01

*Mortality curves that did not differ among themselves by the 95% CI were combined into a single curve.

1 hour after application). The other extracts caused less than 20% mortality at 1 hour after treatment (Fig 1, Table 1).

For *A. subterraneus molestans*, the fastest response was achieved with *S. oleracea* extract, which caused about 60% mortality right after application and 100% mortality 59 hours after application. This was followed by extract of *R. speciosa*, which showed 50% mortality 72 hours after application. The extracts of *C. weberbaueri*, *M. parvifolia* and *S. amazonica* did not differ among themselves and mortality caused by them was less than 40%. *B. guianensis* extract was the least potent (mortality > 20%) against *A. subterraneus molestans* (Fig. 1, Table 1).

Extracts that cause worker mortality in a short amount of time, such as that of *S. amazonica* to *A. laevigata* and *S. oleracea* to *A. subterraneus molestans* are more appropriate for ant control methods that use direct application, such as thermo fogging. On the other hand, if the purpose is to utilize these extracts for bait manufacture, the ideal extract is one that slowly promotes ant death. In this way, worker ants live long enough to take the baits back to the nest and feed it to the colony and queen (Della Lucia & Araújo 2000).

The chemical compounds responsible for the insecticidal effect in the extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, and *S. amazonica* have not been chemically isolated yet but are liposoluble substances, since they were extracted with hexane, a non-polar solvent.

The powdered stemwood of *R. speciosa* is a botanical insecticide known as Ryania (Kuna & Heal 1948). The stem extract contains several structurally related ryanoids, including: ryanodine, 10-(O-methyl)-ryanodine, 9,21-dehydroryanodine, and ryanodol. The most toxic and abundant compounds in the extract are ryanodine and 9,21-dehydroryanodine, and thus, they account for virtually all of the insecticidal activity (Rogers *et al.* 1948). Ryania is highly toxic to the fruit moth (*Grapholita molesta*), coddling moth (*Cydia pomonella*), corn earworm (*Heliothis zea*), European corn borer (*Ostrinia nubilalis*) and citrus thrips (*Scirtothrips citri*), but its efficiency in controlling ants had not yet been investigated (Jefferies 1992, Leslie 1989, Kamrin 1997). In our study, the extract of *R. speciosa* showed moderate toxicity at a concentration of 5 mg mL-1, but the effect in higher concentrations should be further analyzed in prospective studies.

For *S. oleracea* there are several studies of isolation and identification of bioactive compounds. Spilanthol, considered to be one of the most active constituents, was obtained from *S. oleracea* in 1903 (Gerber 1903). This compound is the main constituent of *S. oleracea*, which has been administered as a traditional folk medicine for years to cure toothaches, stammering, and stomatitis. Previous studies have demonstrated its diuretic, antibacterial, and anti-inflammatory activities (Wu et al. 2008), in addition to its insecticidal activity (Phrutivorapongkul 2008). Since the isolation of spilanthol, a number of other *N*-isobutylamides such as 2*E*-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, 2*E*,7*Z*-*N*-isobutyl-2,7-tri-decadiene-10,12-diynamide and 7*Z*-*N*-isobutyl-7-tride-cene-10,12-diynamide, undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acid isobutylamide. have been reported (Nakatani & Nagashima 1992, Ramsewak *et al.* 1999, Saraf & Dixit, 2002). Nevertheless, there are few reports concerning the investigation of insecticidal activity of these compounds and most studies are related to the control of insect vectors of disease (Saraf & Dixit 2002, Phrutivorapongkul 2008).

Our results indicate that *S. oleracea* extract is most promising among the plant extracts studied and should be further investigated as an alternative for control of leaf-cutting ants. The extract of *M. parvifolia* can also be an alternative to control *A. laevigata*, as it induced mortality greater than 80% in this species.

We observed that *A. laevigata* was the most susceptible insect to the plant extracts, suggesting that different leaf-cutting ant species are differently affected by exposure to the same plant extract. Results found by Ribeiro *et al.* (2008) with *A. laevigata* and *A. subterraneus subterraneus*, using *A. conyzoides*, *M. piperita*, *C. verbenaceae* and *R. graveolens* extracts also showed that distinct species of leaf-cutting ants responded differently to plant extracts. Therefore it becomes very important to determine which extract is the most effective against each ant species as well as the optimum concentration to be utilized. Thus, the plant extracts that showed low toxicity at the concentration used in this bioassay should be studied at higher concentrations.

Walking behavior

Tracks representative of the typical walking behavior of ants species on arenas sprayed with extracts are shown in Fig. 2.

The walking behavior of all three ant species on arenas sprayed with extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, *S. oleracea* and *Siparuna amazonica* was similar to the control. However, the extract of *R. speciosa* reduced the distance moved and velocity of the three species (Fig. 3).

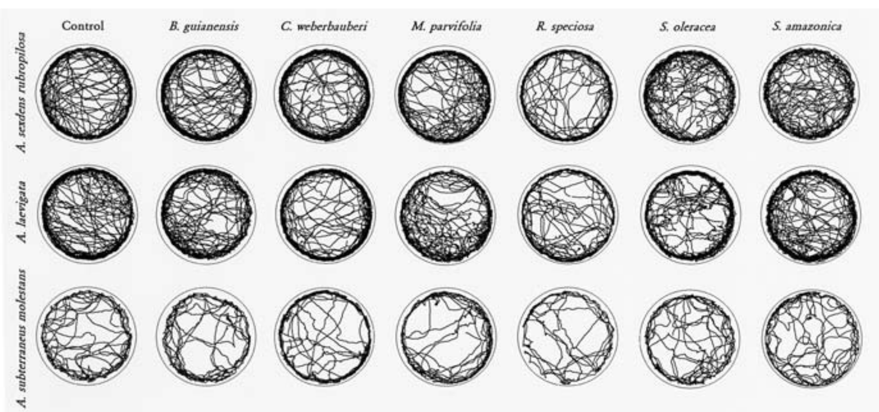

Fig. 2. Representative tracks showing the movement of individual ants from three species of leaf-cutting ants (*Atta sexdens rubropilosa*, *Atta laevigata*, and *Acromyrmex subterraneus molestans*) over a 10min period on arenas fully sprayed with solvent or plant extracts.

The reduced mobility observed with *R. speciosa* extract may result from neurotoxic activity of Ryanodine, the main chemical compound responsible for the Ryania insecticidal effect. Ryanodine induces paralysis in insects by

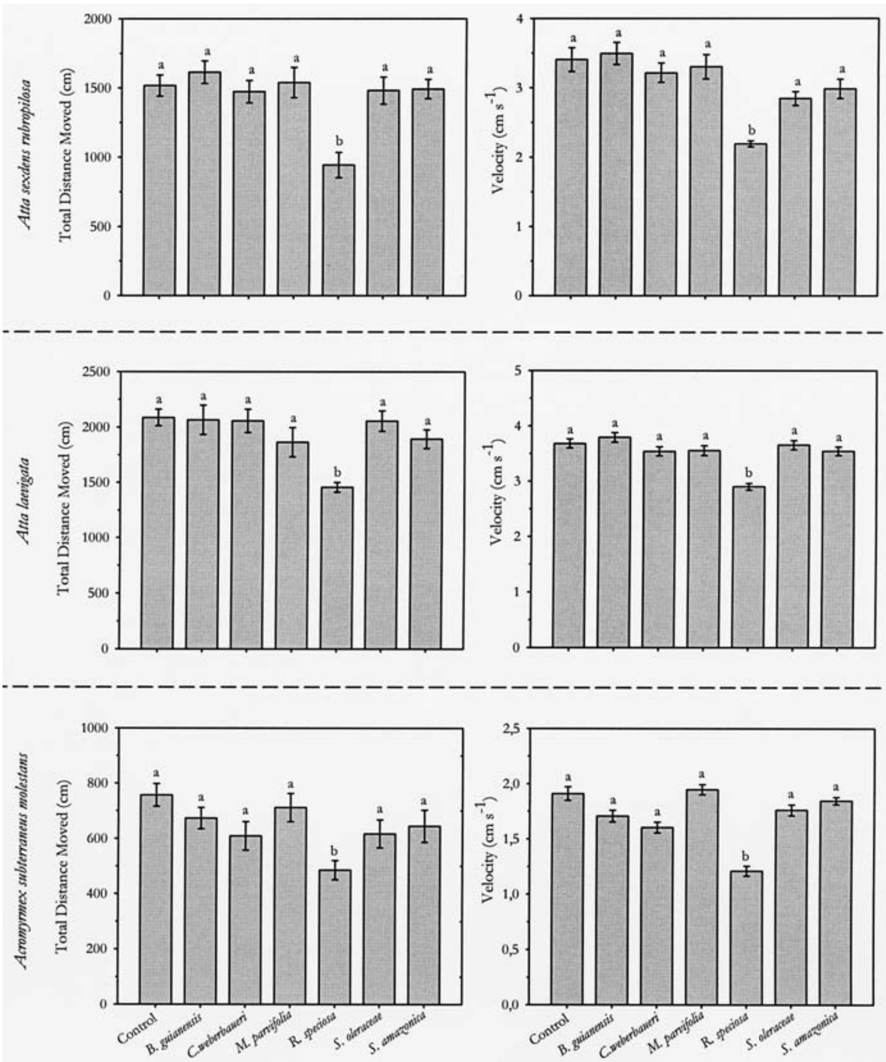

Fig. 3. Total distance moved and velocity (± standard error) by three species of leaf-cutting ants (*Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans*) exposed to arenas fully sprayed with either solvent or plant extracts over a 10min period. Histogram bars with the same letter do not significantly differ by the Scott-Knott test ($P < 0.05$).

causing a sustained contracture of skeletal muscle without depolarizing the muscle membrane. A number of studies have confirmed that ryanodine can irreversibly activate the calcium release channel in the sarcoplasmic reticulum. The irreversible activation of this calcium channel floods the muscle fibers with calcium, inducing the sustained contraction of skeletal muscle and paralysis observed in ryanodine poisoning (Bloomquist 1996).

The sublethal behavioral effects of insecticides are also relevant for leaf-cutting ant management because the species are expected to remain exposed to sublethal concentrations of these compounds during loading of the bait or as a consequence of insecticide degradation. These behavioral effects may modify the foraging activity of workers and so they may not take the baits back to the nest (Boaretto & Forti 1997). Therefore, insecticides that modify the workers' behavior are not indicated for use as bait and should be used in a different formulation. The results of the present study indicate that the extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, *S. oleracea* and *Siparuna amazonica* did not present behavioral effects to the ants. Therefore, the application of these extracts for leaf-cutting ants control in the form of baits may be feasible.

In summary, *S. oleracea* extract in the concentration of 5 mg mL^{-1} was efficient against all ant species, and the extract of *M. parvifolia* was effective only for *A. laevigata*. The extract of *R. speciosa* was the only one that affected the walking behavior of ants, and may not be efficient for use in baits.

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