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Can the extract of *Aristolochia argentina* Griseb affect the foraging decisions of the leaf cutting ant *Acromyrmex lundi* (Guérin)? Preliminary assays

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ABSTRACT
The aim of this study was to evaluate whether foraging decisions of *Acromyrmex lundi* in the field are affected by the extract of *Aristolochia argentina*. A free choice test was performed using *Rosa x hybrida* leaves treated with the extract and control with acetone. Two leaves (treated and control) were presented on either side of a foraging trail. The percentage of removed material was recorded for 90 minutes of observation on two consecutive days. Dry weight and repellency index were calculated. Ant activity, the percentage of carried leaves and weight data were analyzed using a "*t*"-test for paired data, and a General Linear Mixed Model test was used to evaluate the different variables and their interactions. *A. argentina* extract (1%) did not affect the foraging activity of *A. lundi*, whereas the 5% dose caused significant differences in foraging activity as well as between the factors and the interactions. A repellency index of above 95% was obtained at the 5% dose. The extract of *A. argentina* could be considered for future management of this insect.

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Acromyrmex lundi; repellent activity; *Aristolochia argentina*; botanical extracts; field assays

1. Introduction

Leaf-cutting or foraging ants of the genera *Atta* and *Acromyrmex* (Formicidae: Attini) (Ward et al. 2014) is considered the most common polyphagous herbivores in the neotropics (Rico-Gray & Oliveira 2007; Cristiano et al. 2013), causing damage in different systems: agriculture, forestry, gardens and organic orchards (Giménez 2006; Fernandes et al. 2007; Zanetti et al. 2014). The harvested plant material is transferred to the nest and used as substrate for culturing a Basidiomycete fungus of the tribe Leucocoprineae; this fungus, with which ants have developed a symbiotic relationship, provides their main food source (Aubad López 2010). In general, these herbivores prefer to feed on plants containing high nutrient concentrations and low amounts of secondary metabolites that are harmful to them or the fungus (Saverschek et al. 2010; Silva & Vasconcelos 2011). Foraging decisions on the suitability of substrates are very complex, since leaf-cutting ants do not harvest for themselves, but for their symbiotic fungus, whereas adult workers obtain more than 90% of their energy requirements from the sap of the harvested plants (Saverschek et al. 2010).

Numerous studies suggest that ants can not only recognize leaves of different plant species, but also evaluate leaf quality at the foraging site and determine whether this characteristic affects the fungal growth (Saverschek et al. 2010; Saverschek & Roces 2011). Leaf-cutting ants can alter their behavior by rejecting unsuitable substrates; such rejection depends on several factors, such as type and quantity of the material carried, ant species, and size and status of the colony (Herz et al. 2008).

Research efforts have focused on insect management strategies involving options that are environmentally less aggressive than synthetic insecticides, such as botanical extracts. So far, more than 2000 plant species with insecticidal properties affecting the development and behavior of this group of arthropods have been identified (Montesino Valdés et al. 2009; Wondafrash et al. 2012). Thus, Gruber and Valdix (2003) used extracts of *Gliricidia sepium* (Jacq.) (Fabaceae) and *Azadirachta indica* A. Juss (Meliaceae) to manage *Atta* spp. in the field. The extract of this Fabaceae only irritated ants; instead, *A. indica* drastically reduced ant population density. On the other hand, extracts of *Ziziphus mistol* Griseb (Rhamnaceae) and *Hymenaea courbaril* L. (Leguminosae, Caesalpinioideae) significantly repelled the workers of *Acromyrmex striatus* Roger (Pelotto & Del Pero de Martinez 2002) and *Atta cephalotes* (L.) (Hubbell et al. 1983).

Among the different plant families studied so far, some species of the genus *Aristolochia* (Aristolochiaceae) have been reported for their insecticidal and repellent activity (Wu et al. 2004). In choice tests, extracts of *Aristolochia pilosa* were avoided by adult Coleoptera and Hemiptera, but not by lepidopteran larvae, which ingested similar amounts of both

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substrates (Sáenz Bocanegra 2010). Furthermore, extracts of different species of *Aristolochia* produced adverse effects on various insect species (Jbilou et al. 2006; Das et al. 2007; Kamaraj et al. 2010; Baskar et al. 2011). Laboratory tests demonstrated the toxic effects of *A. argentina* extract on adults of *Sitophilus oryzae* (Coleoptera) (Broussalis et al. 1999) and a strong repellent effect on the leaf-cutting ant *Acromyrmex lundi* (Díaz Napal et al. 2015).

According to Bucher (1987), *A. lundi* (Guérin) is one of the most important herbivorous species in the Chaco phytogeographic region of Argentina. These insects can cut various plant parts (leaves, flowers and fruits) and attack almost any crop species (Giménez 2006). In the neotropical region, they produce economic losses on several crops, such as sunflower (Saluso & Xavier 2010), sorghum (Dans et al. 2009), poplar, eucalyptus and pine plantations (Caffarini et al. 2002; Achinelli et al. 2006; Nickele et al. 2012). The injury these insects can inflict is intensified in monospecific systems, where the lack of alternative foods forces ants, facilitated by their great adaptability, to concentrate their attacks on the only implanted species, generating substantial losses (Silva Araújo et al. 2003; Cantarelli et al. 2005).The aim of this study was to evaluate whether the foraging decisions of *A. lundi* in the field are affected by the extract of *Aristolochia argentina*.

2. Materials and methods

2.1. Extract preparation

Aerial parts of *A. argentina* were collected from Sierras Chicas hills in Córdoba province, Argentina (31°41′30.84″S, 64°28′17.05″W). The voucher specimen was deposited in the "Marcelino Sayago" Herbarium of the School of Agricultural Science, Catholic University of Córdoba (UCCOR 191) and was authenticated by the botanist Gustavo Ruiz. Plant material was air-dried at room temperature and crushed, followed by extraction by 48-h maceration with ethanol. The solvent was vacuum evaporated to obtain a syrupy extract (Palacios et al. 2010). Extract solutions at 1% or 5% were prepared by dissolving 100 or 500 mg of extract in 10 mL of acetone, respectively.

2.2. Field evaluation

The *A. lundi* nests selected had active foraging trails and were separated from each other by at least 100 m. The foraging activity of each nest was determined by counting the number of laden workers that walked toward the nest entrance for 3 min (Farji-Brener 1993). Ants of the selected nests were exposed to rose leaves (*Rosa* x *hybrid*) for 10 days to allow them to habituate to this substrate, given its absence in the study site. For the choice test, two rose leaves were placed at each side of the trail; one leaf was treated with extract solution and the other with acetone (control). The extract concentrations used were 1% (100 μg/cm^2) and 5% (500 μg/cm^2), with six and five replications (nests), respectively. Both leaves (treated and control) were placed approximately 50 cm from the nest entrance (Saverschek et al. 2010); the experiment ended after 90 min (Matthews 1997). Moreover, environmental variables (temperature and humidity) were recorded during the assays. This bioassay was performed for each nest on two consecutive days (Jofré & Medina 2012). The percentage of substrate that was not picked up during the test was recorded every 15 min (Caffarini et al. 2006). The remaining leaves were dried (48–60 h at 60 °C) to determine the dry weight (Medina et al. 2012). The repellency index was calculated as (RI) = $[1 - (T/C)] \times 100$, where "T" and "C" are the percentage of treated and control leaves carried into the nest, respectively (Schmidt et al. 1997).

2.3. Data analysis

Ant foraging activity, the percentage of leaves carried and dry weight data were compared between the first and second days using a Student's *t*-test for paired samples. The relationship between ant activity and environmental variables was analyzed using linear regression. A Generalized Linear Mixed Model (GLMM) was performed to test the effect of the studied factors, including the factor "treatment" with four levels: control (1% and 5%) and treated (1% and 5%); "time" with six levels (observations made every 15 min during 90 min for each bioassay) and "day", and interactions days × treatments, days × time, treatments × time, and days × treatments × time. The Tukey test for multiple comparisons was used to determine differences between means (Di Rienzo et al. 2011).

3. Results

Ant foraging activity of each nest before the start of the bioassays was markedly reduced on the second day of observation for both doses evaluated (Figure 1), with a reduction between the first and second days being 2.4-fold for 1% dose (t = 3.59; p = 0.016) and 1.8 for 5% (T = 4.35; p = 0.012).

Ant foraging activity on the first day was significantly and positively correlated with temperature (R^2 = 0.4; p = 0.036) and humidity (R^2 = 0.44; p = 0.026) (Figure 2).

However, on the second day of observation, there was no relationship between ant foraging activity and the environmental variables (humidity: R^2 = 0.01; p = 0.823; temperature: R^2 = 0.13; p = 0.269).

The dry weight of the remaining leaves was compared after the end of the choice tests, with no significant differences being detected between the 1% dose

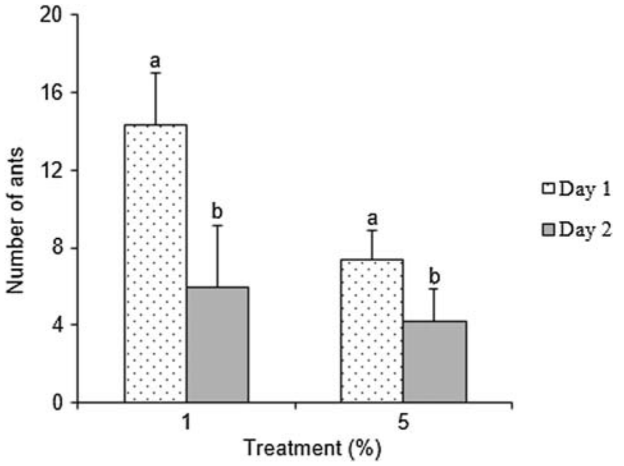

Figure 1. Harvesting activity of *A. lundi* on two consecutive observation days. Bar graphs with the same letters between days are not significantly different ($P = 0.05$).

treatment and days (Day 1: $T = -0.42$, $p = 0.689$; Day 2: $T = 0.79$, $p = 0.465$). Significant differences in weight for both test days were recorded at the 5% concentration (Day 1: $T = -5.5$, $p = 0.005$; Day 2: $T = -6.3$, $p = 0.003$) (Figure 3).

The 1% concentration had no significant effect on the quantity of material carried during the assay, whereas the 5% concentration showed a marked repellent effect, on both experimental days (Table 1).

The analysis of each of the factors included in GLMM showed significant differences between "treatments" ($p < 0.01$), "time" ($p < 0.01$) and "days" of assays ($p < 0.01$). The results of the interactions between the factors "treatments × time" and "days × treatments" showed significant differences in the substrate that was not picked up by ants ($p \leq 0.05$) (Table 2).

The analysis of the interactions between the factors "days × time" and "days × treatment × time" showed no significant results (Table 2). The Tukey test showed

Table 1. Choice test involving *A. lundi* workers exposed to *Rosa × hybrid* leaves treated with two doses of *A. argentina* extract on two consecutive days.

		Leaf area removed (%)				
Day	Dose	Treatment	Control	RI (%)[b]	*t*	*p*
1	1%	29 (16.61)[a]	33 (15.30)	13	0.15	0.88
	5%	4 (4.00)	70.4 (8.16)[c]	95	8.33	0.001
2	1%	0	15 (15.00)	100	1.00	0.37
	5%	0	64 (4.89)[c]	100	13.09	0.0001

[a]Values are mean (±SEM) of the removed area.
[b]Repellency index (RI) = [1 − (*T*/*C*)] × 100 (see Methods).
[c]Significant differences between treated and control leaves, *t*-test for paired comparison.

Table 2. Analysis of the effects of different factors and their combinations on the foraging activity of *A. lundi* using a GLMM.

Factors	Numerator df	Denominator df	*F*	*p*
Treatments	3	192	57.207	0.001
Time	5	192	11.147	0.001
Days	1	192	30.011	0.001
Days × treatments	3	192	2.574	0.055
Days × time	5	192	0.536	0.749
Treatments × time	15	192	3.226	0.001
Days × treatments × time	15	192	0.621	0.855

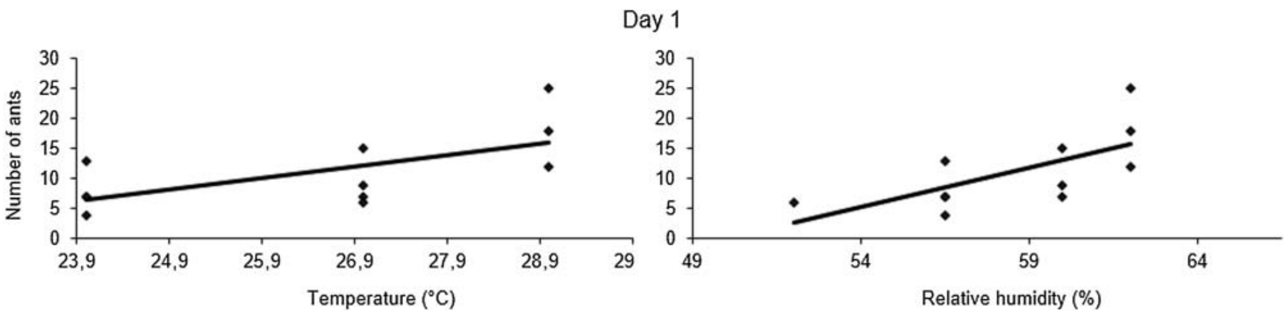

Figure 2. Environmental variables, temperature (A) and relative humidity (B), recorded on the first day of the field trial.

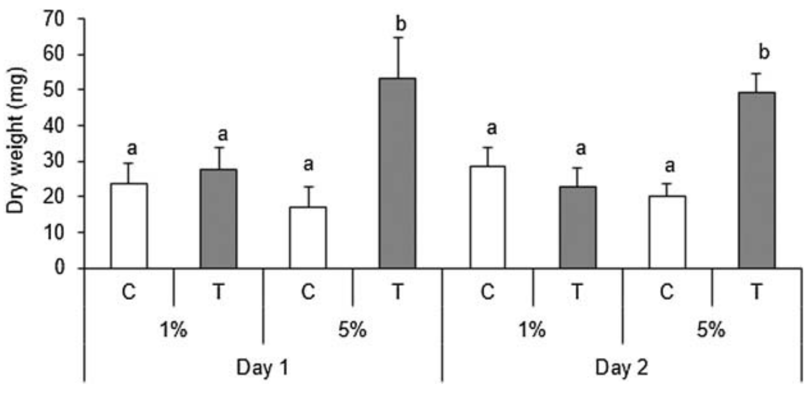

Figure 3. Dry weight of the substrate remaining after two consecutive test days. Bar graphs with the same letters between treatment and control are not significantly different ($P = 0.05$).

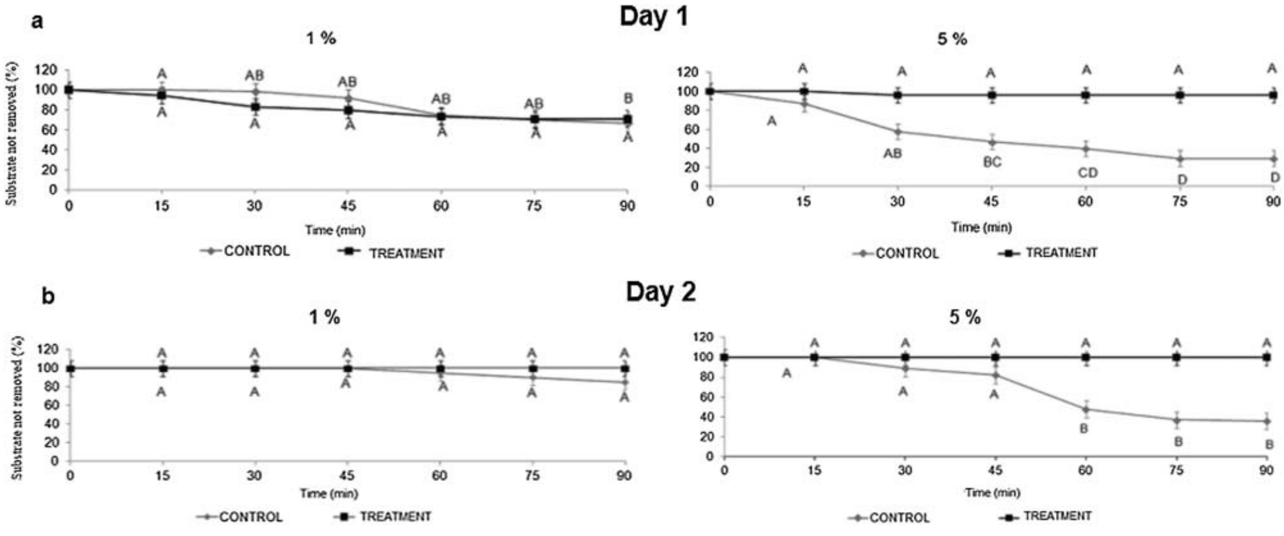

Figure 4. Substrate (%) not removed by *A. lundi* workers during 90 min of observation on two consecutive days: 1 (a) and 2 (b). Substrate was treated with two extract doses (1% and 5%). Different letters between treatment and control, within each graph, indicate significant differences (Tukey test: $P = 0.05$).

differences between the control and 5% treatment, given that only control leaves were carried on both days of the bioassays (Figure 4(a,b)). Leaves treated with 1% concentration and control leaves were equally carried to the nests, with no differences between treatments (Figure 4(a,b)).

4. Discussion

The results of this study indicate that some behavioral aspects associated with foraging decisions on the material carried by *A. lundi* were affected by the presence of *A. argentina* extract.

In the field, the number of laden ants entering the nest at a fixed time is used as an indicator of the foraging activity pattern (Farji-Brener 1993). We noted a marked reduction in foraging activity on the second day of observation with respect to the activity before the start of the assays. The observed changes could be caused by many factors, among them the incorporation of a resource that is unsuitable for the colony. Forti et al. (2000) and Escobar et al. (2002) proposed two possible pathways for ant rejection: either the ants decided not to introduce a new resource to the nest after ingesting the sap emanated from the harvested material, or they found that the incorporation of the material into the nest affected fungus development. In the present work, the available information does not allow us to explain the effects of the extract through either of these possibilities.

Foraging activity in cutter ants from temperate zones is closely linked to environmental variables (Hölldobler & Wilson 1990; Farji-Brener 2000; Guerrero & Sarmiento 2010; van Gils & Vanderwoude 2012). Our findings indicate that *A. lundi* foraging activity was significantly and positively related to temperature and humidity on the first day of assay.

During the choice test, workers had the possibility of choosing between substrates treated either with extract or solvent (control). When offered *Rosa* x hybrid leaves treated with the lower dose, ants carried similar amounts of both substrates. Similar responses were obtained by Pelotto and Del Pero de Martinez (2002) and Medina et al. (2012), who reported a lack of preference of *A. striatus* and *Acromyrmex lobicornis* for any of the food items offered in field tests.

Only 4% of the *Rosa* leaves treated with 5% *A. argentina* extract and presented on the foraging trails were carried to the nest 30 min after the start of the bioassay. This behavior was more marked on the second day of observation, when the substrate treated with the extract was avoided by all colonies. In contrast, the control was carried on both days all throughout the bioassays, transferring 70% and 64% on the first and second days, respectively. *A. lundi* workers probably detected the *A. argentina* extract in 5% treated leaves and considered them unpalatable. Our results in this study agree with findings reported by Hertz et al. (2008) and Saverschek and Roces (2011), who suggest that this behavior is effective in that ants avoid noxious plants and do not carry unsuitable substrates to the colony.

The repellency index shows that the *A. lundi* workers avoided treated leaves at the highest dose of *A. Argentina* extract, within 24 and 48 h of exposure. Herz et al. (2008) reported that *A. lundi* workers subjected to choice tests detected and rejected the presence of fungicide in their food 24 h after its application.

Most experiments studying ants are performed in the laboratory because there are multiple factors affecting foraging behavior (Medina et al. 2012). Once the activity of an extract is tested under artificial conditions, it is important to confirm the effectiveness in the field, where the behavior of both insects and compound will become apparent. It is known that an artificial environment may not only alter ant foraging activity but also the effectiveness of the extract (Caffarini et al. 2006).

Several works reported that toxicity of botanical extracts affecting both fungus and workers may not necessarily be caused by the same active compound (Bigi et al. 2004; Morini et al. 2005). Under laboratory conditions, Díaz Napal et al. (2015) found that argentilactone, the active principle of *A. argentina*, has repellent and antifungal compounds affecting the basidiomycete cultured by *A. lundi*. Future studies should be performed to test if this compound alone is the one responsible for the repellent effect found here.

Thus our work has shown that the crude extract of *A. argentina* affects foraging decisions of *A. lundi*. The fact that our results were obtained under field conditions must be remarked, as this represents an important step towards finding efficient control methods for leaf-cutter ants. Research in natural conditions, with all their implicit complexity, needs to be carried out before active compounds like argentilactone and others being currently studied (Dos Santos et al. 2013) can be available to farmers for the management of *A. lundi*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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