



RESEARCH ARTICLE - ANTS

Interspecific Deterrent Effect of Hydroalcoholic Solution of Nest Refuse from Two Leaf-cutter Ant Species

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Article History

Edited by

Evandro Nascimento Silva, UEFS, Brazil

Received 19 October 2023

Initial acceptance 04 July 2023


Final acceptance 22 September 2023

Publication date 28 September 2023

Keywords

Atta opaciceps, *Acromyrmex balzani*, insect control, deterrence, refuse dumps, contamination.

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Abstract

Leaf-cutting ants manage their waste continuously to avoid risks of colony contamination. The presence of residues or their odor near foraging areas is enough for ants to stop attacking plant tissues. However, to date, no study has demonstrated an interspecific effect of nest waste on the deterrence of leaf-cutting ants. Here, we tested whether a hydroalcoholic extract of nest refuse (NR) of the leaf-cutting ants *Acromyrmex balzani* Emery, 1890 and *Atta opaciceps* Borgmeier (Hymenoptera: Formicidae, Attini) causes an interspecific deterrent effect. Field colonies of *Ac. balzani* (n = 30) and *A. opaciceps* (n = 36) were used. For each colony, we offered baits made with plastic straws covered by citrus pulp and sprayed with a hydroalcoholic solution of 20% (vol/vol) alcohol/water (Control) or an extract of 20% (vol/vol) of NR from *Ac. balzani* and/or *A. opaciceps*. Over 20-30 minutes we recorded the amount of baits removed and data were compared through survival analysis and Linear Mixed Effect (LME) model. Ants avoided collecting baits sprayed with NR resulting in less than 20% of these baits being carried to the nest *versus* 80% of control baits. The deterrence was both intra and interspecific, considering that the response was similar for baits from both species. Such results reinforce the premise that the presence of nest refuse alters the foraging behavior of ants and paves the way for new studies that can verify its effectiveness in reducing foliar consumption on a larger spatial scale.

Introduction

Leaf-cutting ants (LCA) (Hymenoptera: Formicidae, Attini) (genera *Atta* and *Acromyrmex*) cause significant economic damage and are, therefore, considered an important agricultural pest in their areas of occurrence (Holldöbler & Wilson, 1990; Urbas et al., 2007). Several methods for the management of LCA have been used, such as insecticide application, fungicides, and the use of entomopathogenic fungi. However, some of these methods have high costs and adverse effects on the environment and human health (Montoya-Lerma et al., 2012; Della Lucia et al., 2013; Zanetti et al., 2014).

An alternative and less environmentally toxic method is the use of substances with a deterrent effect. These substances

act on the insect's behavior and prevent or inhibit foraging and are generally associated with secondary metabolites produced by plants (Dethier et al., 1960; Lara, 1991).

LCA efficiently manage their waste (here referred to as nest refuse, NR) produced in the colony, since such behavior guarantees the health of individuals and the integrity of their symbiotic fungus (Bot et al., 2001). Previous studies showed that ants avoid cutting tissues from plants that are in contact with dry NR mounds (Zeh et al., 1999; Farji-Brener & Sasal, 2003; Ballari & Farji-Brener, 2006) and, more recently, Sousa-Souto et al (2022) observed that the liquid NR extract also proved to be deterrent against ant attack to the plants. Thus, odors associated with NR may be the main mechanism of deterrent action.



LCA cultivates the same species of symbiont fungus, *Leucoagaricus gongylophorus* (Pagnocca et al., 2011), and maintains similar habits of hygiene, and communication between nestmates. Likewise, the NR produced by different species of these two genera probably has similar chemical characteristics. Given these similarities, it is likely that the deterrent effect of the NR extract is interspecific.

In the present study, we verified the interspecific deterrent effect of NR in liquid formulation (hydroalcoholic extract) on leaf-cutting ants of the species *Acromyrmex balzani* Emery, 1890 and *Atta opaciceps* Borgmeier. For this, the study started from the following hypotheses i) since NR is toxic and avoided by leaf-cutting ants, even attractive baits prepared with this substrate can also be avoided; ii) if substrate-treated baits have an intraspecific deterrent effect, there is a high probability that they also have an interspecific effect.

Material and Methods

Study site and LCA species used

The study was carried out on the campus of the Federal University of Sergipe in São Cristóvão (10°55'33.55 "S 37°6'8.327" W) and the chosen species were the most common and have the highest density in the area.

The species *Acromyrmex balzani* has a wide distribution in disturbed environments, in addition to a high density of colonies in these environments, reaching 900 colonies/ha (Sousa-Souto et al., 2013). This species has the characteristic of discarding NR outside the nest, in piles on the soil surface, adjacent to the nest entrance. Likewise, the *Atta opaciceps* species was chosen because it is easily found in the study area, forming mounds up to 2.5 m in diameter with numerous entrances (Delabie et al., 1997).

Nest refuse (NR) collection

The nest refuse produced by *A. balzani* was collected directly in the field, at the study site, and in the mounds discarded by the colonies. The NR from *A. balzani* colonies is characterized as a light-colored residue in front of the nest entrance. The material was collected with a spoon, deposited in paper bags, taken to the Laboratory, and placed in an oven at 60 °C for 48 hours in order to eliminate possible pathogens or contaminants.

The NR produced by *A. opaciceps* was collected from 10 colonies maintained at the Laboratório de Entomologia Florestal - UFS. This substrate is discarded in plastic chambers arranged for that purpose and has a dark brown color. The material was collected and deposited in paper bags and later taken to the oven at 48 °C for 48 hours for drying.

Hydroalcoholic extracts preparation

Hydroalcoholic extracts (HE) were prepared following the methodology of Barbosa et al. (2006) with adaptations of Sousa-Souto et al. (2022). The HE were prepared with 80mL

of 100% ethyl alcohol, 80mL of distilled water, and 40 mL of crushed NR of *A. balzani* or *A. opaciceps*. The extracts were strained and mixed twice a day, placed at rest at room temperature for 48h when they were then vacuum filtered through a Büchner funnel and filter paper (90 mm diameter), and stored in a refrigerator at 8 °C before field tests.

Preparation of attractive baits

The attractive baits were composed of colored straws impregnated with citrus pulp, allowed to dry and sprayed with a 1:1 control solution (ethyl alcohol and distilled water) or with hydroalcoholic extract of the substrate produced by both species, differentiated by color between treatments: white – control (Cont); pink - *Acromyrmex extract* (ExtAc); blue - *Atta extract* (ExtAt).

The citrus pulp was used in this study because it is reported as a substance with high attractive power for ants and for that reason widely used in ant killer baits, so that ants disseminate the toxic substance inside the colony (Boaretto & Forti, 1997; Carlos et al., 2009).

Field tests

For the tests, 30 *A. balzani* and 36 *A. opaciceps* field colonies at the study site were systematically selected. The colonies were marked with wooden sticks and their location was georeferenced with GPS. We identified the foraging areas of each colony and, following a methodology modified from Moreira & Forti (1999), with 10 attractive artificial baits per treatment offered, totaling 30 baits per colony.

In our study area, *A. balzani* forages without the formation of permanent foraging trails. As a result, ants return to the nest in a scattered manner. For this reason, baits were offered in two different ways: for *A. balzani* colonies, the baits were arranged like a half moon, at 25 cm of distance from the nest entrance (Figure 1A). For *A. opaciceps*, the baits were arranged in a line, parallel to the foraging trail, at 10 cm of distance from the trail and 1 m from the nest entrance (Figure 1B).

After the first contact of a worker with any of the baits, we started the period of 30 minutes of observation for colonies of *A. balzani* and 20 minutes for colonies of *A. opaciceps* and the number of baits collected by leaf-cutting ants was recorded. The difference in the observation time between species was due to the size of the colonies and the form of recruitment (smaller nests and slower recruitment in *A. balzani*). During the observation period, we consider as collected any bait that has been removed from the pile, even if the bait has subsequently been abandoned.

The number of baits remaining between treatments was compared using a Generalized Linear Mixed Model (GLMM) with a Poisson distribution and a logarithmic link function. This modeling approach offers more flexibility for variables that do not follow a normal distribution (Bolker et al., 2009). We used the "lme4" package in R to adjust the GLMM to our data.

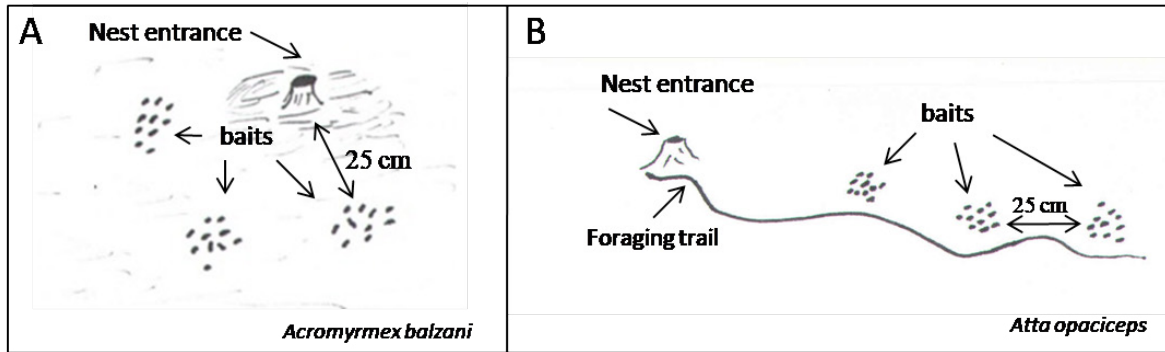


Fig 1. Experimental design indicating the arrangement of the baits near the nests of leaf-cutting ants: For *Acromyrmex balzani* the baits were arranged radially (A). For *Atta opaciceps* nests the baits were placed parallel to the foraging trail (B).

The model consisted of the response variable ‘number of baits remaining’, the fixed effect, represented by the variable ‘treatment’, with three levels (ExtAc, ExtAt, and Control), and the random effect ‘nests’, allowing to evaluate the variation in the number of baits remaining among the different observed colonies, even if the treatment effect is the same for all colonies. In another approach, the “survival” time of the baits (i.e., the time until they were removed) between the different treatments was tested using survival analysis (Kaplan-Meier), followed by pairwise comparisons using log-rank tests, to verify if there was a preference for the collection by a certain treatment level, at a significance level of 5% (Crawley, 2007). The Kaplan-Meier method is widely used to estimate the survival function when dealing with censored data, that is, where the event of interest (bait withdrawal) is not observed for all baits in the group or in all treatments, during the observation period (Barber & Jennison, 1999). Data were analyzed using R software (R Development Core Team, 2023).

Results

Deterrence was observed for extracts from colonies of LCA and this effect differed significantly from control extracts. Both foragers of *A. balzani* and *A. opaciceps* avoided the baits treated with NR extracts, regardless of their origin (intraspecific or interspecific). Ants avoided treated baits even with the attractive effect of citrus pulp. In general, 85% of the baits with NR were rejected, while approximately 80% of the control baits were carried to the nests during the observation period.

For *A. balzani* colonies, the average number of remaining baits (unloaded) was 2.93 ± 0.61 (mean \pm standard error) for the control (Cont), 9.27 ± 0.36 for the intraspecific extract (ExtAc) and 9.6 ± 0.28 for the interspecific extract (ExtAt). Thus, there was a significant difference between the number of remaining baits of Cont x ExtAc ($p = 0.001$) and Cont. x ExtAt ($p < 0.001$), but there was no significant difference between ExtAc versus ExtAt treatments ($p = 0.906$) (Figure 2A).

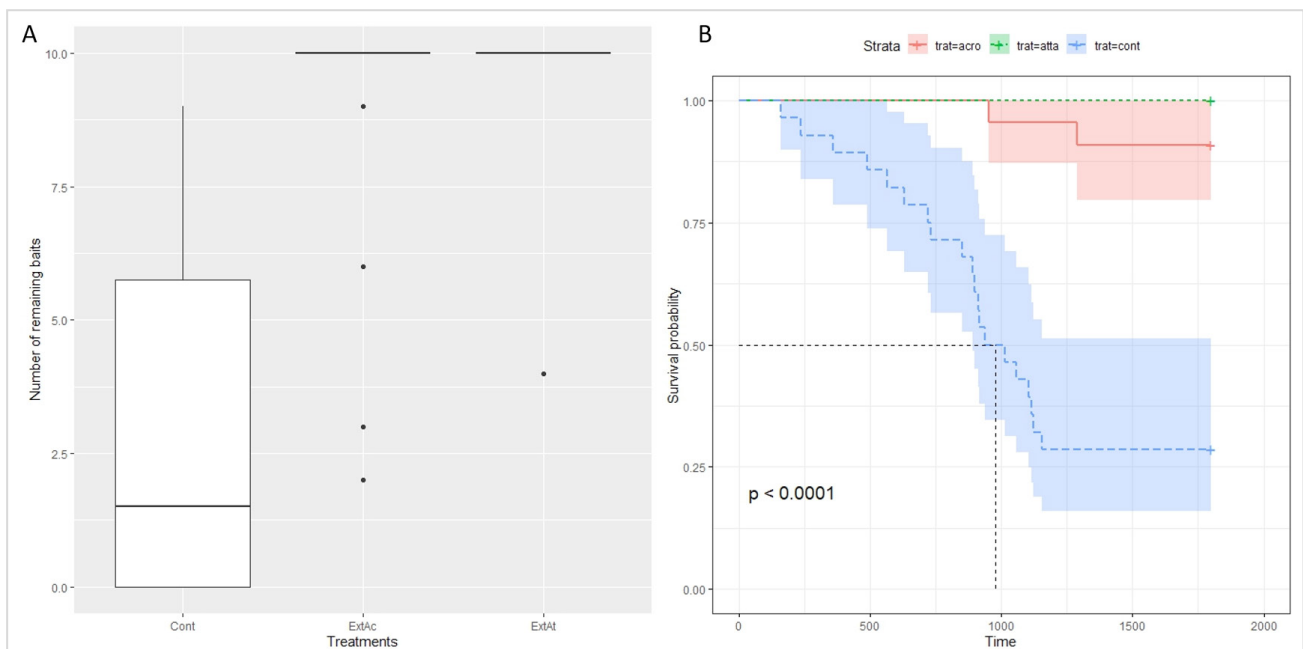


Fig 2. Results of the bioassay with field colonies of *Acromyrmex balzani*. A - Boxplot showing the remaining baits among the three treatments. Horizontal lines indicate median values, the top and bottom box indicate the upper and the lower quartiles, respectively, and dots are outliers. In B, bait survival probability over the observation time of 30 minutes (1800 seconds).

Regarding the “bait survival”, there was a significant difference in the probability of bait survival of the ExtAc versus Control ($p = 0.018$) and ExtAt versus Control ($p < 0.001$) but there was no difference between ExtAc versus ExtAt ($p = 0.508$) (Figure 2B).

Similar results were observed for colonies of *A. opaciceps*. The average number of remaining (unloaded) baits was 1.47 ± 0.36 (mean \pm SE) for control, 6.44 ± 0.57

observed in ExtAc, and 8.19 ± 0.43 for the (ExtAt). There was a significant difference between the NR extracts and the control ($p < 0.001$), and between ExtAc versus ExtAt ($p = 0.015$) (Figure 3A). As observed in *A. balzani*, there was a significant difference in the “bait survival” among the extracts ($p < 0.001$), but no difference between the ExtAc versus ExtAt treatments ($p = 0.52$) (Figure 3B).

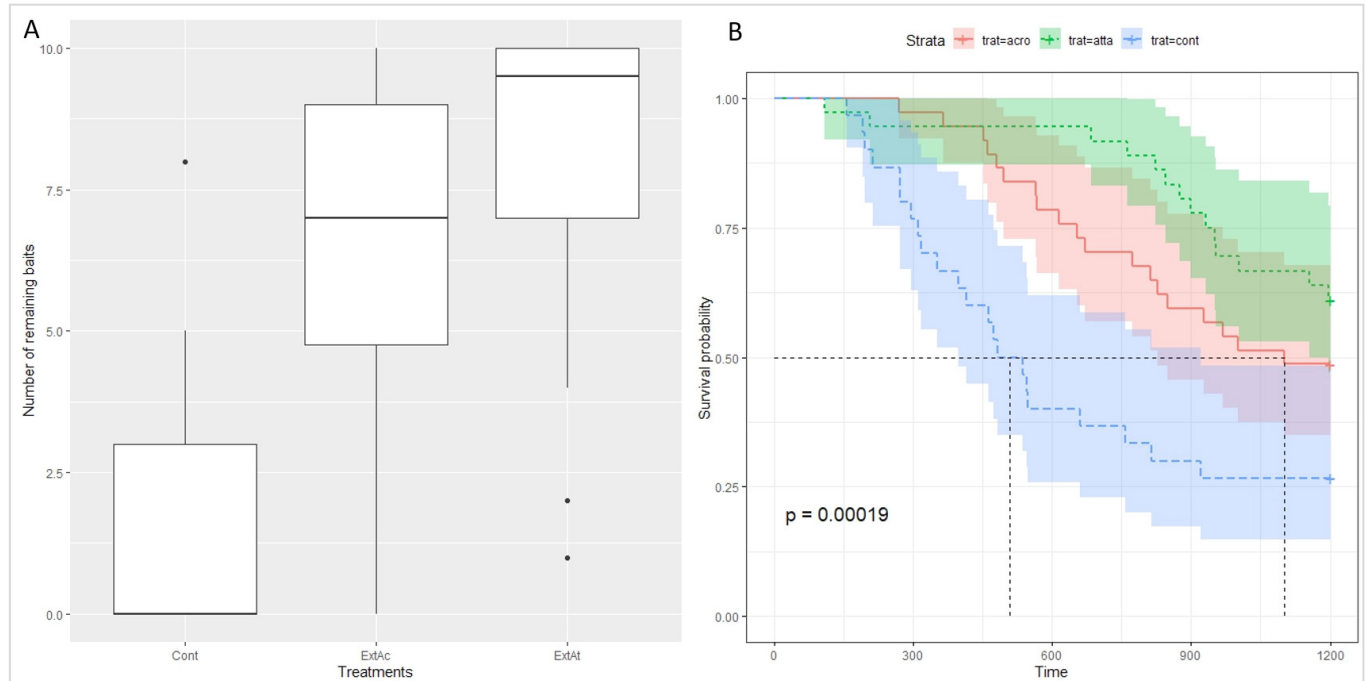


Fig 3. Results of the bioassay with field colonies of *Atta opaciceps*. A - Boxplot showing the remaining baits among three treatments. Horizontal lines indicate median values, the top and bottom box indicate the upper and the lower quartiles, respectively, and dots are outliers. In B, bait survival probability over the observation time of 30 minutes (1800 seconds).

Discussion

The nest refuse from LCA is being better studied and most results have shown multiple uses, either as an additional source of nutrients and organic material to the soil (Cerda et al., 2012; Santos et al., 2018; 2019), or through its deterrent effect against herbivory of the ants themselves (Sousa-Souto et al., 2022) and, more recently, against aphids (Mecenas et al., 2023). Our results show that extracts in liquid form, prepared with the disposal substrate (NR) have both intra and interspecific deterrent effects on the foraging behavior of ants, corroborating similar results using dry waste (Zeh et al., 1999; Farji-Brener & Sasal, 2003; Ballari & Farji-Brener, 2006).

In the present study, hydroalcoholic extracts from *A. balzani* and *A. opaciceps* colonies were efficient in reducing bait collection by ants when compared to the control extract. Almost all control baits were collected by the different sampled colonies and there was little collection of treated baits (less than 40% in the most active colonies). In addition, some behavioral changes were noted, although not measured, as for example, the processing time (touch of antennae and jaws on

the bait) and the start of loading the baits were significantly shorter for control baits when compared to the baits of the ExtAt and ExtAc treatments. Besides the longer processing time of baits with NR by workers, few baits were actually loaded, even after the observation time had ended.

One point to be reported is that many baits with NR registered as “counted” were abandoned by the workers after two or three minutes that were removed from the point of origin. This behavior was not observed in ants that collected the control baits. In addition, the behavior of attempting to remove the citrus pulp inside of the plastic straw was also observed. In fact, many abandoned straws were cut or bitten by ants. This behavior is further evidence that baits with NR showed characteristics that were recognized by the ants as harmful to them, corroborating the hypothesis that odor is the mechanism behind deterrence (Sousa-Souto et al., 2022). In fact, a preliminary analysis through liquid chromatography (HPLC-DAD) of the NR extracts suggests that the substances present may be secondary metabolites from plants foraged by the colonies, such as gallic and p-coumaric acids, two simple phenolic acids (Mecenas et al., 2023). Thus, although the strong

attractiveness of the citrus pulp in the baits treated with NR, the deterrent effect was effective, causing rejection or later abandonment of the treated baits.

Ants select the material to be harvested from the physical or chemical characteristics of the host plant, such as the absence of trichomes in the leaves, nutritional value of the material, levels of secondary metabolites, energy expenditure to incorporate this material into the symbiotic fungus garden (Garcia et al., 2005; Verza et al., 2007). Vegetable materials considered unsuitable for the cultivation of the fungus or which may be toxic are generally rejected at the time of cutting by foragers, or before incorporation into the fungus, by garden workers (Verza et al., 2007). Thus, it is possible that the rejection of baits with NR at the time of foraging shows that the workers were able to associate the odor of the baits with those phenolic acids mentioned above or other potential risks for the colony.

Although LCA shares the same species of symbiont fungus and has similarities in communication between nestmates, the colonies differ through their highly specific chemical profile (Viana-Bailez et al., 2011). The experimental design of the present study, however, does not allow us to conclude that the low collection of baits treated with the interspecific extract was due to the recognition of the baits as material from a rival colony (territorial behavior) or simply due to the inherent odor of the disposal substrate.

Considering the composition of nest refuse between the two species, we can highlight some striking differences between these two substrates (Della Lucia et al., 2013). First, there are differences in the origin of the plant material of both NRs, for *A. balzani* (ExtAc) the NR composition is of herbaceous plants (with a predominance of grasses). The material is totally discarded outside the colony, making it drier and more fragmented. The disposal material of *A. opaciceps* is done mainly through underground chambers, although some colonies deposit their NR in external piles adjacent to the nest (Sousa-Souto et al., 2022). The incorporation of nest waste in the inner chambers makes the substrate texture more compact, with high moisture content and a more pronounced odor. It is reported that residues deposited internally are more susceptible to the proliferation of pathogens, compared to waste discarded outside the nests (Farji-Brener et al., 2016). Even with all these differences, however, the observed interspecific effect of the extracts indicates that the deterrence can be triggered simply by the characteristic odor of the refuse substrate.

Despite advances in knowledge of the potential deterrent effect of nest refuse on ant foraging, it is still unclear whether such effects are long-term and whether its use would be viable on a large scale. Thus, new studies must be conducted to fill in the gaps that still exist, confirming (or not) the use of NR extracts as a new perspective in integrated pest management.

Acknowledgments

We would like to thank Hosana H. Mecnas, for the support of data collection, to Dr. Genésio Tâmara Ribeiro, from the Laboratório de Entomologia Florestal (LEFLO) da Universidade Federal de Sergipe (UFS) and Dr. Yana Teixeira dos Reis, from the Laboratório de Entomologia (UFS) for their help in acquiring the substrate. To Dr. Paulo Cesar de Lima Nogueira, from the laboratório de Química Orgânica (UFS) for his assistance in conducting the chromatographic analyses. To Dr. Sinara M. Moreira for suggestions for improving the methodology. To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), CAPES (PROAP), and CAPES/FAPITEC (88881.157451/2017–01) for funding.

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