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Research Article

Repellent Effect of Leaf-Cutting Ant Waste Against the Aphid *Lipaphis erysimi* (Kaltenbach)

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Abstract

Most conventional methods for controlling agricultural pests cause environmental problems. Thus, sustainable pest management methods have been constantly sought and tested in recent years. The waste substrate produced by leaf-cutting ants (referred to as 'nest refuse') is reported to be repellent to their own foraging. This substrate is avoided by leaf-cutting ants because it presents chemical and physical warning signs to the ants in addition to possible pathogens. In the present study, we experimentally analyzed whether a liquid formulation prepared with the discarded substrate produced by leaf-cutting ants of the species *Acromyrmex balzani* and *Atta opaciceps* would be repellent to the aphid *Lipaphis erysimi*. A total of 103 bioassays were carried out in petri dishes of Θ = 150 mm, with two leaf discs of *Brassica oleracea L.* (var. acephala) sprayed with the control solution (water/alcohol 50%) or with the extract of the nest refuse of one of the ant species. Bioassays were compared using binomial analysis. Ant waste extracts from both species were efficient in repelling aphids ($P < 0.05$). New studies can help fill in the gaps found, such as the identification of substances potentially causing the effect, the duration of the effect, and the dosage and adequate amounts of extract to be applied, as well as other crops to be protected, and other pests to be repelled.

Introduction

The increase in pressure for greater agricultural production and the consequent expansion of this sector have made insecticides the most commonly used method of control in the last 50 years [1]. However, insecticides are expensive and have adverse effects on the environment and human health, which has led to a constant search for sustainable management methods [2,3].

More sustainable management methods include techniques such as the use of resistant cultivars and integrated pest management, including biological control, insecticides, and repellents prepared with organic compounds [2-4]. The repellent effect can be triggered by chemical substances that are usually released by plants, where the herbivore moves in the opposite direction to the source of the substance [5,6].

Recent studies indicate that the waste substrate produced by leaf-cutting ants (Hymenoptera: Formicidae) (genera *Atta* and *Acromyrmex*) has a repellent effect on their own foraging [7-10]. The waste (here referred to as 'nest refuse' or NR) is composed of the remaining particles of plant material transported by the ants into the nest, the remains of dead ants, and the unconsumed and degraded symbiotic fungus [11]. It may contain contaminants such as competing fungi and other pathogens, as well as bactericidal and fungicidal substances produced by ants or by the degradation process [12]. Thus, NR can become toxic and is therefore constantly removed from places where the healthy fungus, queen, eggs, and larvae are present. Depending on the ant species, NR is deposited either in the internal chambers of the colony excavated for this purpose or externally in an area close to the colonies [10,13].

Previous studies have reported an increase in the mortality of workers in contact with disposal chambers [14,15]; thus, contact would be avoided by forager workers [7,9]. More recently, Sousa-Souto et al. (2022) [10] observed a drastic reduction in the herbivory of *Atta sexdens* and *A. opaciceps* in plants treated with liquid extract from NR and pointed to the odor of the material as one of the mechanisms responsible for repellency. At the same time, several substances produced by specific glands in ants have antifungal or bactericidal functions, such as the mandibular and metapleural glands, which are directly related to the hygiene of workers and the colony [16,17]. These substances can remain active in waste, preventing the action of contaminating organisms in the colony. Thus, such substances may also act indirectly to repel insects associated with leaf-cutting ant colonies.

Because ants avoid contact with their own waste, NR could be an alternative for managing other phytophagous insects if the antagonistic behavior is similar. Therefore, the present study verified the repellent effect of NR produced by leaf-cutting ants of the species *Acromyrmex balzani* and *Atta opaciceps* in liquid formulation (hydroalcoholic extract), and tested this effect on the aphid *Lipaphis erysimi* Kaltenbach, 1843 (Hemiptera: Aphididae), a sap-sucking insect. Therefore, the study started with the following hypotheses: i) because NR is avoided by leaf-cutting ants, leaves in contact with this substrate can be avoided by sap-suckers, and ii) if NR-treated leaves are avoided by the aphids, the repellent effect will be observed regardless of the origin of the substrate (*A. balzani* or *A. opaciceps*).

Materials and Methods

Study site and species used

The study was conducted on the campus of the Federal University of Sergipe, São Cristóvão, Sergipe, Brazil (10°55'33.55"S 37°6'8.327"W). The aphid *L. erysimi* is a common pest of Brassicaceae crops such as *Brassica oleracea L.* (var. acephala D.C.), and in times of outbreak, it can cause damage of up to 70% to these crops [18], whether direct damage, consuming the sap, reducing leaf tissue, and consequently photosynthesis or indirectly, through the transmission of phytopathogenic viruses [19].

The species *Acromyrmex balzani* was chosen because it is widely distributed in disturbed environments, in addition to a high density of colonies, reaching 900 colonies/ha in the study area [20]. Its peculiar characteristic is the disposal of organic waste outside the nest on the soil surface, which facilitates its collection and disposal in the environment [21]. Similarly, *A. opaciceps* was chosen because it is easily found in open areas of the hinterland, roadsides, and urbanized areas throughout the Brazilian Northeast, common in Sergipe, forming nest mounds up to 5 m in diameter, with numerous entrances distributed radially as the colony matures [22]. It has underground disposal chambers, but in some cases, colonies dispose of garbage outside the colony [10].

Collection of substrates

The NR produced by *A. balzani* was collected in the field at the study area in mounds discarded by the colonies themselves. In this species, NR is characterized as a light-colored residue in front of the entrance to the nest, collected with the aid of a spoon, deposited in paper bags, taken to the laboratory, and placed in a stainless-steel tray in an oven at 60°C for 48 h to eliminate possible pathogens or contaminants.

The waste produced by *A. opaciceps* was collected from 10 colonies kept in the laboratory and subjected to the same treatment mentioned for *A. balzani*.

Preparation of extracts

The hydroalcoholic extracts were prepared at a concentration of 20% vol/vol, adding 80mL of 100% ethyl alcohol, 80mL of distilled water and 40mL of substrate separately for each species, mixed at the time of preparation and twice a day, left to rest at room temperature for 48h, after which they were filtered and stored at room temperature, following the methodology described by Sousa-Souto et al. (2022) [10].

Aphids and host plants

Cabbage plants were cultivated by sowing commercial genotypes of *B. oleracea (L)* var. acephala D.C. (Georgian Kale, Isla Pak Ltd.) in styrofoam trays with 200 cells. The commercial substrate Vitaplan®, composed of *Pinus* sp. bark, sand for substrate, vermicompost, and vermiculite, was used. One seed per cell was deposited at a depth of 0.5 cm in the substrate. After sowing, the tray was kept in a greenhouse and irrigated twice a day. Thirty days after sowing, the seedlings were transplanted into garden pots (3.6 liters), using two seedlings per pot, and kept in the same place until they were used in the bioassays.

The individuals of *L. erysimi* individuals were collected from cabbage plants grown in a greenhouse at the Department of Agronomy, Federal University of Sergipe. A leaf infested with aphids was removed and transferred to the cabbage plants cultivated for maintenance and placed in screened plastic cages (45 × 45 × 45cm, 149μm mesh) to prevent their escape, with three or four plants per cage. Dead cabbage plants were replaced with new plants to ensure a constant food supply for aphids. One hour prior to the tests, aphids were left deprived of contact with the host plant.

Bioassays

Leaf discs of *B. oleracea* measuring 1.8cm in diameter, were sprayed with the extract on their abaxial and adaxial surfaces using a manual sprayer (0.05 mL of extract per disc). The discs were maintained at room temperature until the extract was dried on the leaf surface. Extracts from the two species were tested separately as controls.

The methodology adopted for the bioassay was adapted from Roobakkumar et al. (2010) [23], using 150 mm diameter Petri dish arenas. A disc was arranged on each side, treatment, and control, drawn at random, and 10 aphids were released at the center of the plate (Figure 1). Each arena was considered as a bioassay. One hour after the aphids were released, they were counted according to the choice between the treatment or control discs, considering a choice if six or more aphids were on one of the discs. A total of 49 bioassays were performed with the *A. balzani* extract and 54 bioassays with the *A. opaciceps* extract.

The results were analyzed using a binomial test [24]. Data were analyzed using R software [25].

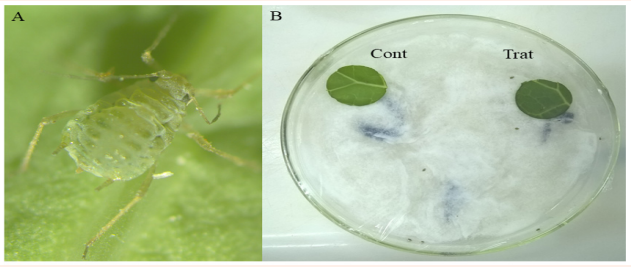

Figure 1: Experimental design of the bioassay for *Lipaphis erysimi* aphid on cabbage leaf (A). Attractiveness bioassay in Petri dishes with two cabbage discs and aphids released to choose between treatments (B).

Liquid chromatography analysis (HPLC-DAD)

The NR extracts were analyzed using liquid chromatography to obtain the chromatographic profile and identify the main compounds present in each extract.

Samples were prepared from approximately 15mg of *A. balzani* and *A. opaciceps* extracts and solubilized in 1mL of HPLC-grade methanol. The resulting solutions were subjected to solid-phase extraction (SPE) using C-18 cartridges (Phenomenex) before chromatographic analyses. The samples were analyzed in a liquid chromatography system with a diode array detector (HPLC-DAD) from Shimadzu (Kyoto, Japan), model Prominence® comprising: CBM-20A communication module, DGU-20A3 gasifier, LC-20AT binary pump system, SIL-20AHT automatic injector, SPD-M20A diode array spectrophotometric detector, CTO-20A column furnace. For the analysis, the elution gradient mode was used, with a 0.1% formic acid solution in water (A) and methanol (B) as the mobile phase, ranging from 5% to 100% (B) for 80 min, remaining in 100% (B) for 20 min, and returning to 5% (B) in 1 min; phenylhexyl stationary phase column 150 mm × 4.6 mm) (Phenomenex, Kinetex®, 5μm) and flow rate of 1 mL/min, oven temperature at 40°C.

Results

Bioassays

In the bioassays carried out with the NR extract produced by *A. balzani,* 35 out of 49 arenas had more than six aphids on the control discs, and in 14 arenas, most aphids were dispersed on cotton. Thus, in 72% of the arenas, most of the tested insects were on control discs (Z = 3.08; p = 0.002). In the bioassays with the NR extract of *A. opaciceps*, the results were similar, with 41 arenas with six or more aphids recorded in the control discs (74%) and 13 arenas with most of them in the cotton matrix (Z = 3.52; p < 0.001). Thus, both extracts showed a significant difference in relation to the control, indicating their repellent effect, regardless of the species (Figure 2).

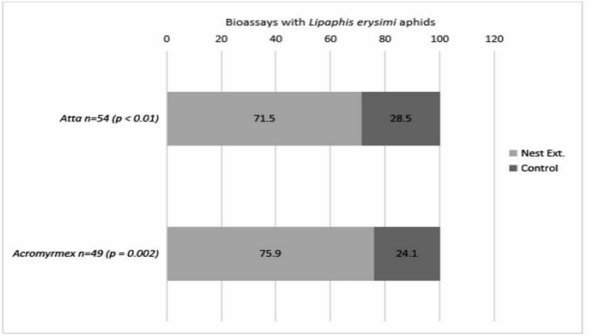

Figure 2: Bioassays with *Lipaphis erysimi* aphids in 150 mm petri dishes with two discs of *Brassica oleracea* leaves, showing a preference for the control disc (water/alcohol 50%) when compared to the disc treated with hydroalcoholic extract of nest refuse (NR) produced by leaf-cutting ants: *Acromyrmex balzani* or *Atta opaciceps*. The numbers inside the bars indicate the percentage of responses.

Analysis of extracts through liquid chromatography (HPLC-DAD)

Chromatographic analyses under the conditions used did not show UV spectra that could reliably indicate the substances present in each extract. The UV spectra suggest that the substances present may be secondary metabolites from plants foraged by the colonies. The UV spectra of NR from *A. balzani* are compatible with the spectra of flavonoids such as p-coumaric acid, a simple phenolic acid. The chromatographic profile of NR from the *A. opaciceps* colonies differed from that of *A. balzani*. The UV spectrum of the main peak was consistent with that of gallic acid, another simple phenolic acid.

Discussion

The hydroalcoholic extract of both species of ants was avoided by the aphid *L. erysimi*, demonstrating its potential use as an alternative method for managing this aphid species under conditions of organic cultivation of *Brassica spp*. Previous studies have already verified the efficiency of liquid extracts applied to leaves, such as tobacco or neem extracts [26,27]; however, this is the first study to address the use of waste produced by leafcutter ants as a natural repellent of an agricultural pest. The response of *L. erysimi* to the extracts of both species was similar, corroborating the idea that the waste substrates of both species can be used as bioactive for this aphid.

During the observation time before choice registration (one hour), the aphids were observed moving randomly in the arena, groping the leaf discs to start feeding. Sometimes, a change in the choice of disc was observed, with the abandonment of the treated disc towards the control disc for feeding.

Although it was not possible to precisely identify the compounds in the chromatographic analysis, the resulting spectra suggested some broader classes that are mentioned in the literature as compounds that act in the chemical defense of plants. The presence of these compounds may explain the behavior observed in aphids and may be the main mechanism acting on the repellency of leaves treated with the NR extract.

Previous studies have reported a variety of insects sensitive to flavonoids, and in many cases, the mechanism of action is due to inhibition of feeding [28- 30]. In addition, several authors have reported that gallic acid causes malfunction of the intestinal tract when consumed by insects, impairing their digestion [31-33].

Other compounds present in NR that were not identified may play an important role in the deterrence of phytophagous insects. For example, substances excreted by the metapleural and mandibular glands of leaf-cutting ants have antifungal and bactericidal actions [16,17] and may act as a compound of strange odor to aphids. The Dufour gland also releases alarm pheromones from ants [34,35]. Such secretions, if present in the NR extract, can also act by stimulating repulsive behavior in the aphids.

The aphid is a sap-sucking herbivore that is sensitive to changes both on the surface of the leaf and on its internal tissues. In this sense, it is possible that the extract acts not only superficially but can also be absorbed by the leaf, causing changes in taste when in contact with mouthparts. Therefore, the effect may be different for other herbivore guilds, such as chewers and miners, reinforcing the need for further studies to verify the repellent potential of these guilds. Thus, additional analyses are necessary, such as different chromatographic approaches, sample preparation with other solvents, or other types of chemical analyses that allow the definitive identification of NR compounds that have the potential to repel or delay the consumption of plant tissues beyond a better understanding. of the mechanisms of action of these compounds.

Our results demonstrate the viability of using NR extracts from leaf-cutting ants in the management of the aphid *L. erysimi* and possibly other agricultural pests, allowing future studies on the application of this method under field conditions. In the present study, 40g of NR was used to produce 200 mL of the extract. Considering the dosage applied to the discs and the responses obtained in the laboratory, it would be possible to use this amount of extract to treat 150 seedlings or 30 adult plants under field conditions. This amount of NR can be easily obtained weekly from just two *A. balzani* field colonies or from a laboratory-maintained *A. opaciceps* colony [36]. It is important to mention that in Brazil, the use of liquid extracts based on natural products is one of the main pest management methods in organic crops and/or family farming, indicating that the use of NR extracts may be well accepted by farmers.

However, field studies are essential not only to determine the dosage and application of adequate amounts of extract, but also to determine the duration of the repellent effect. Furthermore, future studies should test the effect on other host plants and pests, as well as determine which compounds are present and cause the observed effect. Of all the shortcomings, a key point for the viability of this technique is the elucidation of the duration (in days) of the repellent effect and whether abiotic variables (such as temperature and precipitation) influence this duration. In this case, the development of an extract associated with an adhesive or protective compound could increase the permanence time of the solution on the leaf or plant, providing a new perspective for this management method.

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