Research, Society and Development, v. 13, n. 6, e9113645176, 2024
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v13i6.45176

Leaf-cutting ant species (Hymenoptera: Formicidae, Myrmicinae) in three agrosystems and the attractiveness of a natural bait to *Atta laevigata's* biocontrol

Espécies de formigas cortadeiras (Hymenoptera: Formicidae, Myrmicinae) em três agroecossistemas e a atratividade de uma isca natural para o biocontrole de *Atta laevigata*

Especies de hormigas cortadoras de hojas (Hymenoptera: Formicidae, Myrmicinae) en tres agroecosistemas y el atractivo de un cebo natural para el biocontrol de *Atta laevigata*

Received: 02/22/2024 | Revised: 06/10/2024 | Accepted: 06/13/2024 | Published: 06/16/2024

Antonio Mendonça dos Santos
ORCID https://orcid.org/0000-0001-8793-9018
Universidade Federal da Grande Dourados, Brazil
E-mail: antonio.m90bio@gmail.com
Manoel Araécio Uchoa
ORCID: https://orcid.org/0000-0001-5764-1421
Universidade Federal da Grande Dourados, Brazil
E-mail: uchoa.fernandes@ufgd.edu.br
Isaias de Oliveira
ORCID: https://orcid.org/0000-0002-0044-9675
Agência de Desenvolvimento Agrário e Extensão Rural, Brazil
E-mail: isaiasagraer@gmail.com
Elisângela de Souza Loureiro
ORCID: https://orcid.org/0000-0002-9708-3775
Universidade Federal de Mato Grosso do Sul, Brazil
E-mail: elisangela.loureiro@ufms.br
Tamara Santos Ferreira de Farias
ORCID: https://orcid.org/0000-0001-9383-0107
Universidade Federal da Grande Dourados, Brazil
E-mail: tamara.f.farias@gmail.com

Abstract
Leaf-cutting ant species with economic importance are those of the genera: *Atta* and *Acromyrmex*. Natural baits with spores of these entomopathogenic fungi can be used in the biological control of pest species. Therefore, the aims of this research, were: 1) inventory the species of leaf-cutting ants in three agroecosystems; 2) evaluate the attractiveness of the natural bait based on orange peel albedo impregnated with a bioinsecticide; 3) compare the amount of baits carried between the different concentrations of the bioinsecticide, and 4) evaluate the difference in the times spent for the first meeting of the bait and beginning of the loading in the direction of the nest by *Atta laevigata*, in relation to the concentrations of conidia of the bioinsecticide. Active searches were carried out in the anthills to collect adults ("soldiers") and identify the species. The evaluations of the natural baits occurred in four adult nests of *A. laevigata*, being offered 2g of baits per repetition. The position of the treatments was changed at each repetition. We found four species: *Atta sexdens*, *Atta laevigata*, *Acromyrmex rugosus* and *Acromyrmex balsani*. There was a significant difference between the total amount of loaded and unloaded baits, indicating that leaf-cutter ants were attracted by the baits and ignored the presence of *Beauveria bassiana* conidia. The time for first contact and start of loading, did not differ significantly between treatments, nor between loaded amounts of different treatments. The bait containing the bioinsecticide is attractive to *Atta laevigata* workers, regardless of the concentration of conidia.
Keywords: *Acromyrmex*; Fruit growing; Pastures; Entomopathogenic fungi; Orange peel albedo.

Resumo
As espécies de formigas-cortadeiras com importância econômica são as dos Gêneros: *Atta* e *Acromyrmex*. Iscas naturais com esporos dos fungos entomopatogênicos podem ser usados no controle biológico das espécies-praga. Por isso, os objetivos desta pesquisa, foram: 1) inventariar as espécies de formigas-cortadeiras em três agrossistemas; 2) avaliar a atratividade da isca natural à base de albedo de casca de laranja impregnada com um bioinseticida; 3) comparar a quantidade de iscas carregadas entre as diferentes concentrações do bioinseticida, e 4) avaliar a diferença nos tempos gastos para o primeiro encontro da isca e início do carregamento na direção do ninho por *Atta laevigata*, em relação às concentrações de conídios do bioinseticida. Foram realizadas buscas ativas nos formigueiros para coletar adultos ("soldadas") e identificar as espécies. As avaliações das iscas naturais ocorreram em quatro

formigueiros adultos de *A. laevigata*, sendo oferecidas 2g de iscas por repetição. A posição dos tratamentos foi mudada a cada repetição. Ocorrem quatro espécies: *Atta sexdens*, *Atta laevigata*, *Acromyrmex rugosus* e *Acromyrmex balsani*. Houve diferença significativa entre a quantidade total de iscas carregada e não-carregada, indicando que as formigas-cortadeiras foram atraídas pelas iscas e ignoraram a presença dos conídios de *Beauveria bassiana*. O tempo para primeiro contato e início do carregamento não diferiu significativa entre tratamentos, nem entre quantidades carregadas dos diferentes tratamentos. A isca contendo o bioinseticida é atrativa para as operárias de *Atta laevigata*, independente da concentração de conídios.
Palavras-chave: *Acromyrmex*; Fruticultura; Pastagens; Fungos entomopatogênicos; Albedo da casca de laranja.

Resumen
Las especies de hormigas cortadoras con importancia económica son de los géneros: *Atta* and *Acromyrmex*. Los cebos naturales con esporas de hongos entomopatógenos pueden utilizarse en el control biológico de especies plaga. Los objetivos de esta investigación, fueron: 1) inventariar las especies de hormigas cortadoras de hojas en tres agrosistemas; 2) evaluar el atractivo del cebo natural a base de albedo de piel de naranja impregnado con un bioinsecticida; 3) comparar la cantidad de carnadas cargadas entre las diferentes concentraciones del bioinsecticida, y 4) evaluar la diferencia en los tiempos para el primer encuentro de la carnada y inicio de carga en dirección al nido por *Atta laevigata*, en relación a las concentraciones de conidios del bioinsecticida. Se realizaron búsquedas activas en los hormigueros para recolectar adultos ("soldados") e identificar la especie. Las evaluaciones de los cebos naturales se realizaron en cuatro nidos de adultos de *A. laevigata*, ofreciéndose 2g de cebos por repetición. La posición de los tratamientos se cambió en cada repetición. Hay cuatro especies: *Atta sexdens*, *Atta laevigata*, *Acromyrmex rugosus* y *Acromyrmex balsani*, en la region. Hubo una diferencia significativa entre la cantidad total de cebos cargados y descargados, lo que indica que las hormigas cortadoras de hojas fueron atraídas por los cebos e ignoraron la presencia de conidios de *Beauveria bassiana*. El tiempo para el primer contacto y el inicio de la carga no difirió significativamente entre tratamientos, ni entre cantidades cargadas de diferentes tratamientos. El cebo que contiene el bioinsecticida es atractivo para las obreras de *Atta laevigata*, independientemente de la concentración de conidios.
Palabras clave: *Acromyrmex*; Fruticultura; Pastos; Hongos entomopatógenos; Albedo de piel de naranja.

1. Introduction

The leaf-cutting ants with economic importance in the Neotropical Region belongs to two genera *Atta* Fabricius, 1804 and *Acromyrmex* Mayr, 1865, (Hymenoptera: Formicidade, Myrmicinae, Attini), occurring from southern North America to temperate regions of South America (Anjos et al., 1998; Della Lucia & Souza, 2011). The Attini tribe is a monophyletic group, consisting of forty-five valid genera and fossil one (AntCat, 2015). The ants of this tribe depend directly on the fungus gardens they cultivate for their food supply. This cultivation co-evolved in Attini species, indicating that these could have artificially selected more productive fungal cultures (De Fine Licht & Boomsma, 2010).

The activity of the leaf-cutting ants implies the defoliation and removing the live bark of plants, which can cause tree's death in certain cases, making them of great economic importance for agrosilvipastoral activities (agriculture [especially fruit growing], pasture, or forestry). In this case, that species of leaf-cutting ants are considered agrosilvipastoral pests (Cherrett & Peregrine, 1976; Anjos et al., 2008). Most of the leaf-cutting ants' diet is based on the symbiotic fungus *Leucoagaricus gongylophorus* (Agaricales: Basidiomycota) which develops on fresh parts of vegetables that these ants cut, load and supply, such as leaves, flowers, fruits, buds or bark of plants near the anthill (Brandão et al., 2011; Della Lucia & Souza, 2011), although, during the processing plant materials, Attini adult workers can consume sap (Quinlan & Cherrett, 1979; Forti & Andrade, 1999). Some species of Attini can forage until more than 100 m in a circular radius around from their nests.

The Attini phylogeny can be established according to the type of cultivated fungus and the substrate used for such cultivation, being the genera *Atta* and *Acromyrmex* the most derived ones (Schultz & Brady, 2008; Mehdiabadi et al., 2012).

The nests of *Atta* species are characterized by having an underground seat with several tunnels and chambers and an apparent seat that can be formed by mounds of loose earth ("morundum"), in addition to a series of holes (spots) through which foragers leave (Forti et al., 2011) to found plant materials. *Acromyrmex* species has small or inconspicuous nests, which can be superficial or underground, in the latter case, with few chambers and little volume of loose soil (Anjos et al., 1998; Forti et al., 2011). Loose earth mounds are important for the location of leaf-cutting ants' nests in the field, as in some species they

stand out among plantations, and have some typical characteristics. The "morunduns" loose soil area is widely used in chemical control programs for these ants to estimate the age of the nests, as well as the amount of insecticide to be applied. Such an area can be obtained in two different ways: multiplying the greatest length by the greatest width of the area occupied by the mounds of loose earth; or adding the individual areas of each mound (Zanetti et al., 2002).

In agrosilvipastoral ecosystems, leaf-cutting ants attack several plant species in their different stages of development, causing direct damage, culminating in the death of young or adult plants or causing reduced tree growth. Furthermore, leaf-cutter ants causse indirect losses, such as reduced plant resistance to attack by other insects and phytopathogenic agents (Loeck & Grützmacher, 2001; Chong et al., 2007), reducing production and productivity. On the other hand, not all species of leaf-cutter ants cause considerable economic damage, as this is directly related to their foraging habits, degree of endemism and nest size (Fowler et al., 1989). In Brazil, for example, only five species of *Atta* and nine of *Acromyrmex* are considered to be of economic importance (Zanetti et al., 2002). We observed that if a species of plant is defoliated three times in a row in the same month, this culminates in the death of the plant.

The prospecting for non-polluting methods aimed at managing populations of leaf-cutter ant species, which have pest status in agrosilvipastoral systems is of paramount importance. Techniques that use physical barriers to protect trees and shrubs, have shown to be more promising (Jiménez et al., 2022), as well as the population suppression methods with biological weapons, especially entomopathogenic fungi (Loureiro et al., 2022a, 2022b). The coexistence with leaf-cutting ant species should be sought, in order to enable the production of food free of pesticides; without major damage to rural producers, avoiding the local extinction of Attini species, because that last mission, is a task that is not only ecologically undesirable, but also, probably, impossible.

The accurate taxonomy of *Atta* and *Acromyrmex* species that occur regionally in cultivated areas is essential for the development of management methods and population control of these organisms, where they are considered pests. The inventory of species should be the first step of an integrated pest management program, to allow the adequate use of population suppression methods for pest species, making it possible the coexistence among humans and leaf-cutting ants in the agroecosystems.

The control of leaf-cutting ants, mainly in reforestation companies, has been carried out using chemical products, through the use of granulated baits or micro portals based on sulfluramid, thermonebulization and fumigants (Boaretto & Forti, 1997). The systematic use of granulated baits to control leaf-cutting ants alters pre-existing diversity patterns in ecosystems, negatively affecting non-target organisms. Because they are not biodegradable, these chemicals accumulate in the food chain (Corassa et al., 2013).

The risks of environmental contamination and the probability of promote the resistance of leaf-cutting ants to the products used on chemical control, have aroused concern and led to new research aimed at alternative methodologies for the management of leaf-cutting pest species, such as the use of entomopathogenic fungi. In *Eucalyptus saligna*, using a strain of *B. bassiana* in a concentration of 1×10^{9} conidia.g^{-1}, applied directly on nests of a *Acromyrmex* species demonstrated high efficiency, with mortality rates of 87.2 and 83.1%, respectively, up to the 35th day (Diehl-Fleig et al., 1993).

The species *Atta laevigata* (Smith 1858) (Formicidae: Myrmicinae: Attini) establishes its nest in both - shaded or sunny environments (Zanetti et al., 2002) and has the habit to forage in both: monocotyledonous and dicotyledonous plants to supply its symbiont fungus in their colonies (Lima et al., 2002). Due to the economic importance of this leaf-cutting ant species, it is necessary to develop efficient, low-cost, and management techniques (Zanetti et al., 2014), taking care in the biodiversity and physical environment.

The natural occurrence of *Beauveria bassiana* in queens of *Atta sexdens* Forel 1908 was verified by Cardoso et al. (2011). *B. bassiana* and *Metarhizium anisopliae* were isolated from queens of *A. sexdens* (Alves & Sosa-Gomez, 1983) and *B. bassiana* was recovered in winged females of *A. laevigata* and *A. capiguara* Gonçalves, 1944 (Rodrigues et al., 2010). These fungi observed on the queens' cuticles are possibly acquired from the environment, especially when the queens are tunnelling to build up their new nest. Several species of fungi were isolated from leaf-cutting ant queens during excavations in the field and in the laboratory, with predominance of fungi species from the genera: *Beauveria*, *Paecilomyces*, *Isaria*, *Fusarium* and *Aspergillus* (Barcoto et al., 2017).

In the laboratory, isolates of the entomopathogenic fungus *B. bassiana* caused significant mortality for *A. sexdens*, with workers being more susceptible than soldiers. The isolate IBCB 21 also caused high percentages of confirmed mortality, being recommended for use in the biological control of leaf-cutting ants (Loureiro et al., 2022a). Loureiro et al. (2022b) tested five isolates of the entomopathogenic fungus *M. anisopliae* against leaf-cutting ant adults. All isolates were pathogenic for *A. sexdens*, being also more virulent for workers than for soldiers. The isolates IBCB 348, IBCB 410 and IBCB 425 were the most efficient in killing leaf-cutting ants. The isolates IBCB 425 and UFGD 03 caused high percentages of confirmed mortality. Such results showed that the tested fungi have great potential to be used as biological control agents against *A. sexdens*.

Given the above introduced, make clear the need for sustainable techniques to control leaf-cutting ants, and that entomopathogenic fungi are potential biological control agents for Attini pest species. Therefore, the aims of this research were: 1) survey the species of leaf-cutting ants and their distribution in three agroecosystems; 2) to evaluate the attractiveness of a bait based on albedo of orange peel, impregnated with different concentrations of the bioinsecticide Ballveria®; 3) to compare the amount of baits loaded between the different concentrations, and 4) evaluate the difference between the times spent for the first find of bait and the beginning of it loading in the direction of nest by *Atta laevigata*, in relation to the concentrations of conidia of the bioinsecticide.

2. Material and Methods

This research is composed for two steps, being the first one, an inventory on diversity of leaf-cutting ant species in three environments, and in the second step, we evaluate the attractiveness of natural bait containing *Beauveria bassiana* conidia for *Atta laevigata* in a mixed orchard of fruit trees. Both studies were carried out in the Central Brazil (municipality of Dourados, state of Mato Grosso do Sul).

The research occurred in both: 1. laboratory: bait preparation and evaluation of bait quality; 2. field: evaluation of bait acceptance and carrying of different concentration of fungi conidia incorporated in the baits. So, this research can be qualified as qualitative and quantitative (see Alves 1988; Loureiro et al. 2022a, 2022b).

Assay one: Leaf-cutting species in three Agroecosystems

Three environments were evaluated: *Estância D'Torres* (22°12'29.69"S and 54°55'38.23"W) and *Estância Biocenose* (22°12'34"S and 54 °55'33”W) both with areas of approximately 8 and 10ha, respectively, occupied mostly by the grass *Urochloa brizantha* (Stapf.) Webster. The third environment evaluated was *Chácara Dourada* (22°15'59” S and 54°48'23” W) with an area of 2.5 ha formed by a mixed orchard of fruit trees.

During the months of April to July 2014, two assessments were carried out in each of the areas mentioned above. These occurred during the day, through active searches for the entrance holes of the ants (adults in activity) and/or piles of loose soil that were demarcated with a numbered stake for later location. The holes of ant nests were injured with the aid of

hoe or a piece of wire to encourage the exit of larger workers or "soldiers". The soldiers of leaf-cutting ants were collected and placed in 50 mL plastic pots, containing information on the place and date.

All collected adults of leaf-cutting ants was taken to the *Laboratório de Entomologia*, *Universidade Federal da Grande Dourados* (UFGD), Dourados-MS, Brazil, where it was sorted and identified with the aid of a stereoscopic microscope and identification keys by Gonçalves (1961) and Forti et al. (2006), in addition to comparing images with those from the antweb.org and antwiki.org databases.

Assay two: Attractivity of *Atta laevigata* to a natural bait

Bait elaboration

To manufacturing of baits, orange peel albedo (*Citrus sinensis* var. 'Pera Rio') was used as an attractant, extracted with the aid of a juicer. The epicarp was removed and then the mesocarp was placed in aluminium basins to dry in an oven at an average temperature of 50°C for a period of forty-eight hours. After drying, the mesocarp was crushed in a blender until a fine and homogeneous flour was obtained, which was frozen for later use.

Grains of type 1 rice (high quality) were crushed in a blender until obtaining a fine powder that was used as a binding agent to make the formation of pellets possible. Another reason for using rice in baits was the fact that this type of substrate is used for the production of bioinsecticides based on entomopathogenic fungi. In this way, it is expected that the conidia, if they germinate, have a temporary nutritional alternative in the baits, so that they can remain viable for a longer time, before coming into contact with the cuticle of the leaf-cutting ant species.

Another component used for the production of baits was refined soy oil, with the aim of assisting in the homogenization and also in the agglutination of the other components of the bait, making easy the pellets formation. Distilled water was added to give consistency to the dough. To form the baits, the mass produced was passed through a manual machine used to make spaghetti-type pasta, with toothed cylinders with a spacing of approximately 1.7 mm.

In the bait preparation was used: 200g of flour of orange peel mesocarp, 200g of crushed high-quality rice, and the bioinsecticide Ballveria®, active ingredient of the entompathogenic fungi *Beauveria bassiana* (isolated IBCB 66), being all were mixed to make a homogenized with such ingredients. Then, 40 mL of refined soybean oil and sterilized distilled water were added, added little by little, until obtaining a homogeneous and brittle mass, considered the ideal point for preparing the bait pellets.

The dough, in the form of fillets, was packed in aluminium moulds and placed to dry in an incubator oven at an average temperature of 7 °C, for a period of 48 hours, then the bait fillets were placed in plastic bags and manually broken to that were reduced to small pellets of ± 6 mm in length. Finally, the baits were kept in plastic containers with lids and placed in a refrigerator at a temperature of approximately 8 °C, for later use.

Characterization of the experimental area and the bioassay

The experiment was carried out in an orchard with an area of four hectares belonging to the *Universidade Federal da Grande Dourados* (UFGD). The cultivated fruit trees are: mango, avocado, guava and vine (grape). The maximum and minimum temperatures during the period were 29 and 18 °C, respectively. Four anthills of *Atta laevigata* were used, measuring between 46.9 m^2 and 18.4 m^2 of loose soil area, with a minimum distance of 50 m between the colonies (anthills).

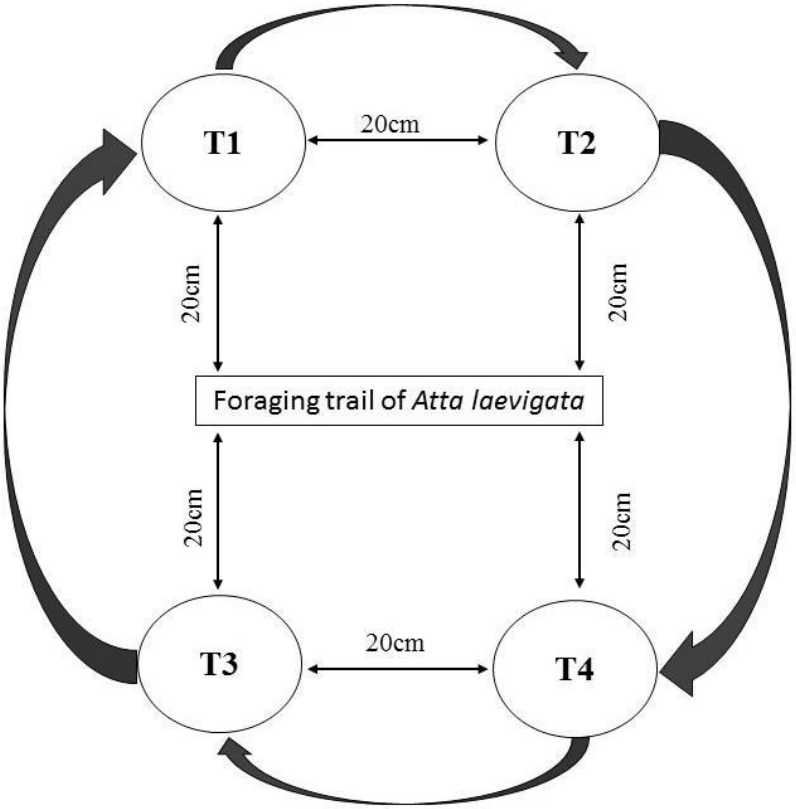
The evaluations on the preference and loading of the baits by the leaf-cutting ant *Atta laevigata* were always carried out after 17:00 hours, which coincides with the period of greatest foraging activity of *A. laevigata*. In the laboratory, the baits were weighed and 2g were placed in autoclaved test tubes and duly identified, for each treatment. Then, the tubes were closed

with hydrophobic cotton and placed in a Styrofoam box, containing ice for transport to the field. For field application, four 6.5 cm diameter filter paper discs were positioned at a distance of approximately 20 cm far from each other, on both sides along the ants' foraging trail of *A. laevigata* (Figure 1). Then, the bait was placed on each of the filter paper discs.

The following treatments were used: 10g, 100g and 200g of Ballveria® with the concentrations of 5×10^{7}, 5×10^{8} and 1×10^{9} con.g^{-1}, of the dry bait, respectively). The experimental design was the Latin Square (Figure 1), with three treatments and four repetitions (different anthills) containing four baits each. In each of the repetitions, the filter paper disks were removed and replaced by new ones, and the treatments were changed in position, always clockwise, so that each treatment was offered at least once in each of the four positions.

The experiment was carried out following the patterns of a design in the sense that each of the concentrations of conidia on the baits corresponded to a treatment, and that each change in the positions of the discs positioned close to the ant trails was considered a repetition. Means were submitted to ANOVA analysis of variance by t test at 5% significance.

Figure 1 - Sketch of the positioning and direction of repetitions for each one of the treatments offered near the foraging trails of *Atta laevigata* (Hymenoptera: Formicidae, Myrmicinae) in the orchard of the *Universidade Federal da Grande Dourados* (UFGD), Dourados-MS, Brazil (Apr. 2015).



Source: Self elaboration.

During the evaluations, the time elapsed until each leaf-cutting ant had the first contact with the baits was measured. This time interval was considered to be the foragers' first touch with their antennae on the granules (pellets), in each treatment. The time spent until the start of loading was also counted; interpreted as the removal of at least one bait from the paper disk, in each of the treatments. The time interval in which the ants carried all the granules (baits) of each treatment was considered as the final loading time.

At the end of each evaluation, which consisted of loading all the baits of the respective treatment, the remaining baits on the other filter paper disks were collected, being placed in their original test tubes (previously identified) so that they could be weighed in the laboratory, with in order to obtain the amount of bait loaded in each treatment.

3. Results

Bioassay one: Leaf-cutting ant species and population density

Four species of leaf-cutting ants occurred in the inventoried areas, being two of the genus *Atta*: *A. sexdens* Forel, and *A. laevigata* Smith, and two of the genus *Acromyrmex*: *A. rugosus* (Smith,) and *A. balzani* Emery. In total, considering all areas surveyed, the species with the highest number of anthills, were *A. sexdens* with forty nests and *A. rugosus* with thirteen.

Atta sexdens occurred mainly in the orchard, with a small nest's abundance; although it also occurred in one of the areas with pasture. On the other hand, *A. laevigata* nests wore found in all surveyed areas, although in smaller numbers of colony in compare to those of *A. sexdens* (Table 1).

The leaf-cutting ants of the genus *Acromyrmex*, occurred in two agroecosystems: *A. rugosus* was frequently found both in the orchard and in one of the areas with pasture, predominating *Urochloa brizantha* grass, and *A. balzani*, on the other hand, occurred only in two areas sampled, with a predominance of *U. brizantha* (Table 1).

Table 1 - Numbers of leaf-cutting ant nests (colonies) and species found in three agroecosystems in Central Brazil (municipality of Dourados, state of Mato Grosso do Sul, April to July 2014).

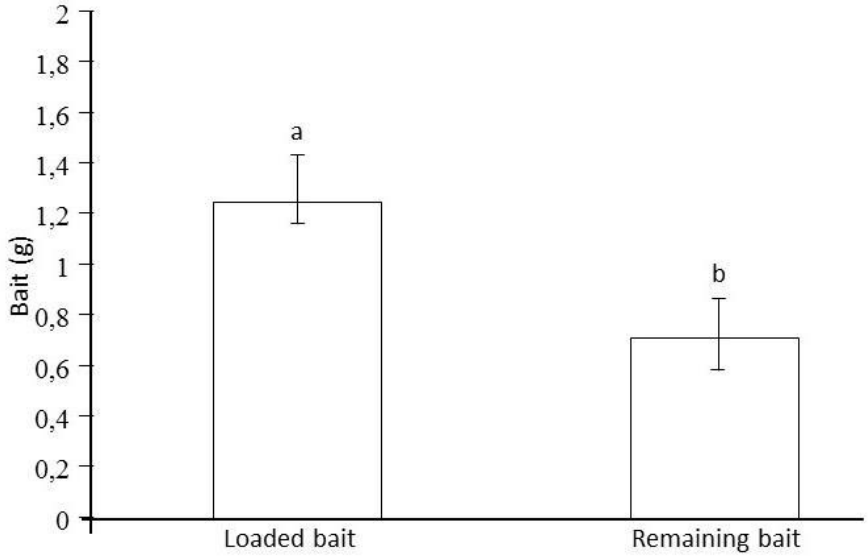
Places	*Atta sexdens*	*Atta laevigata*	*Acromyrmex balzani*	*Acromyrmex rugosus*
Estância Biocenose (Orchard)	5	3	4	9
Estância D'Torres (Pasture)	9	1	1	0
Chácara Dourada (Orchard)	26	3	0	4
Total of Colonies	40	7	5	13

Source: Self elaboration.

Bioassay two: Natural Bait Attractivity to *Atta laevigata*

The mean time to finish loading the baits by *A. laevigata* was 21.17 ± 12.12 minutes. There was a significant difference between the averages of loaded baits compared to the rest of the total bait offered (Figure 2).

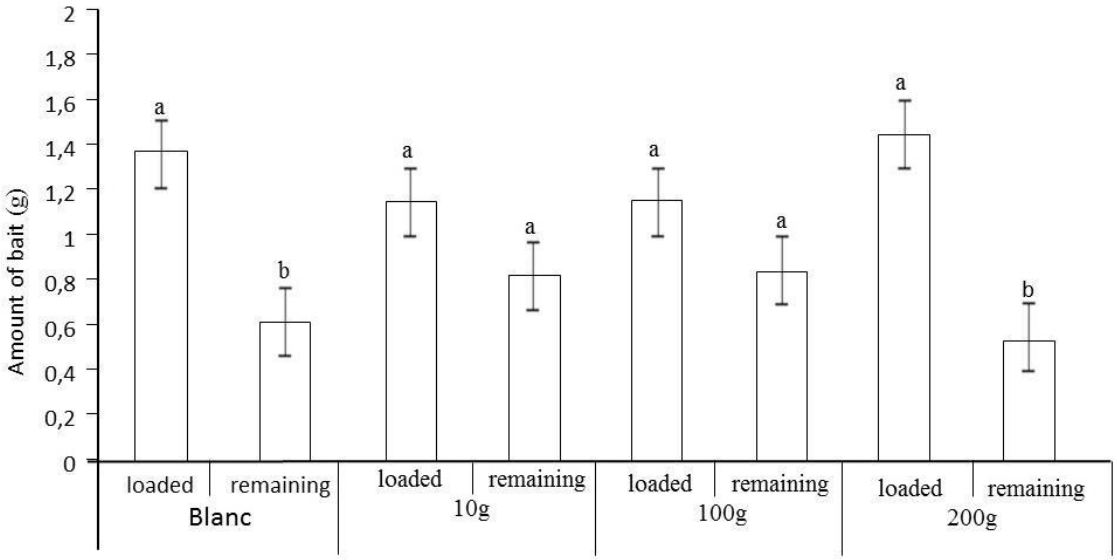
Figure 2 - Analysis by t test ($p < 0.01$) of the mean and standard deviation of the amounts of baits prepared with orange peel albedo, *Citrus sinensis*, containing conidia of the entomopathogenic fungus *Beauveria bassiana*, carried and not carried by the leaf-cutting ant *Atta laevigata* in the orchard of the *Universidade Federal da Grande Dourados* (UFGD), Dourados-MS, Brazil (Apr. 2015).



Source: Self elaboration.

The results of the comparison between the mean amounts of loaded baits and the remaining baits were significantly different, at ($p < 0.05$) for treatments with lower and higher concentrations of conidia of the fungus *B. bassiana*, in relation to those with intermediate concentrations of conidia (Figure 3).

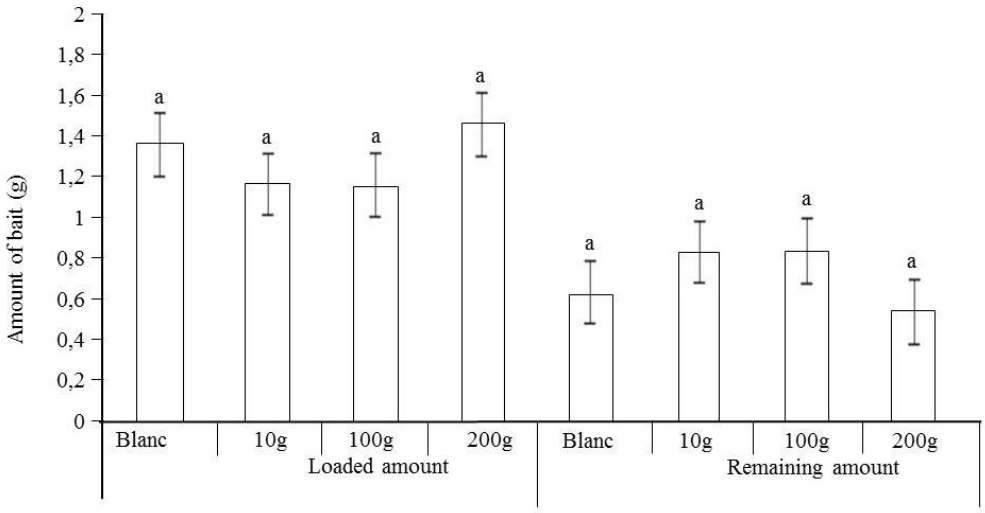
Figure 3 - Analysis by t test ($p < 0.05$) of the difference between the mean amounts for each treatment and standard deviation of baits prepared with orange peel albedo, *Citrus sinensis* 'Pera Rio', containing conidia of the entomopathogenic fungus *Beauveria bassiana*, loaded amounts by the leaf-cutting ant *Atta laevigata* and remaining quantities, in the orchard of the *Universidade Federal da Grande Dourados* (UFGD), Dourados-MS, Brazil (Apr. 2015).



Source: Self elaboration.

There was no significant difference for the amounts of baits carried and remaining by *A. laevigata* in the comparison between treatments, as shown in Figure 4.

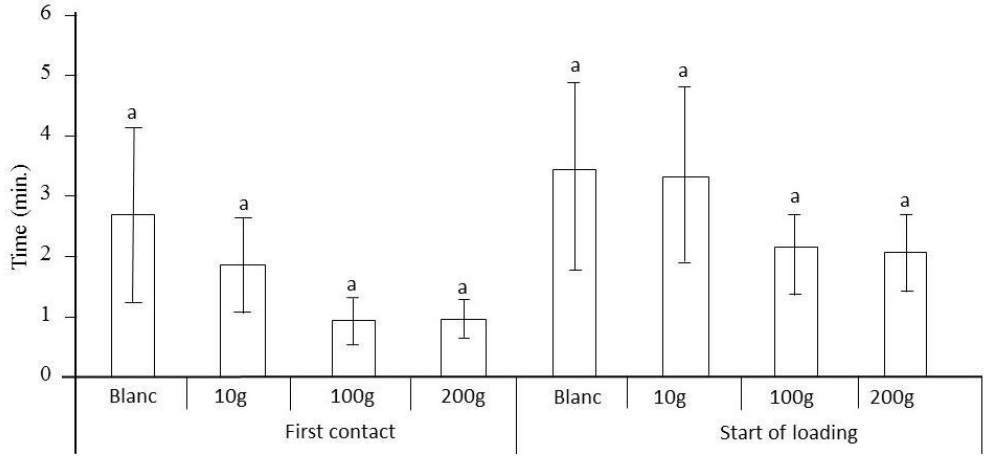
Figure 4 - Analysis by t test ($p > 0.05$) of the mean amount and standard deviation of baits prepared with orange peel albedo, *Citrus sinensis* 'Pera Rio', containing conidia of the entomopathogenic fungus *Beauveria bassiana* of the amounts loaded between the four different treatments offered the leaf-cutting ant *Atta laevigata* in the orchard of the *Universidade Federal da Grande Dourados* (UFGD), Dourados-MS, Brazil (Apr. 2015).



Source: Self elaboration.

In the assessing the attractiveness of the leaf-cutting ant *A. laevigata* by the orange peel albedo-based bait, with different concentrations of *B. bassiana* conidia, there was no significant difference between treatments. Neither the time spent by the workers to have the first contact with the baits, nor the time to start loading the baits impregnated with different concentrations of conidia from the different treatments or concentrations (Figure 5).

Figure 5 - Mean time in minutes for the first contact and start of loading and respective standard errors of baits based on orange peel albedo, *Citrus sinensis* 'Pera Rio', containing conidia of the entomopathogenic fungus *Beauveria bassiana* offered to the leaf-cutting ant *Atta laevigata* in the orchard of the *Universidade Federal da Grande Dourados* (UFGD), Dourados-MS, Brazil (Apr. 2015).



Source: Self elaboration.

The results of this research indicate that there was no significant difference between the treatments by Test t ($p > 0.05$), as shown in Figure 5. So, the leaf-cutting ant *A. laevigata* loaded the baits, regardless of the concentration of conidia of the entomopathogenic fungus *B. bassiana* mixed in the it.

4. Discussion

Bioassay one: The species of leaf-cutting ants in the agroecosystems

In all three locations, the nests of *Atta sexdens* were located under trees, as this species has the behavioural characteristic of nesting in shaded environments, as pointed out by Forti et al. (2011). There is a relationship between the nesting site and the foraging habit, since that ants of this species cut exclusively dicotyledonous plants (Zanetti et al., 2002), which explains the greater number of nests in the orchard and in the pasture area with several trees. In both places, the anthills were concentrated on the edges of the properties, under the trees. This data corroborates the results of Ramos et al. (2008), in *Eucalyptus* plantations, who found that the anthills distribution of *A. sexdens* are concentrated on the edges and, in an aggregated pattern, by the areas where this species occurs.

In this inventory, *A. sexdens* was much more abundant (40 colonies) and widespread (in all areas), compared to *A. laevigata* (7 colonies). This pattern has been found in other regions of Brazil. In work carried out in the south of Espírito Santo state, Brazil, Zanuncio et al. (2010) also reported a low density of *A. laevigata* nests, compared to those of *A. sexdens*, justifying that the first species could have been recently introduced in that region or was undergoing a process of local extinction, due to competition with other organisms. Although *A. laevigata* cut both monocotyledonous and dicotyledonous plants (Lima et al., 2002), the factor that best explains the low number of nests of this species compared to *A. sexdes* in this research, is related to the nesting behaviour of the two species: colonies of *A. laevigata* are commonly found in areas that receive direct sunlight, as highlighted in other studies (Zanetti et al. 2002; Forti et al. 2011), while *A. sexdens* prefers to found its colonies under trees and other shady places.

We found two different species from those previously found on the Pantanal: *Acromyrmex fracticornis* (Forel, 1909) *and A. subterraneus* (Forel, 1893) by Martins et al. (2021). Herein, *A. rugosus and A. balzani* were recorded. *A. rugosus* builds small nests, with one or more mounds of loose earth in an irregular shape and with a central hole, corroborating with the publication by Soares et al. (2006) and Verza et al. (2007). Rando and Forti (2005) reported out the occurrence of this same species in the central and eastern regions of Mato Grosso do Sul and in several other locations in Brazil, mainly foraging dicotyledonous plants, although there are records of *A. rugosus* cutting some monocotyledons, such as corn and other grasses (Poaceae). *Acromyrmex balzani* was found only in places with a prevalence of the grasses *Urochloa brizantha* and *Paspalum notatum* Flüggé. This is related to the foraging behaviour of this species, considered a leaf-cutting ant specialized in cutting grasses, as already pointed out by Gonçalves (1961) and Pimenta et al. (2007).

Bioassay two: Attractivity of *Atta laevigata* to a natural bait

We found that during the assays of this research, the forager workers of the leaf-cutting ant *A. laevigata*, constantly touch the baits with their antennae before loading them. It is known that leaf-cutting ants have sensilla located on antennae, with an olfactory function. Ribeiro and Marinho (2011) point out that leaf-cutting ants use odours to select the substrate that they will cut and to load.

After the forager workers find the bait, they quickly carry it into their anthills. Delabie et al., (2000) point out that this behaviour may be directly related to a better distribution of baits in the symbiont fungus chambers. In a test comparing the attractiveness of *A. sexdens* to baits made with "Jatobá", *Hymenaea courbaril* L., flour and *Citrus* pulp, the mean final time for

baits based on *Citrus* pulp was 11.28 minutes (Teixeira & Santos, 2008). However, Lima et al. (2003) obtained an average final time of 10.2 minutes, evaluating alternative baits for *Atta bisphaerica* Forel 1908. These values are lower than those herein obtained to *A. laevigata*, however the authors above used ten bait granules per treatment, while in this study we offered two grams (2g, around 15 granules) that mean higher number of granules of bait, in compare to published results by Lima et al. (2003).

Herein, times for first contact and the start of loading did not differ between treatments. Similar results were obtained for the species *A. bisphaerica* in relation to its attractiveness to baits with different attractive substrates (Lima et al., 2003). In the work carried out by Teixeira & Santos (2008), the times had significant differences, indicating a greater attractiveness of *A. sexdens* by the bait produced with the flour of *Hymenaea courbaril* L. (Caesalpiniaceae), extracted from their pods. In this research, there was a significant difference in the amount of baits made with orange peel albedo: amounts loaded, in comparison with the amounts of baits that were left after the final evaluation times.

In this research, we found that workers of *A. laevigata*, in their foraging behaviour, ignored the presence of *B. bassiana* conidia incorporated into the baits made of orange's peel albedo. This type of behaviour was also observed by Cardoso et al. (2012) for another species of the genus *Atta*. Diniz & Bueno (2010) reported that leaf-cutting ants are selective in relation to the material carried because they avoid contaminating themselves and their fungus garden.

In this research, for the first time it was verified that orange peel albedo can be used as an attractive bait for *A. laevigata* workers. Orange peel albedo can be impregnated with spores of entomopathogenic fungi to enable the loading of such biological control agents into the nest, aiming to infect the queen, and could, consequently, locally extinguish the anthill (colony). The albedo was an important factor to induce the loading of the baits with the incorporated entomopathogenic fungus. Other researchers have already evaluated other orange by-products as attractive to leaf-cutting ants. Verza et al. (2006) evaluated citrus pulp, noting that it is widely used as a substrate for ant baits. Boareto and Forti (1997) point out that *Citrus* fruits seem to be conducive to the development of the symbiotic fungus cultivated by leaf-cutting ants for food, as *Citrus* are acidic, have high levels of carbohydrates, nitrogen, vitamins and micronutrients.

Beauveria bassiana conidia germinate in 12 to 18 hours, depending on the nutritional and humidity conditions of the environment, as highlighted by Alves (1998). Despite the nutritional versatility of this entomopathogenic fungus, sources of carbon and nitrogen are necessary for the growth of the hyphae, otherwise it becomes unfeasible, as highlighted by Smith & Grula (1981). Fargues et al. (1997) point out that temperature also affects the development of such fungi, having demonstrated that between 25 and 28°C is an optimal temperature range for the development of *B. bassiana*.

In this research, there is the possibility that factors such as temperature and time spent drying the baits may also be related to the fact that there were no significant differences in the loaded and not loaded bait amounts. However, Cardoso et al. (2012), carried out a similar experiment in controlled laboratory conditions. That authors found outs that baits containing entomopathogenic fungi were carried by leaf-cutting ants and caused deleterious effects to *A. sexdens*.

Future research is needed to investigate whether bait of orange albedo-based, impregnated with entomopathogenic fungi can reach the niche of *Atta laevigata*'s queen in the field, and causes her infection and death.

5. Conclusion

Four species of leaf-cutting ants were found in this inventory in three localities at the municipality of Dourados-MS: *Atta sexdens*, *A. laevigata*, *Acromyrmex rugosus* and *A. balsani*.

Atta sexdens uses to fund its colonies in shaded places, while *A. laevigata* establish their nests on sunny locations.

The bait made with orange peel albedo was attractive to workers of *A. laevigata* in their foraging behaviour in the field, regardless of the concentration of the bioinsecticide based on the entomopathogenic fungus *Beauveria bassiana*.

This natural bait has a high potential for use in programs of biological control employing *Beauveria bassiana* conids against *Atta laevigata* in the field.

Research needs: In future researches is necessary evaluate in the field: the ideal concentration of conidia incorporated to the baits, as well as, the amount of baits to achieve the dead of the queen(s) in the ant hills.

Acknowledgments

We thank the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) for the MSc grant to the first author (Santos, A. M.) and a research productivity grant to the second author (Uchoa, M. A. – Grant Process No. 305112/2012-0), and Company Ballagro Agro Tecnologia Ltda, for the donation of some samples of the Bioinsecticide Ballveria® for the biossays of this research in the field.

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