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Colony Behavior of *Atta sexdens rubropilosa* Leaf-Cutting Ants During Biological Control

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Abstract **– Pathogenic fungi occur naturally in ecosystems and can be an excellent tool for biological control in Integrated Pest Management programs. Social insects, however, have developed a large number of defense mechanisms or strategies capable of combat these biological agents. These defense strategies are related to a series of behaviors, such as self-grooming, allogrooming, and secretion of antifungal substances that can inhibit fungal toxins. The present study aimed to observe these behaviors through a new methodology for applying *Beauveria bassiana* (Balsamo) Vuillemin, 1912, and *Trichoderma harzianum* Rifai, 1969, to control *Atta sexdens rubropilosa* Forel, 1908. Said microbial agents were applied through the spraying of a spore suspension and the making of two baits containing 20% (w/w) with *B. bassiana* and *T. harzianum*. This research supports the hypothesis that this new application technology (conidia spray) for the biological control of leaf-cutting ants may contribute to a greater amount of exhibited behaviors, or even to the intoxication, contamination or death of workers in the colony. Intoxication values of 80% were observed for *T. harzianum*, and it was also possible to assess that the nature of the pathogenic fungus interferes with behavioral responses.**

***Keywords – Beauveria Bassiana*, Biocontrol, Pathogenic Fungus, Pathogenicity, *Trichoderma Harzianum*.**

I. Introduction

Leaf-cutting ants of the genera *Atta* Fabricius, 1805 and *Acromyrmex* Mayr, 1865 (Hymenoptera: Formicidae) are eusocial insects found exclusively in the Neotropical region, from the north-east of the United States, latitude 40° N, to central Argentina, latitude 41° S [1]. In Brazil, these insects are relevant from an economic viewpoint, since they represent the main pests in Eucalyptus and Pinus plants [2] and can attack various crops and grazing areas, generating considerable profitability losses [3].

The use of toxic baits is currently the main method for controlling leaf-cutting ants [3]-[4]. In addition to providing excellent control, it is the only method with technical, economic and operational feasibility [3]. The baits are composed of an active ingredient that acts by ingestion; the latter is combined with oil and incorporated into an attractive substrate, usually dehydrated citrus pulp [5].

The active ingredient sulfluramid is the most used in bait formulations, mainly due to the efficiency of this substance in controlling all species of leaf-cutting ants [3]. However, sulfluramid is involved in several environmental issues, especially because PFOSF (perfluorooctanesulfonyl fluoride) is used to produce it, which, from its degradation, produces PFOS (perfluorooctane sulfonate), both considered highly persistent environmental contaminants [6]-[7]-[8].

Exposure to low levels of PFOS and PFOSF are not widely understood [9], but studies conducted by [10] and [11] have shown the relevance of the interactions of these fluorochemicals and their hepatotoxic effects on mice. In addition, animals in which the bioaccumulation of these substances occurred through feeding had their synth-

-esis of fatty acids and serum cholesterol reduced [10].

In view of this problem, in 2006, the Stockholm Convention classified PFOS and PFOSF as persistent organic pollutants in the soil and included them in Annex B, which caused Brazil to apply a National Implementation Plan aiming at eliminating their use [12]. As a result of this possibility that using sulfluramid represents a potential source of toxicity to the environment, researchers began to work towards assessing its impact on ecosystems and present new strategies and perspectives for new control methods against leaf-cutting ants [13].

In recent years, the use of microbial control through pathogenic fungi may represent an alternative for ant control. These organisms include the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin, 1912, and the mycoparasite *Trichoderma harzianum* Rifai, 1969 [14]-[15]. Pathogens to control leaf-cutting ants are usually incorporated into baits with an attractive substrate [16]-[17]-[18]. However, when baits containing fungal spores are used, only the mouthparts come into contact with the spores, and it is in this area where several antibiotic substances that end up inactivating fungal action are found [19]. In addition, in the bait preparation process, ants end up recognizing the contaminating agents and perform hygienic defense behaviors, such as grooming (self-grooming, allogrooming) and nest cleaning that end up preventing the action of pathogens [20]. Thus, when microbiological control agents such as fungi are used, the method for applying them must promote maximum contact with the individuals in the colony and with the individual's cuticle [21].

As hypotheses, we suggest that the application of pathogenic fungi by spraying conidia may interfere with the behavior of the workers, and that the nature of the fungus applied results in different responses in the colony; we also compare the behavioral frequencies observed when the same concentration of conidia is applied in the form of ant killer bait, assessing whether the ants are able to recognize the composition of the contaminated material while handling it, to isolate harmful substances, etc.

Finally, we compare whether this new technology can contribute to a greater number of exhibited behaviors or even to an increase in the intoxication, contamination or death of workers in the colony. Because the frequency of the behavioral acts presented can be an excellent indicative of factors closely related to the social immunity of the colony, this study is relevant to understanding the defense mechanisms of leaf-cutting ants against parasites.

Thus, the objective of this study is to assess the behavior exhibited by workers from colonies of *Atta sexdens rubropilosa* Forel, 1908 exposed to biological control by means of granulated baits in the foraging arena and through the spraying of a suspension on the fungus garden of the colony containing conidia of the fungi *B. bassiana* and *T. harzianun*.

II. Material and Methods

A. *Study Colonies*

Twelve colonies of *A. sexdens rubropilosa* were collected in the city of Botucatu, Sao Paulo, Brazil, and subjected to controlled conditions (temperature of 24 ± 2 °C, relative humidity of 80% and photoperiod of 12 hours of light) in the Laboratory of Social Insects- Pests of the Sao Paulo State University's School of Agronomic Sciences, Botucatu campus, until the bioassay was run. Each colony was housed in a 500 mL plastic container with a 1.0 cm plaster layer at the bottom to keep the fungus garden moist. Daily, leaves of the *Acalypha* spp. Pl-

-ant were provided to maintain the growth of the symbiotic fungus.

B. *Bait Preparation*

Two baits containing 20% (w/w) of two commercial products owned by the company Koppert Biological Systems ® were made. Boveril WP PL63®, composed of 5% of *B. bassiana*, strain PL63 (minimum of 1.0 x 10^8 viable conidia g^{-1}), and Trichodermil SC 1306®, composed of 4.8% *T. harzianum*, strain ESALQ-1306 (minimum of 2.0 x 10^9 viable conidia mL^{-1}).

The baits were handmade. Orange pulps from organic cultivation were dehydrated in an oven at 50 °C for 72 hours, then crushed until a powder was obtained. Subsequently, the powdered citrus pulp (60%), carboxymethyl cellulose (15%), soybean oil (5%) and the commercial product (20% of Boveril WP PL63® or Trichodermil SC 1306®) were mixed in 2.5 mL of distilled water to form pastes with different concentrations of the products being studied. The paste obtained was put in a 60.0 mL syringe for pellet production adapted from [22]. The baits were dried at a temperature of 25 ± 2° C for 24 h, then cut to a length of approximately 2 mm, similar to that of the standard commercial bait.

C. *Suspension Preparation*

Two 15.0 mL suspensions were prepared, one containing 20% (w/w) of Boveril WP PL63®, and the other containing 20% (w/w) of Trichodermil SC 1306®. For the suspensions to be obtained, the commercial product was individually mixed in an aqueous solution of distilled water and 1% Tween 80®, then homogenized for two minutes with the aid of a glass stick adapted from [20]. Afterwards, the suspensions were individually stored in 50.0 mL beakers wrapped in aluminum foil until the product was sprayed.

D. *Behavioral Analysis*

The colonies of *A. sexdens rubropilosa* containing approximately 350.0 cm^3 of fungus garden were distributed into three colonies for each bait and suspension under study, totaling twelve colonies. Subsequently, waste and leaf remains were removed from the waste and foraging chambers, and the colonies did not receive the plant substrate for 24 hours. After this period, each colony received 0.3 g of bait in the foraging arena or had 5.0 mL of suspension sprayed on their fungus garden. In the colonies that received the suspension, spraying was performed with a 500.0 mL hand sprayer, simulating a conventional application of a phytosanitary product. After bait supply or suspension application, the fungus garden container was closed with a transparent glass lid to allow the observation of the behaviors performed in the colony.

The observations were made over six days and followed an adapted scanning proposal described by [22], in which a group of individuals is analyzed quickly, and the behavior of each one is recorded. On the first day, observations were made for five hours at regular intervals of thirty minutes. For the colonies that received the bait, the behaviors analyzed were: transporting the pellet to the fungus garden (1), holding the pellet (2), licking the pellet (3), self-grooming (4), allogrooming (5) and weeding behavior (6). For the colonies that had the suspension sprayed on their fungus garden, the behavioral acts analyzed were: self-grooming (1), allogrooming (2), weeding behavior (3), removal of the contaminated symbiotic fungus (4) and presence of intoxication symptom (5). These behavioral acts were quantified by the frequency of the workers involved in each one. Twenty-four hours after the first assessment day, the plant substrate was again supplied in the foraging chamber to keep the growth of the symbiotic fungus.

From the second to the last day of observations, the abovementioned behavioral acts were analyzed, with observations being made over one hour and at intervals of fifteen minutes. Moreover, the number of dead larvae, pupae and workers was recorded daily, and so was the total weight of dead individuals and of the waste chamber residues.

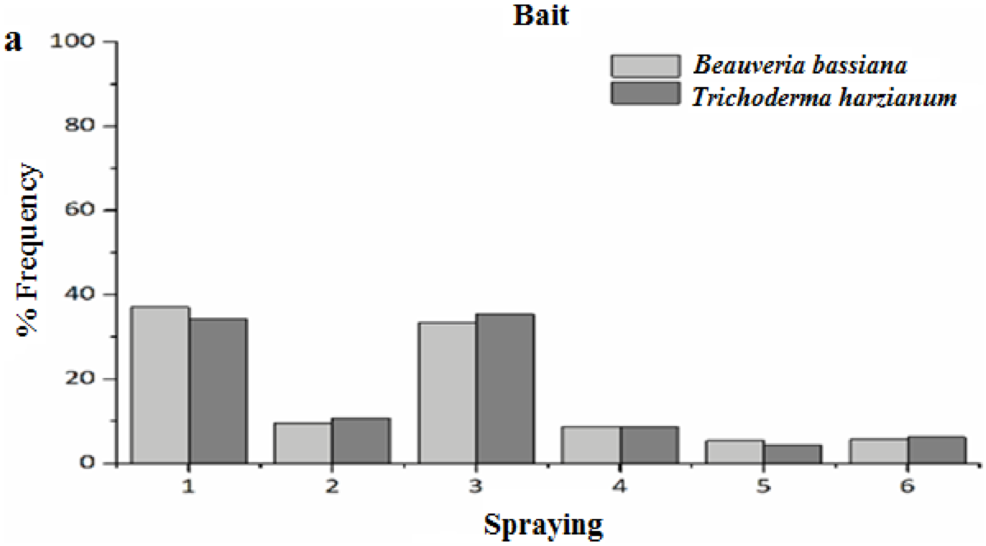
E. *Statistical Analysis*

Both for the colonies that received baits containing the fungi in their foraging chamber and those that had the suspension sprayed on their fungus garden chamber, the Wilcoxon rank sum test with continuity correction (Mann-Whitney U Test) was used to compare the superiority of the frequency referring to the behavioral acts performed by workers between *B. bassiana* and *T. harzianum* ($\alpha = 0.05$). The R environment, version 4.0.0, was used for statistical computing [23].

III. Results

A. *Behavior with Bait*

Among the behavioral acts observed on the first assessment day after bait supply, the one performed most often by the workers in the colonies that received baits containing *B. bassiana* was that of transporting the pellets to the fungus garden (Fig. 1a and Table 1). In contrast, for the colonies that received baits containing *T. harzianum*, the behavior of licking the pellets was the most frequent (Fig. 1a and Table 1). There were no sudden changes in the behaviors of the workers as to the different baits and, therefore, no significant difference was found in the following behavioral acts: transporting the pellet to the fungus garden ($W = 493.5$, $p = 0.5064$), holding the pellets ($W = 378.5$, $p = 0.2863$), licking the pellets ($W = 404$, $p = 0.5001$), self-grooming ($W = 439.5$, $p = 0.8805$), allogrooming ($W = 483.5$, $p = 0.6082$) and weeding ($W = 371$, $p = 0.236$).



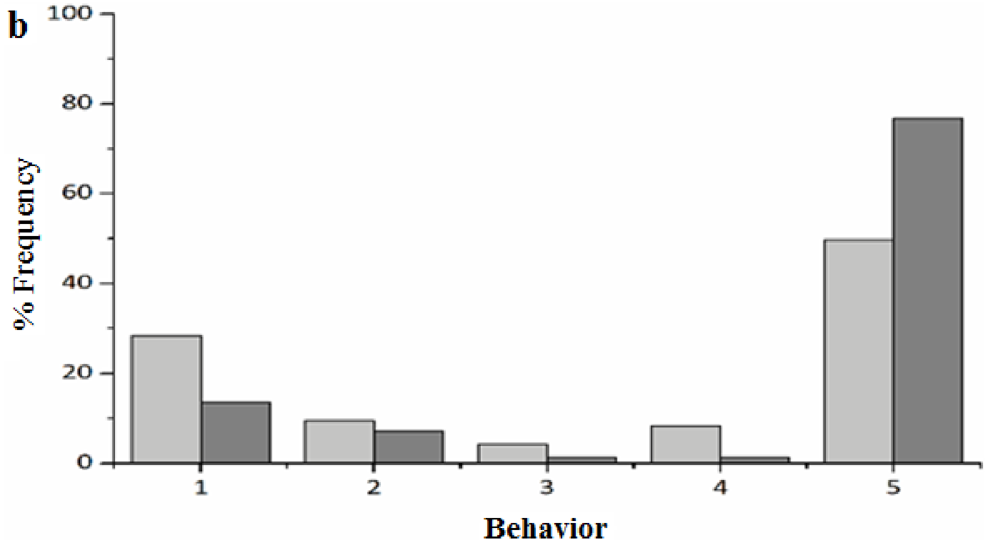


Fig. 1. Percentage of each behavioral act performed by *Atta sexdens rubropilosa* Forel, 1908, on the first assessment day of (a) the colonies that received the baits in their foraging chamber (1: Transporting the pellets to the fungus garden; 2: Holding the pellets; 3: Licking the pellets; 4: Self-grooming; 5: Allogrooming; 6: Weeding behavior) and (b) the colonies that had their fungus garden sprayed with suspensions (1: Self-grooming ; 2: Allogrooming; 3: Weeding behavior; 4: Symbiotic fungus removal; 5: Intoxication symptom).

Table 1. Observed frequency and percentage of behaviors performed by workers of *Atta sexdens rubropilosa* Forel, 1908 (Hymenoptera: Formicidae) after bait supply or suspension spray containing the pathogenic fungi under study.

Baits	**1st day**				**2nd to 6th day**			
	B. bassiana		***T. harzianum***		***B. bassiana***		***T. harzianum***	
Behavior	**Observed frequency**	**Behavior (%)**	**Observed frequency**	**Behavior (%)**	**Observed frequency**	**Behavior (%)**	**Observed frequency**	**Behavior (%)**
Transporting pellets	372.00	37.09	368.00	34.39	-	-	-	-
Holding *pellets*	97.00	9.67	116.00	10.84	-	-	-	-
Licking pellets	336.00	33.51	378.00	35.33	-	-	-	-
Self-grooming	87.00	8.67	94.00	8.79	115.00	53.23	107.00	53.23
Allogrooming	54.00	5.38	47.00	4.39	58.00	32.04	78.00	38.81
Weeding behavior	57.00	5.68	67.00	6.26	8.00	4.20	16.00	7.96
Total	1003.00	100.00	1070.00	100.00	181.00	100.00	201.00	100.00
Spraying	**1st day**				**2nd to 6th day**			
Behavior	**Observed frequency**	**Behavior (%)**	**Observed frequency**	**Behavior (%)**	**Observed frequency**	**Behavior (%)**	**Observed frequency**	**Behavior (%)**
Self-grooming	207.00	28.43	249.00	34.39	133.00	27.82	151.00	26.12
Allogrooming	69.00	9.48	134.00	10.84	103.00	21.55	145.00	25.09
Weeding behavior	30.00	4.12	23.00	35.33	18.00	3.77	12.00	2.08
Symbiotic fungus removal	60.00	8.24	22.00	8.79	49.00	10.25	12.00	2.08
Intoxicationsymptom	362.00	49.73	1418.00	4.39	175.00	36.61	258.00	44.63
Total	728.00	100.00	186.00	100.00	478.00	100.00	578.00	100.00

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Between the second and sixth assessment day, the most frequent behavior in the colonies that received baits made of *B. bassiana* and *T. harzianum* was self-grooming, being statistically similar (W = 112.5, p = 1) (Fig. 2a and Table 1). There were also no significant differences in the allogrooming (W = 121.5, p = 0.7187) and weeding (W = 97, p = 0.4944) behavioral acts. Because all pellets had already been incorporated into the fungus garden, the following behavioral acts were not observed between the second and sixth assessment days: transporting the pellets to the fungus garden, holding the pellets and licking the pellets.

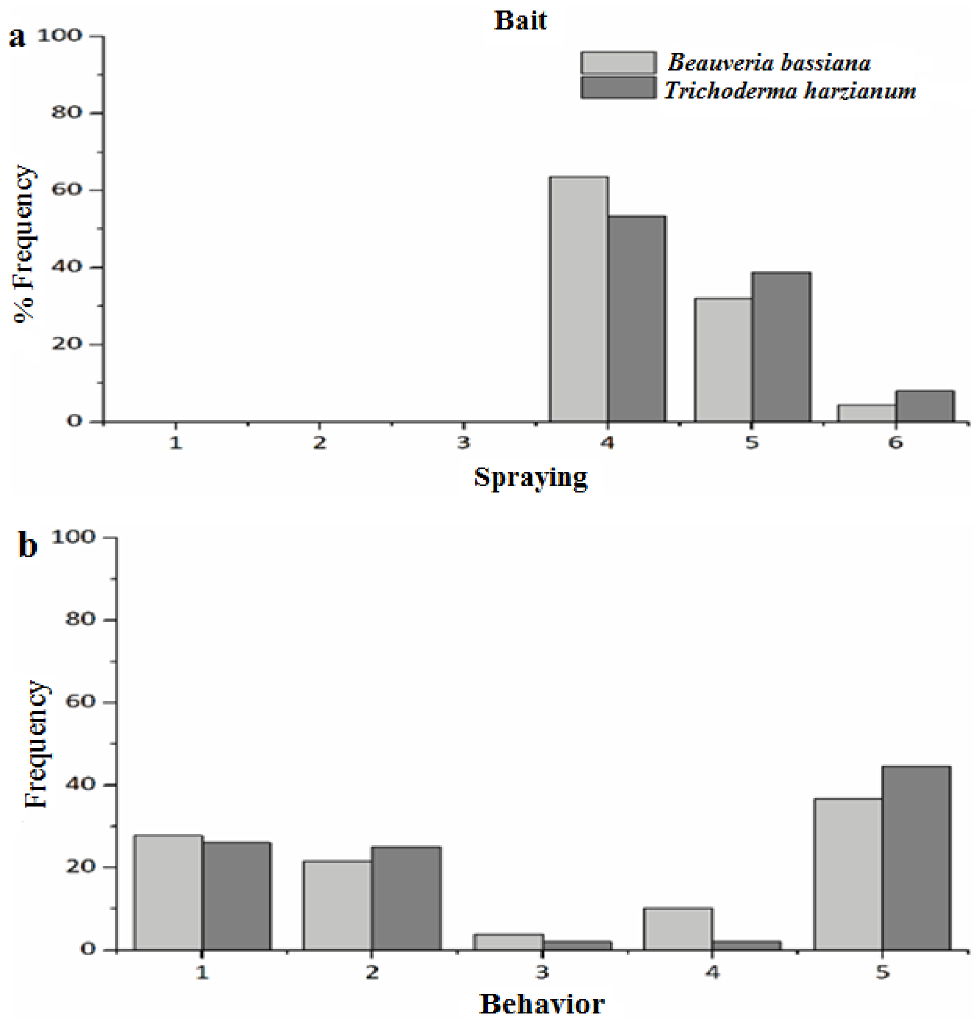

Fig. 2. Percentage of each behavioral act performed by *Atta sexdens rubropilosa* Forel, 1908, from the second to the sixth assessment day of (a) the colonies that received the baits in their foraging chamber (1: Transporting the pellets to the fungus garden; 2: Holding the pellets; 3: Licking the pellets; 4: Self-grooming; 5: Allogrooming; 6: Weeding behavior) and (b) the colonies that had their fungus garden sprayed with suspensions (1: Self-grooming; 2: Allogrooming; 3: Weeding behavior; 4: Symbiotic fungus removal; 5: Intoxication symptom).

No significant difference was found in the number of dead larvae from colonies that received baits containing *B. bassiana*, compared to those that received baits containing *T. harzianum* (W = 112.5, p = 1) (Fig. 3a and Table 2). No dead pupae were found in both colonies. On the other hand, the number of dead workers was higher in the colonies that received baits containing *T. harzianum* compared to those that received baits containing *B. bassiana* (W = 51.5, p = 0.0087). The same was observed for the weight of dead workers, which was superior in the colonies that received bait containing *T. harzianum* compared to those that received bait containing *B. bassiana* (W = 50, p = 0.0079) (Fig. 4a and Table 2). The weight of the waste chamber residues of the colony was statistically similar (W = 147, p = 0.1584).

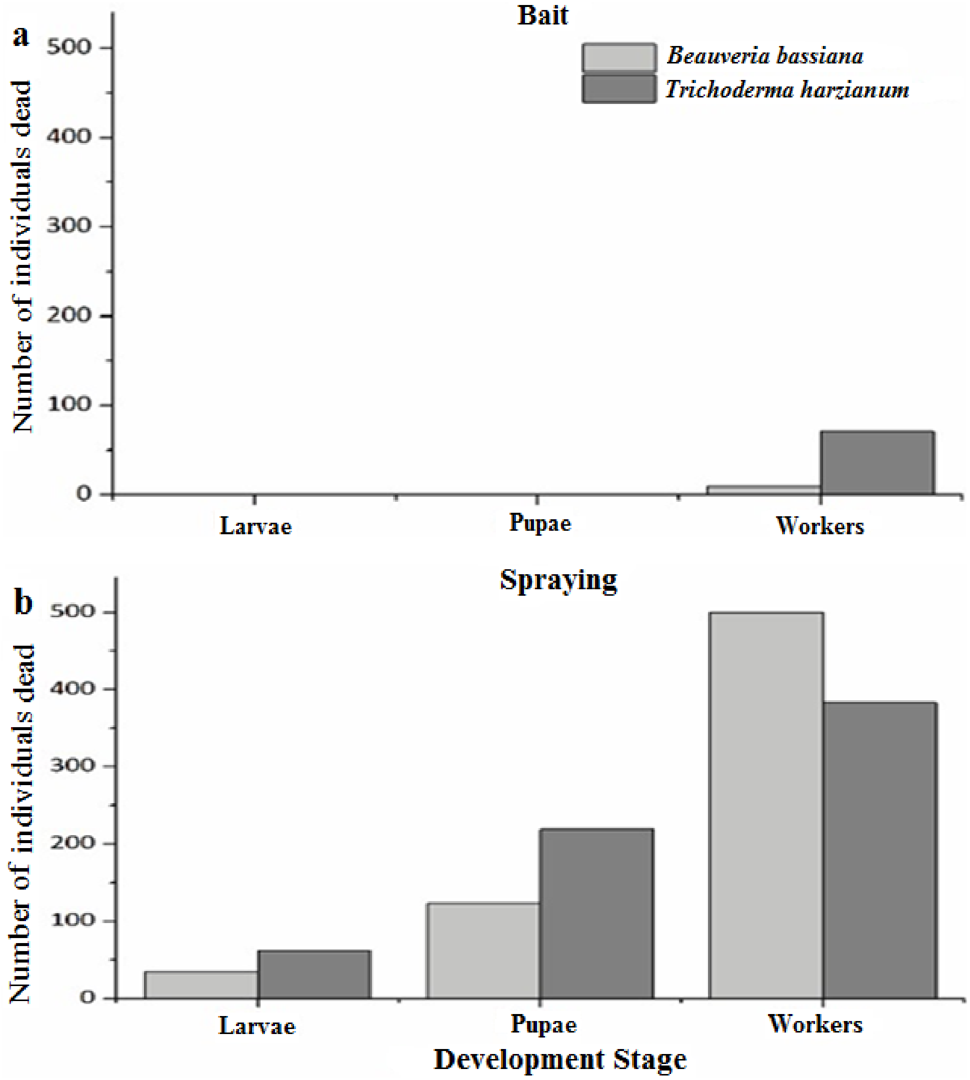

Fig. 3. Number of dead larvae, pupae and workers of *Atta sexdens rubropilosa* Forel, 1908 of (a) the colonies that received the baits in their foraging chamber and (b) the colonies that had their fungus garden sprayed with suspensions.

Table 2. Number of dead larvae, pupae and workers of *Atta sexdens rubropilosa* Forel, 1908, (Hymenoptera: Formicidae), total weight of dead individuals and total weight of residues from the waste chamber in the colonies supplied baits containing the study fungi in the foraging chamber, or that had the fungus garden sprayed with suspensions.

	Bait		**Spraying**	
Pathogens	***B. bassiana***	***T. harzianum***	***B. bassiana***	***T. harzianum***
Number of dead larvae	1	1	34	62
Number of dead pupae	0	0	123	219
Number of dead workers	9	71	500	383
Total number of individuals killed	10	72	657	664
Weight of dead individuals (g)	0.0406	0.2647	2.5771	3.6363
Weight of waste chamber residues (g)	5.9317	4.2877	17.3731	28.1045

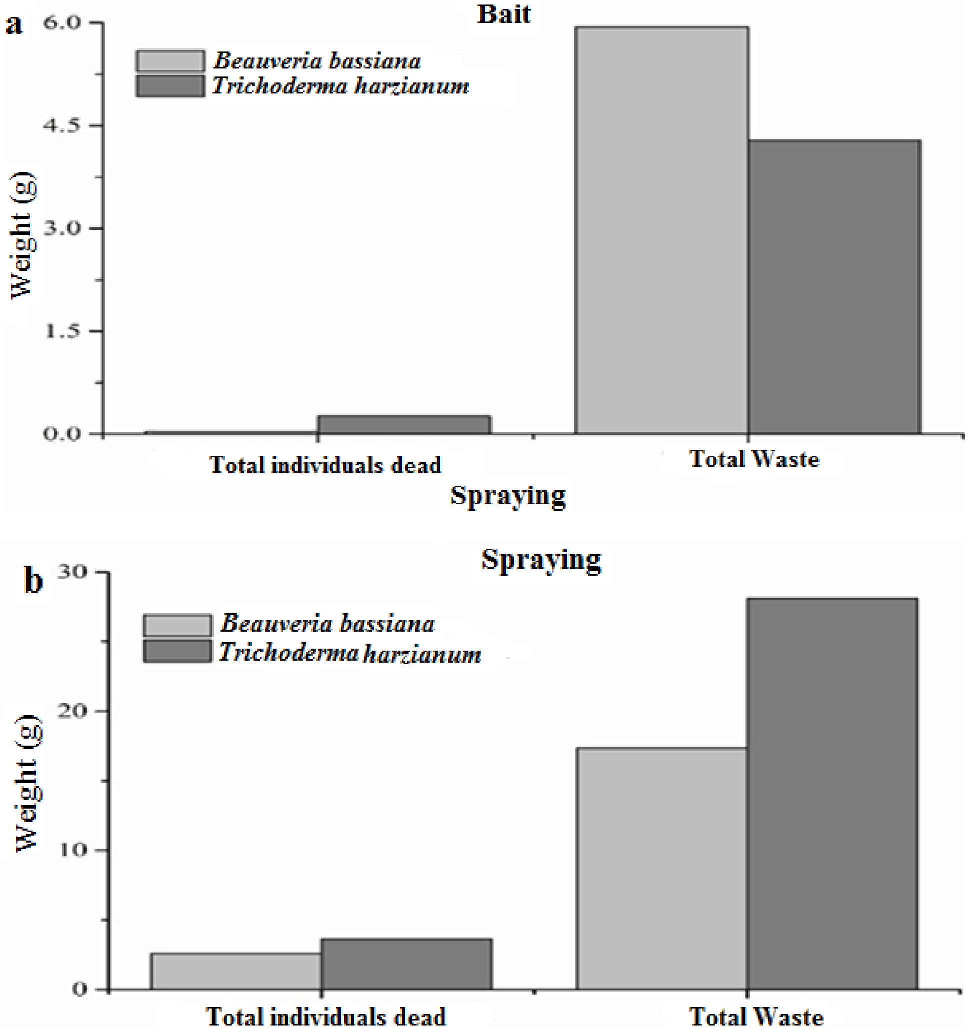

Fig. 4. Total weight of dead individuals of *Atta sexdens rubropilosa* Forel, 1908 and total weight of residues from the waste chamber of (a) the colonies that received baits containing the fungus in their foraging chamber and (b) the colonies that had fungal suspensions sprayed on their fungus garden.

B. *Behavior with Spraying*

On the first assessment day after suspension spraying, the frequency of workers with intoxication symptoms was superior in the colonies that were sprayed with the *T. harzianum* conidia suspension compared to those sprayed with the *B. bassiana* suspension (W = 0, p = 2,886 e-10) (Fig. 1b and Table 1). On the other hand, the frequency of the allogrooming behavior was superior in the colonies that had the *B. bassiana* suspension applied than in those that received the *T. harzianum* suspension (W = 111, p = 8.707 e-6). For the other behavioral acts – self-grooming (W = 279, p = 0.1394), weeding (W = 371.5, p = 0.899) and symbiotic fungus removal (W = 462.5, p = 0.07242) – no significant differences were observed.

Between the second and sixth assessment days, the most frequent behavior in both colonies was the presence of workers with intoxication symptoms (Fig. 2b and Table 1). Only the behavior of removing the contaminated symbiotic fungus showed statistical difference, being superior in the colonies that had the *B. bassiana* suspension (10.25%) applied compared to those that received the *T. harzianum* suspension (2.08%) (W = 174.5, p = 0.0085). The other behavioral acts – self-grooming (W = 97.5, p = 0.5462), allogrooming (W = 108, p = 0.8672), weeding (W = 141.5, p = 0.2079) and presence of intoxication symptoms (W = 76.5, p = 0.1402) –

were statistically similar. There was also no significant difference in the number of dead larvae (W = 99, p = 0.5836), in the number of dead pupae (W = 80, p = 0.1831), in the number of dead workers (W = 126.5, p = 0.575) (Fig. 3b and Table 2), in the weight of dead individuals (W = 82.5, p = 0.2211) and in the weight of residues from the waste chamber (W = 81, p = 0.1985) (Fig. 4b and Table 2).

IV. Discussion

The present study supports the hypothesis that this new technology for applying biological control to leaf-cutting ants may contribute to a greater number of exhibited behaviors and even to the intoxication, contamination or death of workers in the colony.

This investigation indicated the ability of workers to remove pathogenic microorganisms that could potentially destroy their fungus garden. When the colonies were sprayed with spores of fungal pathogens, the workers moved to less infected areas, such as the foraging and waste arenas.

As noted, on the 1st assessment day after suspension spraying, the frequency of workers with behavior resulting from intoxication symptoms was higher in the colonies that had the *T. harzianum* conidia suspension sprayed, compared to those that received the *B. bassiana* suspension (Fig. 1b and Table 1). The number of workers performing the self-grooming behavior increased immediately after spraying in both fungal treatments (Table 1), it was also measured the number of dead larvae, pupae and workers of *Atta sexdens rubropilosa* Forel, 1908, (Hymenoptera: Formicidae), total weight of dead individuals and total weight of residues from the waste chamber in the colonies supplied baits containing the study fungi in the foraging chamber, or that had the fungus garden sprayed with suspensions (Table 2).

On the other hand, the allogrooming behavior was more frequent in the colonies that had the *B. bassiana* suspension applied than in those that received the *T. harzianum* suspension. In this case, it is to be suggested that the amount of toxins suppressed with the aid of defense strategies, such as secretion of antibiotic compounds, in order to reduce the viability of the spores in the cuticle was used more efficiently for the *B. bassiana* suspension, and this can be evidenced by the less significant presence of intoxication symptoms and the increase in self-grooming (Table 1), an indication that, in this way, the workers were trying to perform the disinfection process. When it comes to *B. bassiana*, pathogenicity is also directly related to the production of mycotoxins such as beauvericin [24]. Interestingly, the response in the colonies treated with *T. harzianum* was more drastic, supporting the hypothesis that this fungus competes for space and nutrients [25] and is capable of controlling the growth of basidiomycetes, the phylum to which the symbiotic fungus grown by leaf-cutting ants belongs (*Leucocoprinus gongylophorus* Heim, 1957). Ants, for this reason, in addition to having their death caused by mycotoxins such as trichocenes, can also have problems with their primary source of energy. Furthermore, this biological agent produces metabolites that prevent spore germination (fungistase) and causes cells to die (antibiosis) [26], thus supporting the response hypothesis as to the nature of the fungus applied, since each fungus had a different response in the colony, especially with regard to behavioral acts.

The procedures involved in the substrate preparation for the symbiotic fungus culture include the manipulation of this substrate by the workers (licking, cutting, ingesting exudates), and ants are able to perceive the composition of the material while performing these behavioral tasks [27]. For this reason, the ants were probably able to isolate this contaminated material while handling the bait, supporting our hypothesis that they recognize the contaminated material (Table 1) and showing a high frequency for the transport behavior.

The action of pathogens varies depending on the activities performed by individuals in the colony and on the means of application used in the study, with some individuals being more susceptible than others [28]. As observed in the present study, the means of application was preponderant for effective control, being efficient in the host-fungal pathogen relationship, thus contributing to the occurrence of a large number of dead individuals and residues in the waste chamber in the treatments that received the spore suspension (Fig. 4b). It was possible to observe as well that the treatments that received the spray also presented significant numbers of dead larvae, pupae and workers (Fig. 3b).

The spray application methodology proved to be more efficient than the control using ant killer baits. Since both had the same proportion of formulated product, greater total weights being found for dead ants and waste residues in the treatments with spore suspensions compared to the treatments composed of ant killer baits supports the hypothesis that this may have occurred as a result of the formulation type and of the product application. Additionally, a greater amount of exhibited behaviors, as well as of deaths of individuals, was observed.

In short, microbial control may represent a promising alternative in the near future, but it still has the major challenge of necessarily breaking the mechanisms and strategies developed by leaf-cutting ants. The application of biological agents effectively can bring about countless environmental benefits. Research involving a more sustainable agriculture with less environmental impacts on ecosystems should be encouraged.

The frequency of exhibited behavioral acts can be an excellent indicator closely related to factors such as the social immunity of the colony, and the present study is relevant in the sense of improving knowledge on the defense mechanisms of leaf-cutting ants against biological agents.

V. Conclusion

The evaluated fungi showed their potential as agents for controlling colonies of *Atta sexdens rubropilosa* when sprayed in the fungus garden. This research supports the hypothesis that this new application technology (conidia spray) for the biological control of leaf-cutting ants may contribute to a greater amount of exhibited behaviors, or even to the intoxication, contamination or death of workers in the colony. Intoxication values of 80% were observed for *T. harzianum*, and it was also possible to assess that the nature of the pathogenic fungus interferes with behavioral responses. Thus, representing new perspectives on the use of biological control for colonies of leaf cutting ants. Research and studies should be conducted to verify the efficiency of these biological agents.

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