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Mapping the Adhesion of Different Fungi to the External Integument of *Atta sexdens rubropilosa* (Forel, 1908)

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***Abstract* – Ants of the genus *Atta* are considered important defoliator pests, attacking different crops, pastures, and reforested areas. The entomopathogenic and opportunist fungi, considered natural enemies of these pests, have the potential to be used for biological control. In order to identify the most susceptible regions to conidial adhesion, *Atta sexdens rubropilosa* workers were exposed to fungal suspensions (10^6conidia/mL) of the following fungi: *Beauveria bassiana, Metarhizium anisopliae* and *Aspergillus flavus*, and posteriorly submitted to analysis using scanning electron microscope (SEM). The results obtained for the periods of 24, 48 and 72 hours showed that, after the fungi exposure, the conidia from *B. Bassiana* were the most successful in adhering and germinating, and their preferential site in the ant's body was the region of the clypeus/mandible; while in *M. anisopliae* this preference was restricted to the gaster and, in *A. flavus*, to the antennal fossa. These results strongly suggest that the processes of adhesion and germination of each fungus occur in different moments and places of the insect's body; however, independently of these factors, the infection leads the insect to death. The results of the mortality test showed no significant statistic difference between the fungi *B. bassiana* and *M. anisopliae;* i.e., both were virulent and even when compared with *A. flavus*, the latter presented low virulence potential.**

Keywords* – *Aspergillus Flavus*, *Beauveria Bassiana*, Biological Control, Leafcutter, Integument, *Metarhizium Anisopliae.

I. Introduction

The leafcutter ants of the genus *Atta* (*Fabricius*, 1804) (Formicidae: Attini), known as "Saúva", constitute a group of insects able to cause significant economic losses, partially or totally defoliating important crops for agro- and silviculture [1]. In Brazil, one of the most important species is the *Atta sexdens rubropilosa* Forel, 1908, forages a large variety of crops and is considered one of the main reforestation pest [2]-[3]. These social insects are divided into castes and they work for the maintenance of their fungus garden, which is used by the colony [4]. Other organisms are found in this environment, including the pathogenic fungi [5]-[6].

Studies available in the literature have demonstrated that certain entomopathogenic fungi present real potential to be used in the biological control of leafcutter ants [7]. The entomopathogenic fungi of the species *Metarhizium anisopliae* and *Beauveria bassiana* are more advantageous and efficient than the commercial chemical formicides used to control these insects [8]. Thus, biological control agents have increasingly become an economically and ecologically viable alternative [9]. Furthermore, the possibility of using entomopathogenic fungi and pesticides concomitantly is not excluded, making the integrated management programs more efficient and sustainable [10].

The entomopathogenic fungi are able to infect different stages of the host development; i.e., egg, larva, pupa and adult, and this characteristic is very important when thinking about control. Most entomopathogenic fungi are specialized in penetrating the insect integument, and the stages of this process are commonly classified as follows: adhesion, germination, appressoria formation, penetration peg formation, penetration, colonization and even reproduction and dissemination [11].

The isolation of *B. bassiana*, *M. anisopliae* and *A. parasiticum* from the body of *A. Mexicana* queens in the state of Morelos in Mexico after the nuptial flight is the first report of entomopathogenic fungi in this species [12]. In natural conditions, lineages of *B. bassiana* have already been isolated in queens and workers from other species of the genus *Atta* [13]-[14]-[15].

In general, the entomopathogenic fungi infect the cuticle of the hosts by releasing conidia, which adhere and germinate, forming a number of structures that will help in the penetration into the insect [16]. These fungi commonly produce a variety of enzymes with the capacity to degrade (lyse) the cuticle of the host during the penetration process. The processes of conidial adhesion and formation of appressoria would be of utmost importance, once they represent the first events to stablish the fungi-host relation [17].

The fungus *Metarhizium anisopliae* is an obligate and generalist entomopathogen, once it infects a wide variety of insects, including the leafcutting ants [18]-[19]. This fungus has white mycelia and oval pale greenish-gray conidia mass [12]. The colony of *B. bassiana* is white with hyaline mycelia, with cottony or mealy aspect. The conidia are globose and have smooth walls [12]. Several authors [20]-[21], have reported the presence of this fungus in Atta ants. *Aspergillus flavus* is considered a facultative fungus and has been isolated from leafcutter ant *Acromyrmex echinatior* workers [22]. *Aspergillus flavus* was reported for the first time the presence of this fungus in *A. sexdens rubropilosa* workers discarded in the waste deposit of a laboratory nest [23]. Studying the behavior of *A. sexdens* (L.) workers maintaining the colony fungus garden reported the presence of *A. flavus* with white mycelia [24]. During the sporulation stage, these fungi were yellowish green, with spherical conidia and irregular walls [12].

Considering these data, the present study aimed to map and characterize the preferential regions for conidia adhesion in the body of *A. sexdens rubropilosa* worker ants, after exposing the individuals to fungi isolates (LESF-206 *M. anisopliae*), (LESF-218 *A. flavus*) and (LESF-231 *B. Bassiana*) for 24, 48 and 72 hours, and using scanning electron microscopy techniques.

II. Material and Methods

1) Collection of the Ants

The forager workers of *A. sexdens rubropilosa* used in this experiment were obtained from the colony of the Laboratory of Social-Pest Insects, located in the Fazenda Experimental Lageado of the Agronomic Sciences Faculty - UNESP-Botucatu, SP, Brazil, latitude (22º 50' 48" S), longitude (48º 26' 06" W) and height (817,74 m), maintained according to the methodology proposed by Forti [25].

2) For a better understanding of the insect body regions analyzed in this study, see (Fig.1).

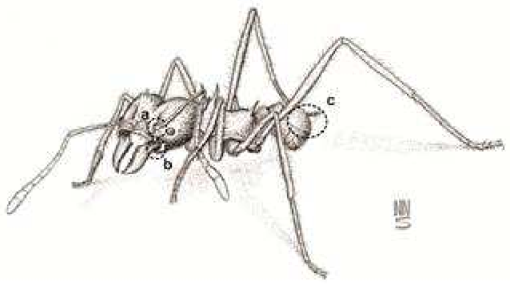


Fig. 1. *Atta sexdens rubropilosa* leafcutter ant: a-antennal fossa, b-clypeus, c-gaster.

3) Collection of the Fungi Isolates:

The isolated fungi used in this study were collected from queens from the Laboratory of Fungal Ecology and Systematics (Laboratório de Ecologia e Sistemática de Fungos - LESF). After inoculation, the isolates were multiplied in 5-cm Petri dishes containing malt agar (2%) and maintained in BOD (temperature+_25°C), for two weeks. When microconidia started emerging on the surface of the dish, serial transfers were performed fortnightly. To prepare the suspension, each dish was open and 10 mL of distilled water + tween 0.001% were added. Then, the conidia were counted using a Neubauer chamber. The ants were exposed to the fungi isolates (LESF-206 *M. anisopliae*), (LESF-218 *A. flavus*) and (LESF-231 *B. Bassiana*) at the concentration of 10^6 conidia/mL.

4) Stablishing the study groups

The treatment groups comprised forager ants (cephalic capsule +- 2mm) individually immersed for 03 seconds in the fungal suspensions (LESF-206 *M. anisopliae*), (LESF-218 *A. flavus*) and (LESF- 231 *B. Bassiana*), adapted from Loureiro and Monteiro [26], after being collected from the same colony using entomological tweezers. Ten workers were placed into each pot (250mL) containing cotton wetted with water, with five repetitions for each suspension. A control group was established, where the individuals were immersed in either distilled water (5 pots containing 10 workers).

5) Statistic Analysis

The mortality test (*Log-Rank test*) was performed to compare the efficiency of each fungal suspension regarding virulence. For this, all the treatment groups (here in described) were maintained in laboratory conditions and were daily assessed until only one individual was left. The data obtained were plotted and statistically analyzed using Biostat 5.0 software with probability 5%.

6) Scanning Electron Microscopy:

For the application of this technique, workers were collected from each treatment group (different fungi and different exposure periods). The whole specimens were fixed in paraphormaldehyde 4% for 15 days and in phosphate potassium buffer at 0.05M and pH 7.4 for 24 hours. Then, three workers were attached with double-sided tape to each aluminum stub in three different positions (dorsal, left lateral and right lateral). The legs and the posterior portion of the antennae were removed for better visualization. Then, the samples were dehydrated in crescent series (70%, 80%, 90% and 95%) of acetone and water solution for 5 minutes each bath, dried at critical point and metallized for 4 minutes with gold in sputtering BAL-TEC model SCD 050. After, the material was taken for analysis and photodocumentation under scanning electron microscope Hitachi TM 3000 in the Electron Microscopy Laboratory of the Bioscience Institute, UNESP-Rio Claro, SP, Brazil.

III. Results

The present study brings the first ultramorphological mapping of the body regions of *A. sexdens rubropilosa* forager workers, here considered the most susceptible to the germination and penetration of fungi (LESF-206 *M. anisopliae*), (LESF-218 *A. flavus*) and (LESF-231 *B.*

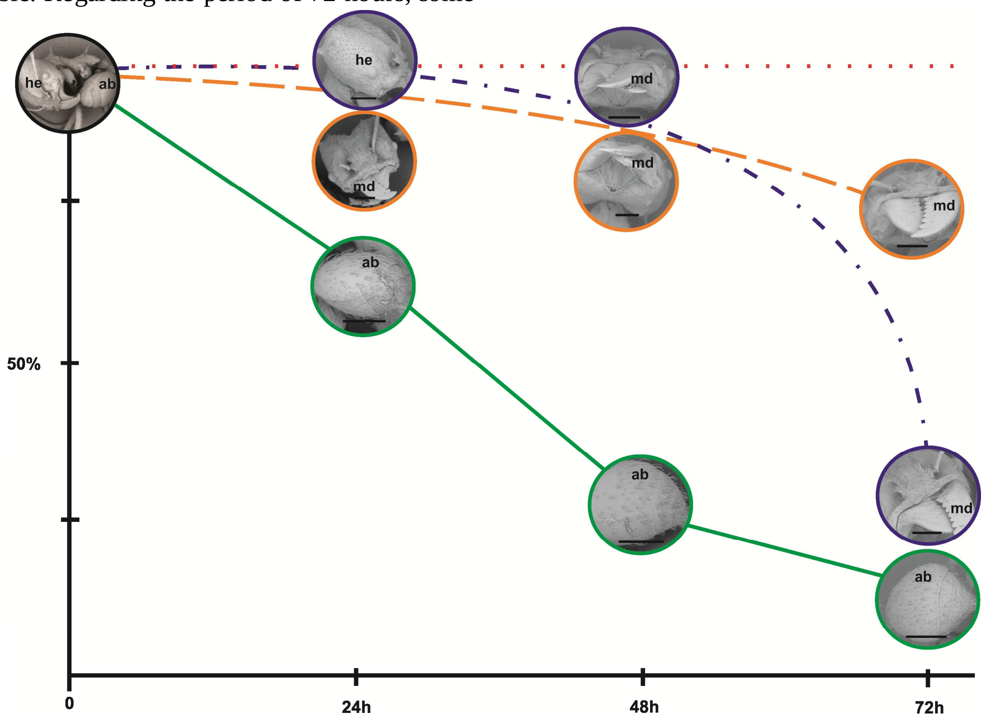
Bassiana), to which the workers were exposed in periods ranging from 24 to 72 hours.

The fungus *M. anisopliae* showed higher levels virulence in the mortality test, eliminating half of the workers in the first 48hours.The same result was obtained for the fungus *B. bassiana* in the period of 72 hours. There was no significant difference when these fungi were compared with each other. Regarding the fungus *A. flavus*, these results were obtained only after 120 hours of exposure (Graph 1).

The morphological results showed that the fungi *M. anisopliae* and *B. bassiana* needed approximately 48 hours to be successful in the germination process; while the fungus *A. flavus* needed a longer period – approximately 72 hours to germinate successfully. In addition, it was stablished that the fungi *B. bassiana* (Fig. 2A, B and C), *M. anisopliae* (Fig. 2D, E and F) and *A. flavus* (Fig. 2G, H and I) preferred to adhere to the regions of the clypeus, gaster and antennal fossa respectively. With specific regard to the fungus *B. bassiana*, after 24 hours of exposure to the spore suspension, a large number of conidia were observed deposited predominantly on the dorsal surface of the insect, and the junction of the clypeus with the mandible is where the largest number of adhered conidia is found (Fig. 2A). It is important to note that, although many conidia adhered to the surface of the insect were observed in the period of 24 hours, it was not possible to detect the germination process in any of them. This same fungus, analyzed 48 hours after the exposure, behaved similarly to the previous period; however, the conidia had already germinated, and were located in the dorsal surface of the insect, preferably in the junction clypeus/mandible. Regarding the period of 72 hours, some conidia preferably adhered to the basis of the sensilla, and others projected the germ tube (Fig. 2C), to attach to the integument, a process that may occur in the most sclerotized region of the body; i.e., the mandible region.

Analyzing the behavior of the fungus *M. anisopliae,* it can be verified that the conidia adhere to all the extension of the dorsal surface of the three segments (head, thorax and gaster) of the insect's body. However, the preferential adhesion region is the gaster, which in the period of 24 hours is seen as a large conidial conglomerate (specifically in the suture between the tergite and the sternite), surrounding the sensilla located there. In this case, it is not possible to observe any signs of germination (Fig. 2D). In the period of 48 hours, germinating conidia predominate, preferably in the dorsal region of the gaster, located closer to the insertion gaster/post-petiole (Fig. 2E). In the period of 72 hours, the infection onset is observed, with the presence of the germ tube in the dorsal region of the gaster, very close to the sensilla or even covering them (Fig. 2F).

For the fungus *A. flavus*, the results showed the accumulation of conidia predominantly in the dorsal region; however, preferably adhered to the antennal fossa (Fig. 2G, H and I). In the 24 and 48-hour periods after the exposure, no signs of germination are observed in this specific site (Fig.2 G, and H); however, the presence of conidia adhered to the integument is identified. In the period of 72 hours, conidia adhered to the region of the antennal fossa are observed, with evidence of germ tube formation (Fig. 2I)



Graph. 1. Mortality curve graph in the first 72 hour. --*B. bassiana* __*M. anisopliae* ---*A. flavus* ...Control
md = mandible **he** = head **ab** = abdômen. **Bars scale**: 0,5mm.

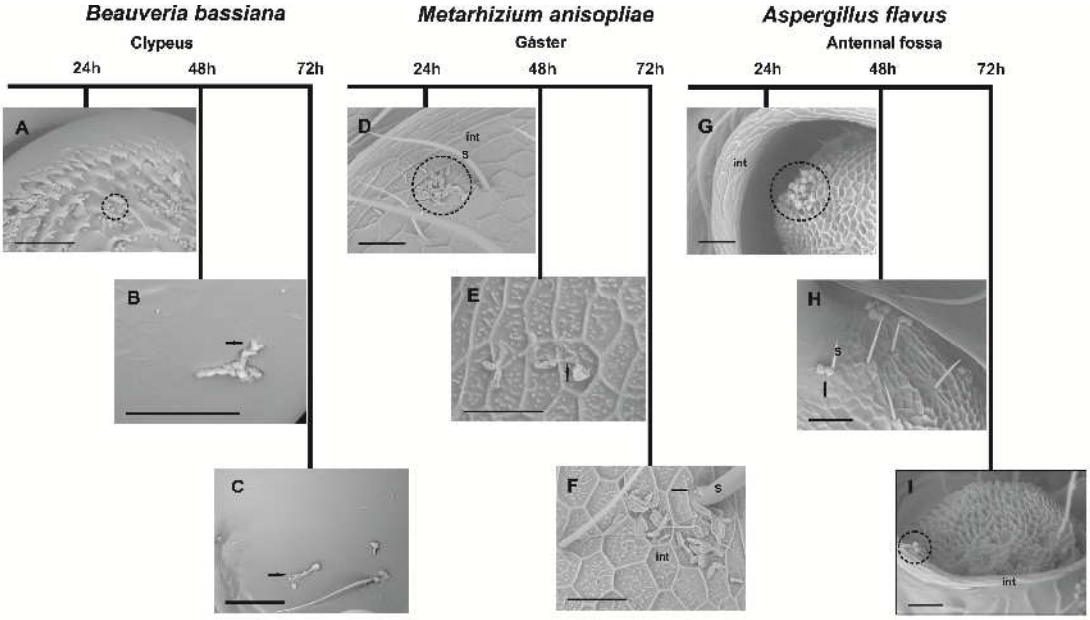


Fig. 2. Body regions of A. sexdens rubropilosa workers, with the presence of *B. bassiana* (**A-C**), *M. anisopliae* (**D-F**) and *A. flavus* (**G-I**) fungi, and the phases (adhesion, germination and penetration) in relation to the exposure periods (24, 48, 72 hours). Dotted circles in **A**= conidia adhered to the integument ornamentation, **D**= conidia conglomerate, **G**= conidia conglomerate, **I**= conidia germinating in the torulus edge **Int**= integument, **s**=sensilla, **black arrow**=conidia **Bars scale** = 20 µm.

IV. Discussion

Regarding the exposures using *M. anisopliae,* the results obtained here for *A. sexdens rubropilosa* corroborate those obtained by Castilho [27], who found higher levels of virulence exposing *A. bisphaerica* soldiers to this fungus in the period of 48 hours. However, these results oppose Diehl- Fleig [8], who studied *A. sexdens piriventris* soldiers and found that 72 hours of exposure would be necessary to obtain 50% of mortality. Accordingly, Loureiro and Monteiro [26], and other authors found that *B. bassiana* and *M. anisopliae* would present a mortality rate higher than 50% in the exposure of *A. sexdens sexdens*. In this case, the period of exposure was shorter than 72 hours, noting that in natural conditions the high mortality of the individuals would occur only after 168 hours (7 days). Regarding the fungus *A. flavus*, the present study showed that an average of 120 hours (5 days) would be necessary for 50% of mortality, corroborating Ribeiro [28], who studied the fungus *A. ochraceus* applied in *A. bisphaerica* workers.

The ongoing search for strategies to control pest insects has been currently stimulated, once the use of chemical synthetic agents have caused several problems to non-target organisms, including the accumulation of residues in the environment and the development of resistance by the insects. In this sense, the development of studies with the potential to find efficient and sustainable ways to control or even minimize the damages caused by these pests is of utmost importance.

Thus, the strategy to control these insects using entomopathogenic fungi is a promising alternative, and this study was developed based on this assumption. The interaction fungal conidia/target insect cuticle is a frequent process and occurs due to the action of glycoproteins, extracellular enzymes, and electrostatic and hydrophobic forces [29]-[30]. The adhesion process depends on the esterase and protease enzymes present in the surface of the conidia. These enzymes affect the surface of the insect's integument, favoring the nutrition and the germination of the fungus [16]. This event is followed by the penetration and infection of the insect, already studied by St. Leger [17], in fungi from the species *M. anisopliae.* The authors observed that the penetration of the germ tube (structure used by the fungus to infect the host) in the target insect's integument (*Manduca sexta)*, followed by the colonization, generalized infection and posterior death of this insect, would occur within 3-10 days after the initial contact [31]. These interactions would occur through enzymatic activities [32], and the mechanical pressure exerted by the germ tube [33]. Although these processes are commonly observed in the environment, they depend on other potentially limiting factors, such as: the presence or lack of humidity and nutritional components of the cuticle (necessary for the chemical reaction to occur); the action of mycotoxins [34]-[35], the fungal virulence factor and the susceptibility of the host insect to the infection [36]-[29].

The literature has reported that the conidial adhesion sites in the insect's body as well as in the intersegmental membranes can vary. Depending on the adhesion site, the process of penetration into the insect can be easier or more difficult [37]. Furthermore, the topography of the insect's cuticle surface would influence the formation of appressoria; i.e., the growth of the germ tube tip, which has previously been studied in *M. anisopliae* fungi. Although the ornamentation of the cuticle is an important factor for conidial adhesion and some studies showed that the germination could successfully occur either in irregular or smooth surfaces; however, the formation of appressoria would always be more successful on smooth surfaces [17].

The insects in general have the so-called natural apertures; e.g., the buccal and the anal cavities. These sites can be considered important for the fungal infection onset [38]-[39]-[40]. Bittencourt [41], [42], verified through three distinct methodologies that the penetration of the fungus *M. anisopliae* (in ticks) would probably occur via integument. Broome [43], had already reported this result studying fire ant larvae (*Solenopsis*) exposed to *B. bassiana* suspension, showing that even with the presence of these apertures the fungus prefers to penetrate into the insect through other sites.

When searching for new efficient strategies for biological control, in addition to analyzing important parameters, such as viability and availability (easy to be obtained) of the organism and storage conditions for further use, it is necessary to take into consideration the time needed for these organisms to act successfully. In this sense, Moino [26], who studied underground termites (*Heterotermes tenuis)* exposed to *B. bassiana* and *M. anisopliae* conidia using SEM techniques, reported the presence of these structures in the adhesion phase in the insect's surface and some of them were even in process of germination in the period of 24 hours. Studying bed bugs exposed to *M. anisopliae* after 24 hours, other authors quantified 5-20% of germ tubes in the conidia adhered to the surface of the insect [45]. In this sense, this study used *A. Sexdens rubropilosa* leafcutter ant workers and demonstrated that in the period of 24hours (regardless the fungi species) after the exposure to the spore suspension, conidia were always present; however, not all of them reached the germination stage (Table 1). It is important to emphasize that the workers studied here; i.e., the leafcutter ants *A. sexdens rubropilosa* have been considered important pests for the agriculture in general; and also that the literature has reported that external integumentary surface of these insects is irregular (with the presence of sensilla, gland apertures, etc.), offering important adhesion sites for entomopathogenic fungal conidia.

The present study mapped the different body regions of workers from this ant species, verifying that some regions of the insect's body were important and preferential for the fungi, both for conidial adhesion/germination and for the penetration of hyphae into the insect's body. Thus, it was observed here that the clypeus/mandible, gaster and antennal fossa of *A. sexdens rubropilosa* would be preferential for the adhesion and germination of conidia from the three species of fungi analyzed: *B. bassiana, M. anisopliae* and *A. flavus*, respectively.

With specific regard to *A. flavus*, when studied by other authors, this fungus was considered to have characteristics of pathogenicity; however, specifically concerning the exposure bioassays performed in ants of the genus *Acromyrmex*, a strong correlation between this exposure (crescent doses of spores) with an increase in ant mortality [18]. On the other hand, it is known that fungi of the genus *Aspergillus* in general do not display pathogenic characteristics; moreover, these fungi have been abundantly found in tropical soils, playing the role of decomposers [46]. Therefore, the species *A. flavus* could be considered a facultative pathogen found in different species of plants and animals [47]-[48]. In this study, the *A. flavus* displayed slower germination in comparison with the entomopathogens analyzed.

Regarding the fungus *B. bassiana*, also studied here, the region elected for conidial adhesion was the clypeus, structure that delimits the margin of the buccal cavity and has the function of aggregating the mouth parts, including the mandible. Veen [49], reported the presence of hyphae in grasshopper palps (*Schistocerca gregaria*) after the contact with the fungal suspension of the species *M. anisopliae*. The clypeus has an irregular surface, with sulci and cavities that ensure the mobility of the mandible [50]-[51]. These characteristics of the clypeus favor the accumulation of conidia in this region. In addition, after using the antennae for the body's hygienization, the workers accumulate the excess of conidia in the infrabuccal cavity [52]-[53]-[54], which facilitates the exposure of this region to the entomopathogen, in this case, the *B. bassiana*.

The bioassays performed in this study clearly demonstrated the preference of the fungus *M. anisopliae* to adhere the conidia in the gaster region, located in the distal part of the insect's body and comprised of the reproductive, excretory and digestive systems. It is important to emphasize that many natural apertures are found in this region, corresponding to glands and spiracles, which are, due to their morphology, candidates for the adhesion and germination of conidia.

Data in the literature suggest that the insects whose body segments are highly sclerotized usually suffer fungi invasion via arthrodial membranes or even spiracle apertures [55]-[34]. In ants, particularly *A. sexdens rubropilosa*, the gaster has irregular texture and is comprised of segmented plates, tergites and sternites. Sensilla are observed, mainly concentrated in the distal end [56]. Specifically concerning the junction of the gaster and the post-petiole, the present study demonstrated that this is the adhesion site of *M. anisoplia* conidia, corroborating Sosa-Gomez [45], who studied bedbugs *Nezara viridula* and verified the presence of *M. anisopliae* conidia in areas containing a large number of bristles.

The insect antennae are sensorial organs of extreme importance in the communication and localization of the substrate for the tribe *Attini*. The antennae are also considered important apparatus for the hygienization and removal of microorganisms from the insect's body [57]-[4], constituted as geniculate organs with a basal bulb placed in a cavity named antennal fossa [58]-[59]. This study established that this site also favors the concentration and accumulation of *A. flavus* conidia, and this is the first time this data is reported in the literature. This information is of utmost importance for the elaboration of sustainable strategies for the development of biological control methods.

For better visualization, the results are summarized in Table 1.

Table 1. Body regions of *A. s. rubropilosa* workers, with the presence of *B. bassiana, M. anisopliae and A. flavus* fungi, and the phases (adhesion, germination and penetration) in relation to the exposure periods (24, 48, 72 hours).

Fungus/Time	24 hours	48 hours	72 hours
Beauveria bassiana **Mandible/Clypeus**	Conidia adhered	Conidia germinanting and hyphae penetrating in the mandible	Conidia germinating and hyphae penetrating in the mandible
Metarhizium anisopliae **Gaster**	Conidia adhered	Conidia germinating	Conidia germinating/penetrating
Aspergillus flavus **Antennal Fossa**	Conidia adhered	Conidia adhered	Conidia germinating

V. Conclusion

In broad terms, the present study mapped and analyzed the body regions of *A. sexdens rubropilosa* workers ants, targeting the development of biologic control strategies using fungi with entomopathogenic characteristics. The results showed that, according to the type of fungus studied and the exposure periods established for the bioassay, the three different fungi showed distinct preferences concerning the region of the insect's body and also that these organisms need different time for the processes of conidial germination and hyphae penetration to occurs. Thus, this study is the first to demonstrate that *B. bassiana* and *M. anisopliae* fungi need approximately 48 hours to adhere, germinate and penetrate the insect, preferring the regions of the clypeus/mandible and the gaster respectively; while *A. flavus* fungi need approximately 72 hours, and prefer the antennal fossa to fulfill the same processes.

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