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Characterizing Naturally-Occurring Entomopathogenic Fungi in Reproductive Females of *Atta spp*.

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***Abstract* – Leaf-cutter ants are considered agricultural pests in the Neotropical region, and great is the demand for a biological control of their populations. The present study isolated entomopathogenic fungi of winged reproductive females (queens) of the genus *Atta* and assessed their *in vitro* characteristics, in order to provide resources for future studies in search of biological-control agents against these insects. The ants were collected during their nuptial flight and kept in laboratory for three months. The dead individuals were evaluated, from which eight species of fungi were isolated: *Beauveria bassiana, Purpureocillium lilacinum, Isaria fumosorosea, Metarhizium anisopliae, Fusarium oxysporum, Fusarium solani, Aspergillus flavus* and *Aspergillus niger*. The studied isolates produced conidia with high viability and high counts, from $1.25x10^6$ to $7.14x10^6$ conidia mL^{-1}. The isolates of *P. lilacinum* and *I. fumosorosea* showed the highest rates of mycelial growth, followed by the isolates of *B. bassiana*. Therefore, despite these being just *in vitro* results, these fungi proved to have potential as biological-control agents against leaf-cutter ants.**

***Keywords* – Biological Control, Leaf-Cutter Ants, Mycelial Growth, Winged Females.**

I. INTRODUCTION

Leaf-cutter ants, genera *Atta* and *Acromyrmex* (Hymenoptera: Formicidae) are considered pests of major importance for the losses they cause in agriculture, livestock farming and forestry [1], [2]. The control of leaf-cutter ants is performed by means of synthetic insecticides, due to the unavailability of highly effective alternatives [2], [3].

Generalist entomopathogenic fungi, such as *Metarhizium* and *Beauveria*, potential biological-control agents against insects, are frequently found in the soil, e,g., as saprobes, and can attack many insects at different stages [4]-[7]. In the case of leaf-cutter ants, the occurrence of *Metarhizium* and *Beauveria* has already been reported, as well as of other fungi such as *Purpureocillium*, *Fusarium* and *Aspergillus*, in queens of *Atta sexdens rubropilosa*, *Atta laevigata* and *Atta capiguara* [8]-[12]. However, these studies, many times, isolated fungi on the surface of the body, a place where growth of opportunistic fungi cannot be ruled out.

In leaf-cutter ants, the pathogenic action of microorganisms varies greatly depending on the studied ant caste and the application means [4]. In addition, these insects have several defense mechanisms, such as removing contaminated material from the nest [13], [14]. Such facts make it difficult to control these colonies and show the importance of better understanding the characteristics of potentially useful fungi, so that it may increase the efficiency of those in control of leaf-cutting ants.

Studies on fungi occurring in leaf-cutter ants can potentially enable the microbial control of these pests, reducing the environmental impacts of using synthetic insecticides. Thus, the objective of the present research

was to isolate and characterize naturally-occurring entomopathogenic fungi in reproductive females of the genus *Atta*, collected during the nuptial flight period; an inner portion of their thorax tissue was removed for fungus isolation, thus minimizing the likelihood of isolating non-entomopathogenic fungi.

II. Material and Methods

A. *Collection of Reproductive Females for Fungal Isolation*

Collection was performed in 2007 and 2008, totaling 1,000 specimens. Each year, on the day of the nuptial flight, a sample of 500 winged reproductive females (queens) of *Atta* spp. was collected, mainly *A. sexdens rubropilosa*, shortly after they descended to the soil to start their own nests, in a small area of *Eucalyptus* spp. (~1.3 ha) [15], located at Lageado Farm (22°49'53.25"S and 48º25'24.22" W), Faculty of Agronomic Sciences - FCA / UNESP, Botucatu, SP, Brazil. The newly collected females were stored in plastic containers measuring 11 cm in diameter and 8 cm in height, with a 1 cm plaster layer at the bottom; the temperature in the laboratory was maintained at around 24 °C. The humidity in the container was controlled through addition of distilled water to the plaster.

B. *Fungus Isolation and Identification*

From collection until 90 days later, the mortality of the reproductive females was monitored daily at the Laboratory of Social Insects-Pests (FCA/UNESP). The dead ants were superficially disinfected for fungus isolation: immersion for 2 minutes in a 70% alcohol solution, in 2% sodium hypochlorite and, subsequently, distilled water. Afterwards, an inner portion of their thorax tissue was removed with a sterile scalpel. This material was chosen to minimize the isolation of non-entomopathogenic fungi that could possibly be on the surface of the insects. This portion was sown in PDA (potato dextrose agar) medium supplemented with the antibiotic penicillin-G and incubated at 25 °C, in the dark, over three to four days. The fungi that developed in these fragments were isolated and purified. Representative isolates were identified through classical methods described in the literature, mainly those based on the colony morphology and asexual reproductive structures of fungi [16]-[18], and stored at 9 °C.

C. *Fungus Preservation and Maintenance*

The isolates were preserved in glass tubes with tilted agar, at a temperature of ~ 5 °C. Additionally, the fungi were preserved by the method of Castellani [19], which consists of preserving fungi isolates in glass vials containing 4 mL of sterile distilled water. The vials were hermetically closed with rubber lids and an aluminum band, and preserved at ~ 5 °C.

D. *Characterization of the Isolates*

As parameters for isolate characterization, the number, size and viability of the conidia, as well as the mycelial growth of the fungal colonies were assessed. To this end, the fungi were first grown in PDA medium at 27 ± 1 °C, relative humidity above 80%, and photophase of 12 hours, for 12 days. These isolates were also characterized as to the general aspect of the colony and the conidia.

E. *Number and Size of the Conidia*

For the conidia to be counted, suspensions of each isolate were prepared: a suspension aliquot was put inside

a Neubauer chamber, with the conidia being counted under a microscope at 400x magnification.

For the size of the conidia to be measured, 4 slides of each isolate were prepared, and 30 conidia per slide were measured using the DN2 Microscope Image Processing System image capture system at 400x magnification. The assessed characteristics were the size of the conidia and their length/width ratio; the shape of the conidia was determined in accordance with the method of Feitosa [20]: Ccc = cylindrical, very short, ratio lower than 2.00; Cc = cylindrical, short, ratio between 2.00 and 2.50; C = cylindrical, between 2.50 and 3.00; f = fusiform, between 3.00 and 3.50; ff = fusiform, very long, ratio greater than 3.5.

F. *Viability of the Conidia*

The conidia were assessed through microculture and examination directly in the slide. Five circles were drawn on the underside of each slide. In each circle, 0.1 mL of fungal suspension was inoculated with 10^5 conidia mL^{-1}. The slides were incubated at 27 ± 1 °C, with no light, for 18 hours. After this period, a drop of lactoglycerol dye was put on the area of the circle to stop germination and facilitate the visualization of the conidia. Then, 150 conidia were counted in each area, using a 400x magnification. Germinated and non-germinated conidia were counted, after which the germination percentage was determined.

G. *Mycelial Growth of the Colony*

To assess the mycelial growth of the colony, a 7-milimeter-wide cylinder of PDA containing mycelium from the colony of each isolate was inoculated in PDA medium, and growth was quantified through measurements (in millimeters) of two diameters previously marked on the outside of the bottom of the Petri dish. Measurements were taken every three days, from the 3rd to the 18th day after inoculation. For each isolate, four repetitions were performed.

To verify the sporulation capacity of the colonies whose mycelial growth was assessed, a suspension of conidia was prepared with the addition of 10 mL of sterile distilled water to each dish, and a 10 µL aliquot was removed for conidia verification in Neubauer chamber.

H. *Statistical Analysis*

Using the Sisvar software, the results were subjected to analysis of variance (ANOVA), and the treatment means were compared by Tukey's test at 5% probability.

III. RESULTS

A. *Isolates Obtained and Characterization*

Among the isolates obtained, there was a predominance of the genera *Beauveria*, *Fusarium*, *Purpureocillium*, *Isaria* and *Aspergillus*. In total, 19 isolates of *B. bassiana*, 6 of *I. fumosorosea* and 12 of *P. lilacinum* were found, in addition to several isolates of *F. oxysporum* (complex *oxyspurum*), *F. solani* (complex *solani*), *A. flavus* and *A. niger*. Some of these are not considered to be entomopathogenic. The isolates of three entomopathogenic species were given codes and were characterized: *B. bassiana* (codes BBOT01, BBOT04, BBOT05, BBOT06, BBOT07, BBOT10, BBOT11, BBOT12 and BBOT18), *I. fumosorosea* (IBOT25), and *P. lilacinum* (PBOT31, PBOT33, PBOT35 and PBOT36).

B. *General Aspect of the Conidia and the Colony*

The conidia were identified as subhyaline, with a smooth wall; they may vary from globose to ellipsoidal. Generally speaking, the studied conidia match the description provided by [6]: globose or semi-globose conidia of *B. bassiana*, with length and width ranging between 2x2 and 3x2 µm; elongated and ovoid conidia of *I. fumosorosea*, with size smaller than or equal to 3 µm, and *P. lilacinum* presenting ellipsoid to fusoid conidia with 2 to 3 µm.

The colonies of *B. bassiana* presented white colonies, with hyaline mycelium, raised and powdery center due to intense sporulation (Fig. 1a); on the reverse, a colorless or light yellow aspect was found at the center of the dish for most isolates.

Most isolates of *P. lilacinum* showed a colony with flaky mycelium, a burgundy shade and colorless reverse (Fig. 1b), abundant pinkish sporulation, and ellipsoid conidia with smooth walls (Fig. 1d). The colony of the fungus *I. fumosorosea* was abundantly pink, also flaky, with orange reverse, (Fig. 1c) and with fusiform and cylindrical, hyaline and smooth-walled conidia.

C. *Number, Size and Viability of the Conidia*

Among the isolates of *B. bassiana*, the number of conidia ranged from $1.25x10^6$ to $5.64x10^6$, with size ranging between 2.29x1.71 µm and 2.51x2.02 µm. For *P. lilacinum*, the number stood between $2.31x10^6$ and $7.14x10^6$, and the size, between 2.30x1.90 and 2.40x2.91 µm (Table 1). The only statistically significant difference was that the isolate BBOT07 (*B. bassiana*) grew less than the isolates PBOT33, PBOT35 and PBOT36 from *P. lilacinum*. As for the length/width ratio, the conidia of all assessed fungi were cylindrical, very short (Ccc), as the ratio was always below 2.00 [20].

For all studied isolates, the viability of the conidia stood above 90% after 17 hours of inoculation, being significantly higher for isolates IBOT33 (98.40%), IBOT35 (97.07%) and IBOT36 (97.20%).

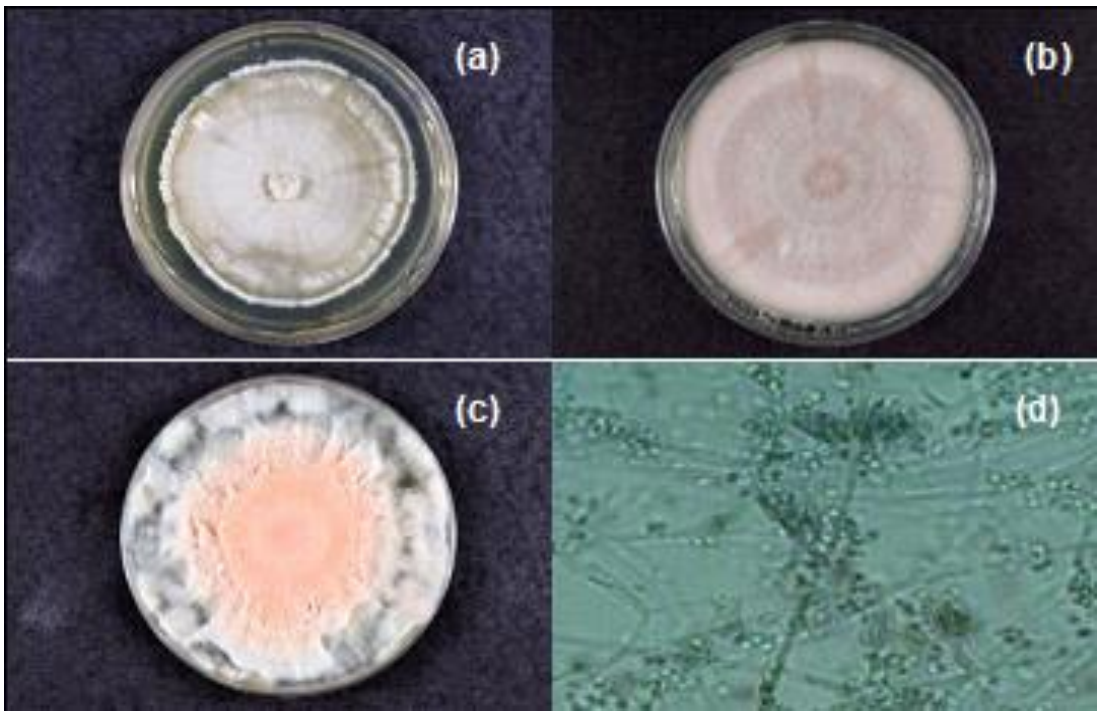

Fig. 1. General aspect of the colonies of (a) *Beauveria bassiana*, (b) *Purpureocillium lilacinum* and (c) *Isaria fumosorosea*; (d) *P. lilacinum* conidiophores and conidia. Culture medium: PDA. Botucatu (SP), 2009.

Table 1. Number, size and viability of conidia of *Beauveria bassiana*, *Isaria fumosorosea* and *Purpureocillium lilacinum* re-isolated from *Atta sexdens rubropilosa* workers. Botucatu (SP), 2009.

Isolates	Fungus	Number (10^6)	Size* (µm)	Viability** (%)
BBOT01	*B. bassiana*	1.25	2.29x1.71	93.73 ab
BBOT04	*B. bassiana*	2.16	2.00x2.01	95.73 ab
BBOT05	*B. bassiana*	4.26	2.33x1.85	92.80 ab
BBOT06	*B. bassiana*	3.00	2.43x2.01	96.00 ab
BBOT07	*B. bassiana*	3.73	2.34x1.83	90.13 b
BBOT10	*B. bassiana*	3.70	2.37x2.07	93.47 ab
BBOT11	*B. bassiana*	3.47	2.38x1.86	94.40 ab
BBOT12	*B. bassiana*	2.22	2.51x2.02	92.80 ab
BBOT18	*B. bassiana*	5.64	2.29x1.85	94.27 ab
IBOT25	*I. fumosorosea*	1.42	2.70x2.01	93.60 ab
PBOT31	*P. lilacinum*	2.31	2.30x1.90	93.60 ab
PBOT33	*P. lilacinum*	2.17	2.46x1.91	98.40 a
PBOT35	*P. lilacinum*	7.14	2.40x2.91	97.07 a
PBOT36	*P. lilacinum*	5.16	2.75x2.14	97.20 a

*Size of the conidia: Length x Width. **Means followed by the same letter do not differ from each other (p = 0.05, C.V%: 3.23).

D. *Comparing Mycelial Growth between Isolates*

Mycelial growth did not differ much between isolates of the same species, unlike what happened when comparing isolates of different species.

Among the isolates of *B. bassiana*, difference was found only 15-18 days after inoculation, with BBOT11 being superior to BBOT01, BBOT04 and BBOT07 (Table 2). The isolates of *P. lilacinum* and *I. fumosorosea* showed differences in mycelial growth in most of the assessment periods, with a decrease trend over the inoculation time, reaching similarity in average diameter in the last assessment period (15-18 days).

Overall, *P. lilacinum* and *I. fumosorosea* grew faster than *B. bassiana* (significant differences, $p<0.0001$). In the beginning, after 3 days, most isolates did not show a significantly different growth, with only PBOT31 and PBOT33 growing more than the others. Over time, however, isolates of *B. bassiana* showed a slower radial expansion compared to the others, and, on the 15th day, all isolates of this fungus presented a smaller diameter compared to all isolates of *P. lilacinum* and *I. fumosorosea*.

Isolate PBOT35 (*P. lilacinum*) reached the maximum diameter allowed by the size of the dish (80 mm) after 9 days, while isolate IBOT25 (*I. fumosorosea*) reached 80 mm after 12 days. The other isolates reached or were close to 80 mm in diameter 18 days after inoculation.

Table 2. Comparing mycelial growth between colonies of *Beauveria bassiana*, *Isaria fumosorosea* and *Purpureocillium lilacinum* re-isolated from *Atta sexdens rubropilosa* workers, in PDA medium, by inoculation time. Botucatu (SP), 2009.

	Mycelial growth (mm)					
	(Days)					
Treatment	**3**	**6**	**9**	**12**	**15**	**18**
	Beauveria bassiana					
BBOT01	12.62 d	15.19 cd	20.25 e	22.00 d	32.44 c	37.81 c
BBOT04	12.00 d	15.87 cd	22.25 e	23.37 d	35.87 c	41.37 c
BBOT05	12.19 d	14.50 d	25.68 e	34.22 d	40.62 c	59.20 bc
BBOT06	11.94 d	15.44 bcd	26.56 e	32.62 d	36.75 c	47.37 bc
BBOT07	12.75 d	17.44 bcd	23.50 e	31.56 d	33.94 c	45.75 c
BBOT10	12.06 d	16.31 cd	26.56 e	27.50 d	32.62 c	47.37 bc
BBOT11	13.94 cd	21.19 bcd	28.25 e	40.12 cd	42.31 c	58.21 b
BBOT12	13.25 d	20.06 bcd	28.50 de	35.44 d	40.19 c	50.25 bc
BBOT18	12.69 d	18.87 bcd	27.81 de	35.12 d	40.12 c	48.25 bc
Mean	**12.60 ± 0.66**	**17.21 ± 2.34**	**25.85 ± 2.88**	**31.33 ± 5.95**	**37.21 ± 3.73**	**47.40 ± 5.75**
	Isaria fumosorosea					
IBOT25	16.00 cd	25.25 bcd	79.12 a	80.00 a	80.00 a	80.00 a
	Purpureocillium lilacinum					
PBOT31	38.00 a	49.94 a	58.19 bc	70.25 ab	75.00 ab	79.25 a
PBOT33	21.56 b	33.00 b	47.62 bc	59.87 b	76.37 ab	78.00 a
PBOT35	16.81 cd	52.00 a	80.00 a	80.00 a	80.00 a	80.00 a
PBOT36	16.94 cd	22.62 bcd	63.75ab	65.00 ab	76.50 ab	80.00 a
PBOT38	14.44 cd	21.50 bcd	45.12 cd	55.62 bc	65.37 b	78.75 a
Mean	**21.55 ± 9.55**	**35.81 ± 14.56**	**58.94 ± 14.02**	**66.11 ± 9.49**	**74.65 ± 5.50**	**79.20 ± 0.86**
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CV%	12.21	25.66	17.14	16.35	9.09	7.96
LSD	4.97	17.97	16.06	19.67	12.39	12.44

Means followed by the same letter in the column do not differ statistically among the fungus isolates.

IV. DISCUSSION

The characteristics of entomopathogenic fungi, such as germination, sporulation and radial growth, are important to define virulence and their potential for pest control [21], [22]. In the present study, all isolates

performed well as to these parameters, showing that they may have potential for biological control.

For all isolates, germination occurred quickly, within 17 hours after inoculation in PDA, which is a good result, because, according to the literature [6], germination time may vary from 18 to 24 hours for fungi without storage or freezer stored. The higher the germination speed, the greater the viability and pathogenicity of the isolate [6], [22]. Rapid sporulation is an important criterion for the selection of fungal isolates, as speed assists in spreading epizootics, in addition to being an advantage in relation to the humoral immunity of insects. Moreover, the faster the conidia germinate, the less time they will be exposed to abiotic factors such as temperature and humidity, which can impair pathogenesis [6], [22], [23]. Also, all studied isolates showed a high percentage of sporulation, revealing high viability, which is recommended for biological control.

Radial growth is also used to assess strains in terms of virulence, as the rate of radial growth is directly associated with speed of infection in the host [24]. The isolates of *P. lilacinum* and *I. fumosorosea* grew faster than those of *B. bassiana*. Difference in mycelial growth was found between *P. lilacinum* isolates, however, according to the literature [25], growth differences between isolates of the same species are common. Although this mycelial growth speed alone does not fully represent the virulence potential of a given isolate, it is still a good indication that the latter may present a higher speed of infection when inoculated to the host, an aspect that can be investigated in future research.

Thus, the morphological and growth characteristics of fungi in culture media, such as the one in the present study, may help further investigations, such as pathogenicity and virulence tests, in the selection of isolates with greater potential for insect control. By the assessed parameters, all isolates in this research have good characteristics for microbial control, especially those of *P. lilacinum* and *I. fumosorosea*, as they grew faster.

On the other hand, other parameters, particularly the ecological interactions of entomopathogenic fungi with their hosts and with the environment, are very important as well [3], [13], [24]. For instance, some species of ants can actively avoid *Beauveria* or try to sanitize the area around their nests, resulting in the absence of this fungus in the surrounding soil, while other species of ants can simply cohabit in the soil with this fungus [26], [27]: in *Monomorium floricola* worker ants, *Beauveria* was typically more pathogenic than *Metarhizium*, the latter of which did not kill a significant number of workers [26].

On the other hand, it was evident that neither leaf-cutter ants nor the *Metarhizium* fungus seem to be actively looking for or avoiding each other [27]. The interesting thing is that this pattern is inverted for fire ants (*Solenopsis*): soils containing *Beauveria* conidia do not trigger any avoidance response or affect the overall colony mortality rate [26]. Therefore, a better assessment of the potential for biological control requires *in vivo* studies that complement the present one (*in vitro*).

V. Conclusion

All isolates in this research showed favorable characteristics for microbial control, especially those of *P. lilacinum* and *I. fumosorosea*, as they grew faster. Furthermore, considering the literature, it is also evident that the isolates of *B. bassiana* in the present investigation should also be included among the most promising ones.

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