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Research Article

Diversity of Fungi Associated with *Atta bisphaerica* (Hymenoptera: Formicidae): The Activity of *Aspergillus ochraceus* and *Beauveria bassiana*

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The grass-cutting ant *Atta bisphaerica* is one of the most serious pests in several pastures and crops in Brazil. Fungal diseases are a constant threat to these large societies composed of millions of closely related individuals. We investigated the occurrence of filamentous fungi associated with the ant *A. bisphaerica* in a pasture area of Viçosa, Minas Gerais State, Brazil. Several fungi species were isolated from forager ants, and two of them, known as entomopathogenic, *Beauveria bassiana* and *Aspergillus ochraceus*, were tested against worker ants in the laboratory. The two species were highly virulent, achieving 50 percent worker mortality within 4-5 days. It is the first time *A. ochraceus*, a commonly found fungal species, is reported to infect *Atta* species at a high prevalence. Possible uses for the fungus within biological control are discussed.

1. Introduction

Leaf-cutting ants (*Atta* and *Acromyrmex* genera) are considered dominant herbivores in the Neotropics [1] and also a severe pest when attacking crops. The leaf-cutting ants include a number of species which cut predominantly monocotyledonous plants and are known as grass-cutting ants. Knowledge of the morphological, biological, behavioural, and breeding characteristics of these species is still lacking, and are important by the difficulties in controlling their colonies. Among the nine *Atta* species occurring in Brazil, *Atta bisphaerica* Forel, *Atta capiguara* Gonçalves, and *Atta vollenweideri* Forel cut mainly monocots, and one, *Atta laevigata* (Smith), cuts both monocots and dicots.

Atta bisphaerica, or "saúva mata-pasto" as it is commonly known in Brazil, stands out as a major pest of pastures and sugar cane [2], currently occupying a leading position as pest species in São Paulo sugar cane plantations and pastures of southeastern Brazil. The control of this species through granular baits is not appropriate as the baits, which are available in the market, attract with the citrus pulp, which is unattractive to grass-cutting ants. This fact has stimulated the search for new materials for a bait production which is more attractive to these grass cutters [3, 4] and also the demand for natural control agents for leaf-cutting ants in general, including fungi [5] and parasitoids [6, 7].

The main advantage of biological control would be the reduction of the use of insecticides that pollute the environment and may constitute a barrier in the export of Brazilian products. Furthermore, in November 2009, the Forest Stewardship Council (FSC) approved the use of the sulfluramid, fipronil, and deltamethrin active ingredients of insecticide baits for another five years in the Brazilian eucalyptus plantations [8], which provides some time for the forestry companies to find alternatives for pest control.

The potential of using fungi to control leaf-cutting ants is large, considering that several entomopathogenic species have been found in worker ants, nearby or within the nests of the ants [9–13] and may cause the death of the worker ants or of the fungus garden. While leaf-cutting ants are constantly in contact with a diversity of entomopathogenic fungi, they do not appear to be normally infected by them. This supports the view that they are effectively protected against these parasites by behavioural mechanisms such as intensive self-grooming or allogrooming [14, 15] and utilization of antibiotics derived from glands or symbiotic bacteria. Knowing the diversity of these fungi and the ants' resistance mechanisms is essential if one wants to implement safe and efficient microbial control methods for the pest control.

The purpose of this study was first to isolate and identify the filamentous fungal species associated with *A. bisphaerica*. Then, we tested the entomopathogenic activity of two species found in the first part of our study presented here: an *Aspergillus ochraceus* isolate, which was the most observed, and an isolate of *Beauveria bassiana*, a species widely used in microbial control of insects and also found in worker ants of *A. bisphaerica*, were tested in the laboratory.

2. Material and Methods

2.1. Collection of Ants in the Field and Research of Pathogenic Fungi. The ants were collected in a pasture in Viçosa, MG, Brazil (20° 44′S, 42° 50′W) on May 27, 2010, between 2:00 and 4:00 PM during their foraging time. We collected 100 foraging worker ants, which were on their return to the nest with grass fragments, each of three colonies (I, II, and III), which demonstrated foraging activity, totaling 300 workers ants. The ants were taken to the laboratory and isolated in Petri dishes supplied with a honey-water solution (1 : 1) and sterile distilled water, being monitored daily until death. The dead worker ants were washed in series with a solution of 70% ethanol, 4% sodium hypochlorite, and distilled water. After washing, they were transferred to sterile Eppendorf tubes, containing moist cotton and kept in the incubator at a temperature of 25 ± 1°C, a relative humidity of 70 ± 10%, and a 12-hour photoperiod, until the external appearance of fungal hyphae. Then the isolation of the fungi was performed in a vertical laminar flux hood. The fungi were inoculated in Petri dishes (9 cm diameter) with a potato dextrose agar (PDA) culture containing antibiotics (penicillin, 0.5 g/L, streptomycin, 0.5 g/L). After obtaining pure cultures, they were subjected to microscopic analysis by performing the identification and classification of the fungi with the aid of taxonomic keys [16–18] and then confirmed at the generic and species level by Dr. Harry Evans.

2.2. Infection of the Ants in the Laboratory. To assess the potential pathogenic fungi *B. bassiana* and *A. ochraceus*, isolated from the workers collected in the field as described in Section 2.1, we tested the mortality of the *A. bisphaerica* worker ants taken from colonies maintained in the laboratory and inoculated with a spore suspension. The colonies were collected 10 days before at Viçosa, Minas Gerais State, Brazil, and maintained in the laboratory according to the methodology developed by Della Lucia [19]. They were maintained at 25 ± 5°C, relative humidity of 75 ± 5%, and a 12 : 12 light : darkness regime. On a daily basis, they received cut leaves of *Hyparrhenia rufa* (Poales: Poaceae) and *Zea mays* (Poales: Poaceae), in addition to clean water. Five hundred and fifty workers (head width ∼3.2 mm) were collected in roughly equal proportions from the three colonies (A, B, and C, maintained under laboratory conditions) in the fungus garden, which probably were less likely to be contaminated. Conidial suspensions of the two fungi species were prepared from a Tween 80 solution of 0.05% at concentrations of 10^5, 10^6, 10^7, 10^8, and 10^9 conidia/mL. For each concentration, 50 worker ants received 1 μL of the suspensions on the pronotum. One μL of a 0.05% Tween 80 solution was applied to the control ants. This control group was used in the comparison with the two isolates since the experiments were conducted simultaneously. After receiving the treatments, the insects were held individually in Petri dishes supplied with a honey-water solution (1 : 1) and sterile distilled water renewed every two days. The mortality of the worker ants was daily monitored. The dead worker ants were washed as in the previous experiment and transferred to sterile Eppendorf tubes, containing moist cotton and kept in the incubator at temperature of 25 ± 1°C, a relative humidity of 70 ± 10%, and a 12-hour photoperiod, until the external appearance of fungal hyphae. Then we proceeded with the isolation of the fungi, which were randomly selected in all the used concentrations with 15 isolate plates for each individual concentration. This was done to confirm whether the death was due to the applied fungi.

2.3. Survival Analysis. Survival curves were generated as a function of the observation time through the Kaplan-Meier method. Firstly, we performed a comparison of multiple groups, and thereafter we compared the survival of two groups applying the nonparametric Log-rank test at 5% significance level with the aid of the Statistica software, version 7.0.

3. Results

The survival of the *A. bisphaerica* worker ants, which were brought from the field, did not vary among the three analyzed colonies ($\chi^2 = 3.06$, $P = 0.21$), and the maximum survival was 25 days (Figure 1). It was possible to isolate and identify 10 fungi species that may have deleterious effects on the worker ants. Among them, three *Aspergillus*, three *Penicillium*, one *Cladosporium*, two *Mucor*, and one *Beauveria* species were identified (Table 1). The most frequent fungus found in the worker ants was *A. ochraceus*, with 44 occurrences. It was found in individuals who had died about 5 days after the beginning of the analyses. The fungi that had not sporulated were classified as *Mycelia sterilia* (Table 1). The percentage of worker ants, which were infected with known entomopathogenic fungi, was 15.33%, namely, *A. ochraceus* (14.67%), *A. niger* (0.33%), and *B. bassiana* (0.33%).

During experimental infection, we found a significant difference in survival time of the worker ants subjected to different concentrations of both suspensions *A. ochraceus*

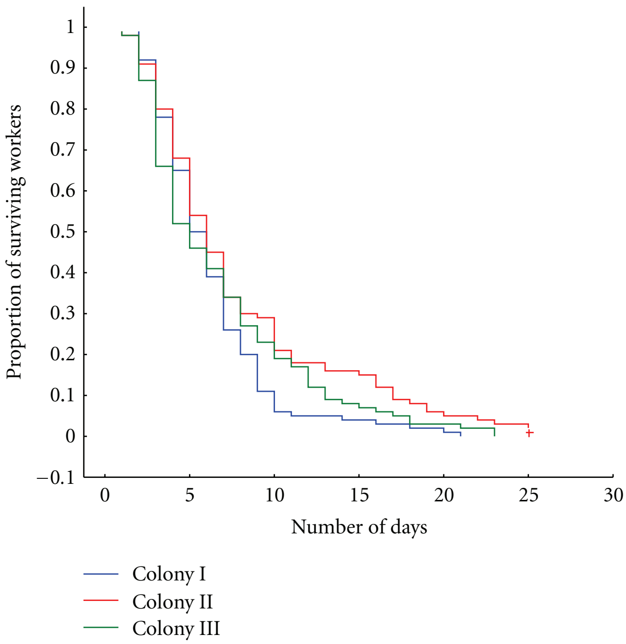

FIGURE 1: Cumulative time-related survival proportion (Kaplan-Meier curves) of the *Atta bisphaerica* forage worker ants from three different colonies.

TABLE 1: Species and frequencies of the fungi isolated from 300 foragers of *Atta bisphaerica*.

Fungus species	Frequency of occurrence (in 300 workers)
Aspergillus niger	1
Aspergillus ochraceus	44
Aspergillus sclerotiorum	5
Beauveria bassiana	1
Cladosporium sp.	15
Mucor hiemalis	4
Mucor racemosus	10
Penicillium lilacinum	2
Penicillium sp. 1	2
Penicillium sp. 2	1
Mycelia sterilia	5

($\chi^2 = 42.27$, $P < 0.001$; see Table 2 for comparing two survival curves with a Log-rank test) (Figure 2) and *B. bassiana* ($\chi^2 = 94.03$, $P < 0.001$; see Table 3 for comparing two survival curves with a Log-rank test) (Figure 3). The shortest survival time was observed at a concentration of 10^9 conidia/mL in both treatments, with an LT_{50} of four days at a 10^9 concentration of *A. ochraceus* (Figure 2) and an LT_{50} of four days for concentrations of 10^8 and 10^9 of *B. bassiana* (Figure 3). The LT_{50} of the control group resulted in 10.5 days.

4. Discussion

Since certain entomopathogenic fungi present a high potential to control the ants, pathogenicity tests have been

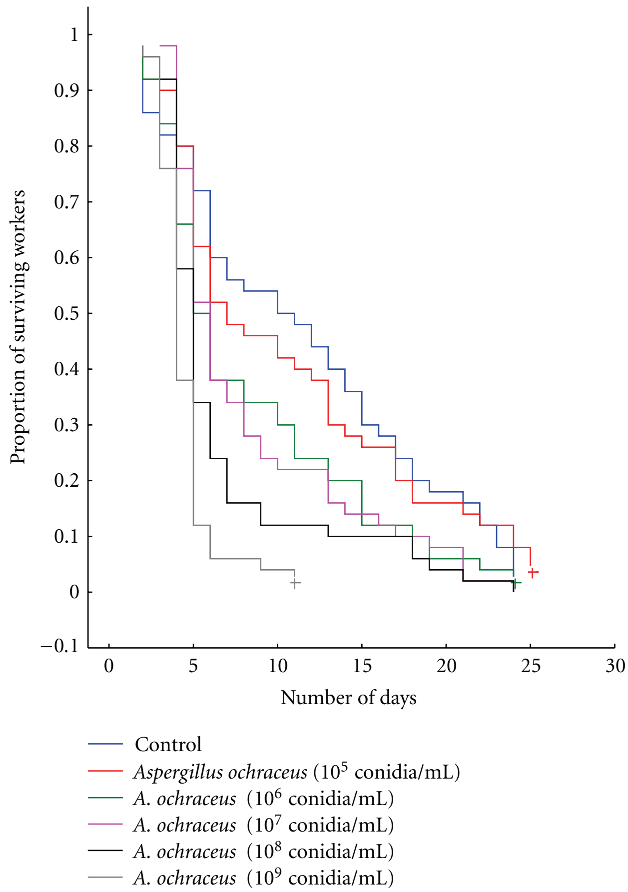

FIGURE 2: Cumulative time-related survival proportion (Kaplan-Meier curves) of the *Atta bisphaerica* worker ants after being treated with different suspension concentrations of the *Aspergillus ochraceus* fungus.

performed with fungi widely used within the microbial control of insects, such as *Metarhizium anisopliae* (Metsch.) and *Beauveria bassiana* (Bals.). They have proved to be highly virulent in the laboratory, although field tests do not reproduce the same results [20–22]. In our study, *M. anisopliae* was not found and *B. bassiana* was obtained only once. The *Aspergillus ochraceus* was the most frequent and, for the first time, reported to infect ants frequently. The mortality test results of ants infected with *A. ochraceus* and *B. bassiana* are similar to those obtained in other pathogenicity tests of *B. bassiana* or *M. anisopliae* against leaf-cutting ants [22–24]. Although the high control mortality indicates that the ants were stressed in some way (e.g., social isolation), and thus probably more susceptible to diseases, the parasite treatments caused significantly increased mortality. This allows us to say that *A. ochraceus* could be also a promising biological control agent of ants. Surely, more research should be undertaken before significant field use of the pathogen. Even though that *Aspergillus* are unusual pathogens of most insects, these fungi produce a diverse range of compounds that can be potent insect toxins and potentially useful as pesticidal [25]. However, the safety of these compounds is a major concern and more studies are

Table 2: P values of Log-rank test to compare two Kaplan-Meier survival curves for *Atta bisphaerica* workers treated with different conidial concentrations of *Aspergillus ochraceus*. P values less than 0.05 were considered significant.

Conidial concentrations	0	10^5	10^6	10^7	10^8	10^9
0	—					
10^5	$P = 0.90$	—				
10^6	$P = 0.06$	$P = 0.07$	—			
10^7	$P = 0.02$	$P = 0.04$	$P = 0.93$	—		
10^8	$P < 0.01$	$P < 0.01$	$P = 0.15$	$P = 0.10$	—	
10^9	$P < 0.0001$	$P < 0.0001$	$P < 0.001$	$P < 0.0001$	$P < 0.01$	—

Table 3: P values of Log-rank test to compare two Kaplan-Meier survival curves for *Atta bisphaerica* workers treated with different conidial concentrations of *Beauveria bassiana*. P values less than 0.05 were considered significant.

Conidial concentrations	0	10^5	10^6	10^7	10^8	10^9
0	—					
10^5	$P = 0.80$	—				
10^6	$P = 0.03$	$P = 0.02$	—			
10^7	$P < 0.0001$	$P < 0.0001$	$P < 0.01$	—		
10^8	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	—	
10^9	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.77$	—

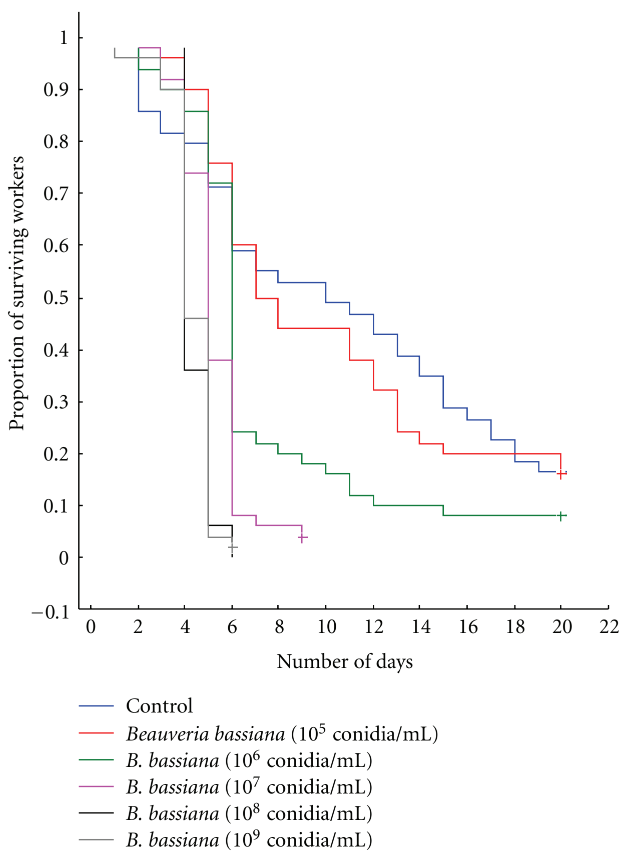

Figure 3: Cumulative time-related survival proportion (Kaplan-Meier curves) of the *Atta bisphaerica* worker ants after being treated with different suspension concentrations of the *Beauveria bassiana* fungus.

needed to evaluate potential risks to humans and nontarget species.

Cladosporium fungi are phytopathogens [26] and may be transported by various insects causing their death. In a study of leaf-cutting queen ants, the *Cladosporium* fungus was prevalent in *Atta laevigata* and in *A. capiguara* [27]. It is believed that these fungi are frequently found due to their cosmopolitan distribution, being acquired from the environment and dispersed during the founding of a colony by queens or forage worker ants carrying leaves. The role of the *Cladosporium* species inside the ant colonies is not known for sure, but they have been considered potential antagonists of the fungus garden [28]. The same kind of reasoning can be adopted for the *Mucor* fungi species, which have also been found in the nests of leaf-cutting ants, however, without knowing exactly how they interact with these ants or the fungus garden. The fungi of this genus are considered invertebrate pathogens. For example, *Mucor hiemalis* can cause the mortality of *Cupiennius* and *Ischnothele* spiders [29]. Toxic metabolites of these organisms present an insecticidal activity against the adults of *Bactrocera oleae* and *Ceratitis capitata* (Diptera: Tephritidae) [30].

Considering the diversity of fungi found in this study, the importance of new surveys and tests of entomopathogenic fungi isolates with the potential of being used in microbial control of these ants becomes evident. *Beauveria* and *Metarhizium* are the species which have been most studied and which have been evaluated on tests on their pathogenic potential. It is evident now that *A. ochraceus* should be further investigated for its pathogenic potential for leaf-cutting ants as well as other fungi genera found here.

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