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Susceptibility of *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae) to *Beauveria bassiana* (Bals.) Vuill.

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Abstract
Entomopathogenic fungi are frequently used as an alternative method for insect pest control. However, only a few studies have focused on the effect of these fungi on bees and on the selectivity of fungi to beneficial organisms in agroecosystems. The objective of the present study was to assess the susceptibility of worker bees of the species *Melipona scutellaris* (locally known as "uruçu") to the isolate (Biofungi 1) of the entomopathogenic fungus *Beauveria bassiana*. The experiment was carried through indirect contact between the fungal suspension and newly-emerged bees and topical application of the fungal suspension on the back of newly-emerged bees. The sampling design was completely randomized and comprised five treatments, which included four different concentrations of the fungus: 1 x 10^5, 1 x 10^6, 1 x 10^7, 1 x 10^8 conidia/ml, and a control composed of distilled water. Each treatment had five replicates. The mortality data were subjected to an analysis of variance and a probit regression analysis, which provided an estimate of the lethal dose to 50% of the population (LD_{50}). The adjustment of the curves to the model was tested with a chi-squared test and differences between curves were tested with a test for parallelism. *Beauveria bassiana* was virulent to uruçu bees, killing the bees at the lowest dose used. These findings may help minimize the impact of this entomopathogen and, therefore, contribute to the maintenance of natural populations of these insects.

Introduction

The improper use of agricultural chemicals has led to studies on alternative methods of sustainable pest control. Among these methods biological control with entomopathogenic fungi stands out as a broadly used method of pest control in agroecosystems (Messias 1989; Marques et al. 2004).

These fungi are highly viable, because they are able to preserve parasitoid, predator, and pollinator populations, and represent an important factor in integrated pest management (Neves et al. 2001; Oliveira 2008).

However, some authors state that these fungi can be pathogenic to bees. Espinosa-Ortiz et al. (2011) studied the susceptibility of larva and adult honeybees (*Apis mellifera)* to three types of isolates of entomopathogenic fungi and observed high mortality (90-100%) caused by the fungus *Beauveria bassiana*, when applied at the dose of 1 x 107 conidia/ml. Bee mortality by entomopathogenic fungi was also recorded by Butt et al. (1994, 1998).

Al mazra'awia (2007) studied the impacts of *B. bassiana* on *A. mellifera* and concluded that bees exposed to high concentrations of this fungus show high mortality. However, he also affirmed that the beehives exposed to high densities of inoculum of the same pathogen had low mortality. According to Hokkaner et al. (2003), the temperature is higher inside than outside the beehives, which makes them safer to fungal infection.

When Hokkenen et al. (2003) assessed the impacts of *Metarhizium* and *Beauveria* on bees they observed that different strategies for the application of these fungi should be considered due to the risks they could bring to insects in the natural ecosystem. The conservation of pollinators requires the attention of scientists due to the large number of problems faced by them in natural ecosystems, including death by pesticides (Otterstatter & Thomson 2008; Freitas & Pinheiro 2010; Rocha 2012).

There are few robust data on the effect of entomopathogenic fungi on social bees (Nogueira-Neto 1953; McGregor

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1976; Ferraz et al. 2006; Braga et al. 2010) and the impact of these fungi on beneficial insects associated to crops (Espinosa-Ortiz et al. 2011; Kanga et al. 2002).

Ferraz et al. (2006) stated that, in spite of not having unequivocal data at hand, it is possible that the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae*, and *Metarhizium flavoviride* cause the death of indigenous bees.

The objective of the present study was to assess the susceptibility of worker bees of the species *Melipona scutellaris* to *B. bassiana* isolates at different concentrations and contact forms.

Material and Methods

The experiment was conducted at the laboratory of the Center for the Study of Insects (INSECTA), at the Federal University of Recôncavo da Bahia (UFRB), with newly-emerged worker bees of the species *M. scutellaris*, locally known as uruçu. The bees were provided by the rearing facilities of INSECTA/UFRB. In the treatments we used the commercial isolate of the fungus *B. bassiana* (Biofungi 1), produced in the Laboratory of Research and Production of Microorganisms/Biofactory, State University of Southeast Bahia (UESB), Vitória da Conquista, State of Bahia, where this pathogen has been successfully tested for the control of crop pests.

Collection and sampling

Brood combs of *M. scutellaris* were removed from the colonies of the rearing facilities at INSECTA and maintained in growth chambers of the B.O.D. type (Biologic Oxygen Demand) at a temperature of 28 ± 2ºC, relative humidity of 70% ± 2%, and a photoperiod of 12h for a possible emergence of bees (Espinosa-Ortiz et al. 2011).

Preparation of different concentrations of conidia and application

One-gram samples were randomly removed from the fungal substrate and added to 10 ml of sterilized water containing Tween 80 adhesive spreader at 1% (v/v). To obtain a homogenized suspension, serial dilutions (10^2) were made, so that the conidia could be counted in a Newbauer chamber under a microscope (100x). The preparation of the suspensions followed Alves (1998b). The treatments included four fungal concentrations: 1 x 10^5, 1 x 10^6, 1 x 10^7, 1 x 10^8 conidia/ml^{-1} and composed of distilled water.

Newly-emerged bees were anaesthetized for 1min in a refrigerator at 16°C to facilitate the handling of worker bees. Bees were exposed to the fungal suspension through topical application on the dorsum and indirect contact. In the topical application, 1µl of each treatment was applied to the dorsum of each bee with a sterile 10µl micro syringe (BD Plastipak). In the exposure by indirect contact, the bees were placed on filter paper sheets slightly moistened with 1 ml of each treatment for 5 min (Rother et al. 2009). After each treatment, the worker bees were placed in plastic containers (6.0 cm x 8.0 cm) and transferred to an acclimatized chamber.

Five worker bees were placed in each plastic container, totaling 125 individuals. The bees were fed with honey at 10% (100g/1000ml distilled water), placed on a sterilized wad of cotton to prevent contamination (Rother et al. 2009).

Data analysis

The experimental design was completely randomized. It was composed of five treatments and five replicates, totaling 25 plots. The mortality of worker bees was monitored at 24h intervals for 10 days. The results were corrected considering natural mortality in accordance with the Abbott formula (Alves 1998).

The corrected mortality data were subjected to an analysis of variance. For this analysis, the data were transformed using the formula $X' = \arcsin (X_i / N)$, where X' is the datum after the transformation, X_i is the mortality observed in the replicate *i* and N is the total number of insects in the experimental plot. Data normality was assessed with a Kolmogorov-Smirnov test and variance homogeneity was assessed with a Levene test.

Mortality data from different treatments were subjected to a probit regression analysis (Sokal 1958) in Statistica (StatSof Inc.). This analysis provided an estimate of the lethal dose to 50% of the population (LD_{50}). The adjustment of the curves to the model was assessed with a chi-squared test and differences between curves for the exposure methods were assessed with a test of parallelism (Alves 1998).

The mortality data at highest dose were used to build survival curves for the two exposure methods following the Kaplan-Meier method (Blanford et al. 2005). Based on these curves the time to mortality (or survival) of 50% of the insects (S_{50}) was estimated. The curves were compared using a logrank test ($P = 0.95$) and a Gehan-Breslow-Wilcoxon test (GBW) in GraphPad Prism 5.0 (Motulsky 1995).

Results and Discussion

Under the experimental conditions, there was no significant effect ($p > 0.05$) of the exposure methods on the mortality of uruçu bees (*M. scutellaris*). However, there was a significant interaction between exposure methods and the doses applied (DF = 4.396; F = 17.68; $P < 0.001$). The corrected mortality caused by different doses of the fungal suspension was higher when applied on the dorsum of the bees than when bees got in indirect contact with the fungal suspension (Figure 1). Even at the lowest dose (10^5 conidia ml^{-1}), the worker bees were affected: they lost mobility and had an average mortality of 56%.

These results differ from those of Butt et al. (1994), who tested the pathogenicity of *Metarhizium anisopliae* to adult bees of *A. mellifera* and observed significant mortali-

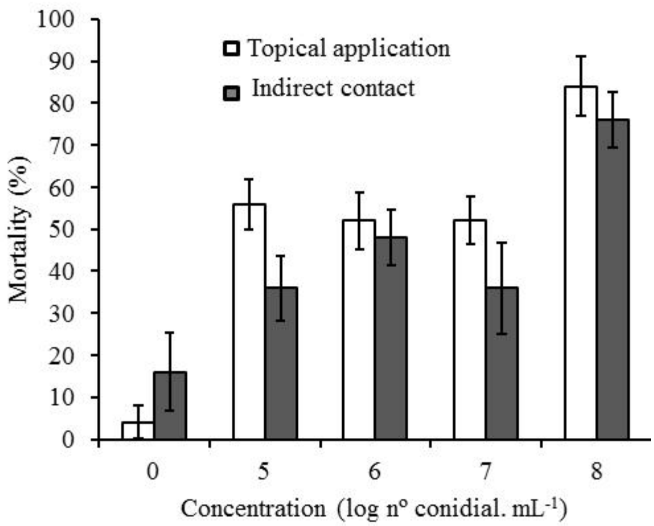

Fig 1 – Corrected mortality (%) of uruçu bees (*M. scutellaris*) ten days after exposure to fungal suspensions with increasing concentration of *B. bassiana* conidia by the methods of topical application and indirect contact.

ties only at very high doses. In a similar study, Kampongo et al. (2008) reported a mortality rate of 42 - 45% for *Bombus* sp. bees exposed to high doses of *B. bassiana* ($2x10^{11}$ conidia ml^{-1}). Espinosa-Ortiz et al. (2011) tested the virulence of different commercial isolates of *B. bassiana*, *M.anisopliae*, and *P. fumosoroseu* on worker bees of the species *A. mellifera* and obtained a mortality rate of 90% - 100% with a dose of 10^7 conidia ml^{-1} of *B. bassiana*. Therefore, it is possible that the isolates exhibit specificity to bee species.

The topical application of the fungal suspension on the dorsum of bees caused high mortality, with low variability between replicates and uniformity among treatments. The data obtained from this method adjusted well to the probit regression model (χ^2 = 2.897; DF = 2; P = 0.235). The isolate of *B. bassiana* used in the experiments was pathogenic to uruçu bees and caused high mortality even at low doses. Based on the results obtained, a LD_{50} of $2.04x10^5$ conidia ml^{-1} was estimated ($7.95. 10^3$; $3.70.10^5$) (Figure 2).

The data obtained from the exposure method of indirect contact with the fungal suspension at different doses showed high variability among doses, and did not fit the probit regression model (χ^2 = 26.811; DF = 2; P < 0.001). With this result, it was not possible to estimate the LD_{50} or make a comparison between exposure methods through the assessment of the parallelism of curves. For the comparison between different exposure methods, the survival curve at the two highest doses was compared with Mantel-Cox and GBW tests.

Data analysis through the construction of survival curves using the Kaplan-Meier method made it possible to assess mortality details over time, and allowed the identification of differences in the survival curve between exposure methods. We observed that the indirect contact method resulted in a lower mortality rate at the lowest doses in the beginning of the observation period, but in the end of the observation period the total mortality was also high.

Figure 3 shows that the topical application of the fungal suspension of *B. bassiana* (10^8 ml^{-1}) resulted in high mortality in the end of the experiment, with a survival of only 20.4% (± 5.1) of the bees; whereas the exposure method through indirect contact resulted in higher survival (69.1% ± 4.2). The survival curves obtained for different methods of exposure to the fungus were significantly different from the control and from one another when compared by Mantel-Cox (Log Rank Test) and GBW tests (Table 1).

The mean survival value (S_{50}) estimated for the topical application method was 216.0 days (Table 1). For the con-

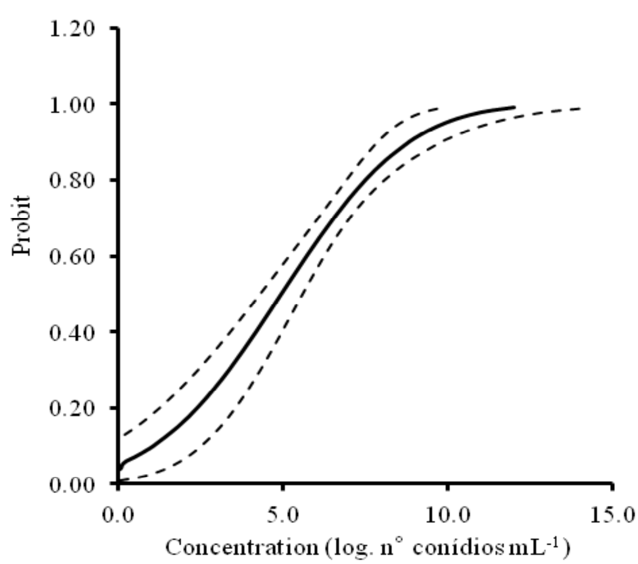

Fig 2 – Dose-response curve resulting from the methods of topical application of fungal suspensions containing *B. bassiana* conidia on uruçu bees (*M. scutellaris*). Dotted lines represent the fiduciary limits of the estimated doses.

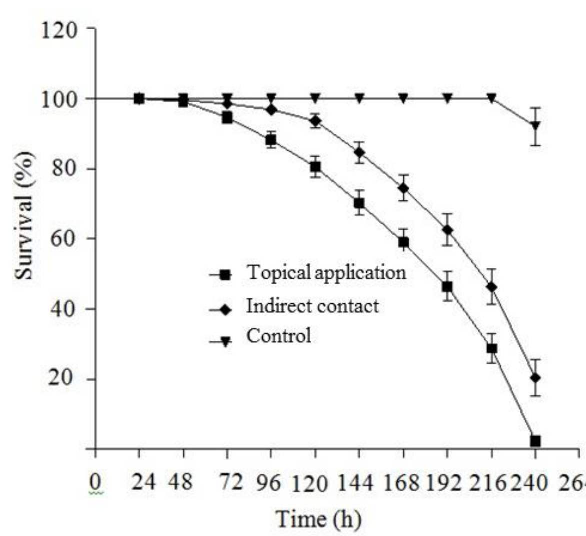

Fig 3 – Kaplan-Meier Survival curves of uruçu bees (*M. scutellaris*) subjected to two exposure methods to fungal suspensions containing *B. bassiana* conidia (10^8 ml^{-1}). The fungal suspensions were applied topically on the dorsum of bees or by indirect contact of the bees with a surface previously sprayed with the suspension.

trol treatment or application by indirect contact, the amount of S_{50} could not be estimated. For the control treatment or exposure method by indirect contact, S_{50} could not be estimated because bee mortality did not exceed 50%, and the use of the Kaplan-Meier model to calculate S_{50} is limited by the increased survival time of the individuals studied. The estimated value of hazard ratio (HR) between the two exposure methods was 0.35, between the topical application and the control it was 8.48, and between the indirect contact and the control it was 5.96 (Table 1). The hazard ratio estimates the difference in mortality between treatments based on the slope of the respective survival curves. In this particular case, the average mortality estimated for the topical application exposure method was consistently 35% higher throughout the experiment in comparison with the indirect contact exposure method.

Table 1 – Comparison of survival curves estimated for uruçu bees (*M. scutellaris*) exposed to topical application or indirect contact with the fungal suspension of *B. bassiana* conidia (10^8 ml^{-1}).

Mantel-Cox Test (Logrank)	
Topical application x Control	76.96**[1]
Indirect contact x Control	21.23**
Topical application x Indirect contact	26.17**
Gehan-Breslow-Wilcoxon Test (GBW)	
Topical application x Control	57.51**
Indirect contact x Control	19.58**
Topical application x Indirect contact	7.69**
Median Survival (S_{50})	
Topical application	216
Indirect contact	192
Control	-
Hazard Ratio (HR)	
Topical application x Control	8.48 (5.26 to 13.67)[2]
Indirect contact x Control	5.86 (2.76 to 12.45)
Topical application x Indirect contact	0.35 (0.22 to 0.61)

[1] Significant at $P < 0.001$; [2] Confidence interval estimated (CI)

Delaplane & Mayer (2005) reported that methods used to apply the compounds may interfere with the results of toxicity assessment of pesticides on non-target insects in the laboratory; there may be an interaction between the active ingredients and exposure methods. Carvalho et al. (2009) tested four methods to assess the toxicity of pesticides to *A. mellifera* and found different responses according to the active ingredient used. Thiamethoxam and methidathion were highly toxic, with low median lethal time (LT_{50}) for topical application, supply of contaminated food, and indirect contact of bees to previously sprayed surfaces. Abamectin showed lowest LT_{50} when provided in contaminated food, whereas deltamethrin showed highest toxicity when the insects were exposed to previously sprayed surfaces (Carvalho et al. 2009).

Pest control with entomopathogenic agents has the advantage of leaving no toxic residues, and therefore can be used for long periods with low environmental impact (Alves 1998). However, the susceptibility of stingless bees to other commercial isolates of entomopathogenic fungi should be tested in future studies, with special attention to the concentration. Only by knowing the effects of entomopathogenic agents on bees, it will be possible to achieve greater efficiency in pest control with minimal impact on these beneficial insects.

Beauveria bassiana (Biofungi 1) was highly virulent to uruçu bees (*M. scutellaris*), killing them at the lowest dose used. This information is important because the use of biological products for insect pest control has been growing, and these products require good management to avoid damage to beneficial insects.

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