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Effect of entomopathogens on Africanized *Apis mellifera* L. (Hymenoptera: Apidae)

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ABSTRACT

This study aimed to evaluate the effect of commercially used entomopathogens on Africanized *Apis mellifera* L. (Hymenoptera: Apidae). Four bioassays were performed: 1) pulverized entomopathogens on *A. mellifera*; 2) entomopathogens sprayed on a smooth surface; 3) entomopathogens sprayed on soy leaves; and 4) entomopathogens mixed with candy paste (sugar syrup). Five treatments were prepared: sterile distilled water (control), distilled water sterilized with Tween® 80 (0.01%), and the commercial entomopathogens *Metarhizium anisopliae* E9 (1.0×10^9 conidia mL^{-1}), *Beauveria bassiana* PL63 (1.0×10^8 conidia mL^{-1}) and *Bacillus thuringiensis* var. kurstaki HD-1 (3.0×10^8 spores mL^{-1}). Each treatment consisted of five repetitions, with 20 workers per repetition, which were stored in a plastic box and, later, in a biological oxygen demand (B.O.D.) incubator (27 ± 2 °C, RH of 60% ± 10%, 12-h photophase). The mortality of the workers was evaluated from 1 h to 240 h, and the data were analyzed using Bayesian inference. The workers killed by the ingestion of candy paste contaminated with the pathogens (products) were randomly separated and selected for the removal of the midgut. Each midgut was fixed in Bouin's solution and prepared for histology. *B. bassiana* was verified to reduce the survival of *A. mellifera* workers in all bioassays. Moreover, *M. anisopliae* reduced the survival of *A. mellifera* workers directly sprayed, on a smooth surface and mixed with candy. *B. thuringiensis* reduced *A. mellifera* survival on a smooth surface and mixed with candy paste. However, its effects were lower than that observed by *B. bassiana*. The treatments with the biological products did not induce morphometric alterations in the midgut of *A. mellifera*.

Introduction

Apis mellifera L. (Hymenoptera: Apidae), the honey bee, is a pollinating bee found in the most varied environments, and has outstanding importance in the cross-pollination of several plant species (Imperatriz-Fonseca et al., 2012). Aside from its important role in pollination, *A. mellifera* produces honey, propolis, wax, pollen, royal jelly and apitoxin, all products of interest to humans. The commercialization of these products helps in providing income to many producers and beekeepers (Wolff et al., 2008).

Beekeeping is a prominent occupation in the Brazilian economy, with around 350,000 beekeepers, most of them family farmers (SEBRAE, 2014). One of the factors of this success is the correct management of the colonies and the specialization of the labor force, which remains incipient.

Despite the care taken in the apiary at the moment of foraging, worker bees may come into contact with plant products that are toxic to them, to the colony, or are contaminants for honey (Codling et al., 2016). They can also be contaminated by products used to control the mite *Varroa destructor* Anderson and Trueman (Acari: Mesostigmata) (Medici et al., 2012) or the wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) (James, 2011; Ferreira et al., 2017). In addition, chemicals used in disease and insect control (Lu et al., 2012) and beekeeping pest control may contribute to colony collapse disorder (CCD) (Rucker and Thurman, 2012).

In this sense, studies have also been carried out to evaluate the safety of biological insecticides on *A. mellifera*, especially on indirect action on the health and development of the colony. The main biological insecticides tested are the entomopathogenic fungi *Beauveria bassiana* (Alves, 1998; Shaw et al., 2002; Al Mazra'awi,

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2007; Meikle et al., 2009; Hamiduzzaman et al., 2012; James et al., 2012; Abdelaal and Hany, 2013) and *Metarhizium anisopliae* (Alves et al., 1996; Shaw et al., 2002; Kanga et al., 2002, 2003, 2006, 2010; Hamiduzzaman et al., 2012; Abdelaal and Hany, 2013) and the entomopathogenic bacterium *Bacillus thuringiensis* (Alves, 1998; Brighenti et al., 2007; Alquisira-Ramírez et al., 2014; Lez et al., 2014; D'Urso et al., 2017). While several tests have been conducted, few have reported the effects of these entomopathogens on Africanized *A. mellifera*, which is the species used in Brazilian beekeeping.

While these entomopathogens, of natural origin or applied in the environment, are considered safe, studies are necessary to evaluate the different methods in which these entomopathogens may come into contact with Africanized *A. mellifera* and interfere with their biology. In addition, different isolates must be tested, since the market for organic products is not static, and new isolates are constantly found, tested and made available. The aim of this study was to evaluate the effect of the entomopathogens *B. bassiana*, *M. anisopliae* and *B. thuringiensis* on Africanized *A. mellifera* in different contact methods.

Material and methods

The Africanized *A. mellifera* workers were obtained from brood frames from Langstroth hives for capped brood, from the Experimental Apiary of the *Unidade de Ensino e Pesquisa – Apicultura* (UNEPE) at UTFPR-DV. The brood frames were allocated to hives selected based on the quality and quantity of the queen's oviposition and were provided a daily artificial diet (17.5 g of soy protein isolate, 4.0 g of flax oil, 4.0 g of palm oil, 17.5 g of yeast extract, 40.9 g of sugar, 10.0 g of honey, 5.0 g of pollen, 1.0 g of soy lecithin and 0.1 g of vitamin nucleus) until oviposition began. When the presence of 1-day-old eggs was observed, feeding occurred three times a week and counting continued until the 21st day (usually the time when the workers emerge) (Couto and Couto, 2002). On the 19th day, the brood frames were removed from the apiary, packed in Kraft paper bags (60 cm × 70 cm, 50 mm thick), sealed, drilled and transported to the Biological Control Laboratory II. The brood frames were placed in a B.O.D. incubator (30 ± 2 °C, RH of 70 ± 10% and 12-h photophase) to simulate the environment of the origin hive, until its emergence, thus providing workers of standardized age. After 48 h, the bees were collected (age zero) with the aid of a tube using the suction method. To feed the bees, pure candy paste was prepared by mixing 50 g of icing sugar with 10 mL of pure honey, until a homogeneous mass was formed.

The entomopathogens (treatments) used were *M. anisopliae* E9 (1.0×10^9 conidia mL^{-1}), *B. bassiana* PL63 (1.0×10^8 conidia mL^{-1}) and *B. thuringiensis* var. kurstaki HD-1 (3.0×10^8 spores mL^{-1}), which were obtained from commercial products and used at concentrations recommended by manufacturers for target pest insects.

Four bioassays were used, employing direct spray techniques, contact with treated surfaces (soy leaves and plastic boxes) and the supply of a contaminated diet. As a control, sterilized distilled water was used in the bioassays of direct spray techniques and contact with treated surfaces. In the bioassays that incorporated entomopathogens with candy paste, pure candy paste was used as a control.

Entomopathogens sprayed on A. mellifera

Each group, containing *A. mellifera* workers previously anesthetized with carbon dioxide (CO_2) (120 s), was sprayed with 1 mL of the treatment using a PneumaticSagyma® airbrush coupled with a Fanem® constant pressure pump (1.2 kgf cm^2). The same procedure was performed for all treatments/repetitions. The bees sprayed with the treatments were transferred into plastic boxes ($11 \times 11 \times 3.5$ cm, $L \times W \times H$) sealed with voile fabric, on which a piece of cotton wool soaked in distilled water and candy paste was laid.

All bioassays in this study were maintained in a B.O.D. incubator (27 ± 2 °C, RH of 60 ± 10%). The mortality of the workers was evaluated from 1 h to 240 h after the beginning of their exposure to the treatments to estimate the survival period (methodology adapted from Baptista et al., 2009). The experimental design was completely randomized with four treatments and five repetitions with 20 bees each. Each bee was considered an experimental unit.

The dead workers, verified in the treatments with the use of the entomopathogenic fungi, in all the bioassays, were placed in a humidity chamber to confirm mortality by fungus (Alves, 1998).

Entomopathogens on a smooth surface

One milliliter of the treatment was sprayed onto the base of a sterilized plastic box using a Pneumatic Sagyma® airbrush coupled with a Fanem® constant pressure pump (1.2 kgf/cm^2). The box was subsequently arranged in a horizontal laminar flow chamber for the evaporation of water. The *A. mellifera* workers, previously anesthetized, were housed inside each box, in groups of 10 individuals, for the entire experimental period. These boxes were sealed with voile fabric, on which cotton soaked in water and candy paste was laid.

Entomopathogens on soy leaves

Soy leaves (*Glycine max*) were immersed for 5 s in each treatment, and then placed in a horizontal laminar flow chamber for the evaporation of water. These leaves were subsequently arranged in plastic boxes. The *A. mellifera* workers, previously anesthetized, were housed inside each box in groups of 10 individuals. These boxes were sealed with voile fabric, on which cotton soaked in water and candy paste was laid.

Entomopathogens mixed with candy paste

Using the calculated dosage as a function of the weight:volume ratio, the entomopathogens were incorporated into honey. Icing sugar was then added to the honey to obtain a homogeneous diet. The *A. mellifera* workers, previously anesthetized, were housed in groups of 10 individuals, inside plastic boxes sealed with voile fabric, on which cotton soaked in water and candy paste with the entomopathogens was laid.

Histological analysis of the A. mellifera *midgut*

To perform histological analysis by repetition/treatment, the midguts of five bees from the bioassay with candy paste incorporated with entomopathogens were used. The samples were fixed in Bouin's fixative and stored in 70% alcohol. They were then dehydrated (alcohol), diaphonized (Xylol), paraffinized and embedded in Histological Paraffin (Cruz-Landim, 2009).

The material embedded in paraffin was cut [2–7 μm] in a manual rotating microtome and subsequently stained using hematoxylin/eosin (H/E). The cuts were observed in a TNB-40T-PL Trinocular Opton Light Microscope, and the images were captured with a digital camera (ScopePhoto 2.04).

A quantitative evaluation was performed, by measuring the heights of the bees' ventricular cells, and a qualitative evaluation was performed by observing tissue alterations. Compared with the samples from the control, they had no alterations in the midgut tissues.

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