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Effect of different *Bacillus thuringiensis* strains on the longevity of Africanized honey bee

Efeito de diferentes linhagens de *Bacillus thuringiensis* na longevidade de *Apis mellifera* L africanizada

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Abstract

The aim of this study was to assess the effects of three strains of *Bacillus thuringiensis* (Bt) on the longevity of workers of Africanized honey bee. Solutions at a concentration of 3.0×10^8 spores mL^{-1} (dosage) were prepared for each strain of Bt (IPS 82, BR 81, and BR 147). Three bioassays were performed as follows: spraying on the bees, contact with the sprayed surface, and candy paste incorporated with Bt. The bees of the Bt bioassay were submitted to histological analysis of the mesenteron. The longevity of workers was assessed from one to 120 hours using different ranges. It was found that the bees that were exposed to the strain of Bt IPS 82, in the spraying test, exhibited a reduced longevity. In the contact test, the BR 147 strain reduced the longevity of the bees. In the food test, in turn, the three studied strains reduced the longevity of the bees as follows: Bt IPS 82: 64.5 hours; Bt BR 81: 64.5 hours; and Bt BR 147: 60.0 hours. The Bt BR 81 strain was considered the most selective of the evaluated strains on *Apis mellifera*, reducing the longevity of this bee only when it came into contact by the method of ingestion.
Key words: Africanized honey bee. Entomopathogenic bacteria. Selectivity.

Resumo

O objetivo deste trabalho foi avaliar o efeito de três linhagens de *Bacillus thuringiensis* (Bt) sobre a longevidade de operárias de *Apis mellifera* africanizadas. Para isso foram preparadas soluções, na concentração de 3.0×10^8 esporos mL^{-1} (dosagem comercial), para cada linhagem de Bt (IPS 82, BR 81 e BR 147). Foram realizados três bioensaios: pulverização sobre as abelhas, contato com superfície pulverizada e pasta Cândi incorporada com Bt. As abelhas do bioensaio de Bt incorporado à alimentação foram submetidos à análise histológica do mesêntero. A longevidade das operárias foi avaliada de uma até 120 horas utilizando diferentes intervalos. Verificou-se que no teste de pulverização, as abelhas que entraram em contato com a linhagem de Bt IPS 82 apresentaram redução de longevidade. No teste de contato, a linhagem BR 147 reduziu a longevidade das abelhas. No teste de alimentação as três

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linhagens estudadas reduziram a longevidade das abelhas Bt IPS 82: 64,5 horas; Bt BR 81: 64,5 horas e Bt BR 147: 60,0 horas. A linhagen Bt BR 81 foi considerada a mais seletiva, dentre as avaliadas, reduzindo a longevidade da abelha *A. mellifera* apenas quando ingerida.
Palavras-chave: Abelha africanizada. Bactéria entomopatogênica. Seletividade.

Introduction

Bacillus thuringiensis Berliner (Bt) is one of several groups of entomopathogenic bacteria used in biological control. This bacterium is used as bioinsecticide for agricultural pest control, particularly caterpillars, in crops of tomatoes, cotton, citrus, manioc, soybeans, wheat and corn (ANGELO et al., 2010; BRAVO et al., 2011). Entomopathogenic microorganisms, such as bacteria, need to be tested before they are applied in the field since, apart from insect pests, they can affect non-target organisms such as pollinators (GALLO et al., 2002). One of these pollinators is the Africanized bee, *Apis mellifera* L. (Hymenoptera: Apidae).

Apis mellifera is a bee that is highly regarded as a pollinator of commercial crops (STRAUB et al., 2016). There is interest in maintaining these colonies, especially in agriculture, because they boost production, leading to high productivity and improved seed and fruit quality, besides producing honey, Royal Jelly, and propolis (VIUDA et al., 2008; RANGBERG et al., 2012; MARINI et al., 2015). Moreover, the absence of pollinators results in the low production of fruit and a lack of standardization in terms of form, appearance, taste, size and shape, which can reduce revenue and profit for producers (COBRA et al., 2015).

Bacillus thuringiensis var. *kurstaki* (commercial name Dipel®PM) proved toxic to adults of *A. mellifera* in the laboratory when sprayed on the workers and when added to the candy paste (BRIGHENTI et al., 2007). However, *B. thuringiensis* sub sp. *kurstaki* (Btk) when aerially sprayed does not affect the performance of the brood of *A. mellifera* bee colonies or interfere in the health of the colony and the behavior of bees. In addition, spray application did not change the behavior of the colonies, compared to before, during and after spraying the brood (LEZ et al., 2014).

D'Urso et al. (2017) found that *B. thuringiensis var. aizawai* and *kurstaki* (strain GC 91) (at the dosage recommended by the manufacturer) did not interfere with the survival of *A. mellifera* and did not cause morphological changes in the midgut of the worker bees.

However, with the advent of new products based on *B. thuringiensis* and the use of different strains on insect pests, selectivity must be constantly reviewed to verify the safety of these products for non-target organisms. The aim of this study was to assess the effects of three strains of *Bacillus thuringiensis* (Bt) on longevity of workers of Africanized honey bee *Apis mellifera*.

Materials and Methods

This study was conducted at the Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos (UTFPR-DV), at the Laboratory of Biological Control I and II and in the Apiculture Teaching and Research Unit (UNEPE-Beekeeping).

Obtaining A. mellifera and B. thuringiensis

Africanized honey bee: The frames containing 19-day-old worker bee larvae were obtained from colonies at the apiary at the Teaching and Research Unit (UNEPE) – Apiculture. The frames were transported to the Laboratory of Biological Control. At the laboratory, the frames were wrapped in Kraft paper bags, sealed, perforated, and kept in a heated BOD-type incubator (34 ± 2 °C, RH 60 ± 5%) for two days. The conditions ensured the even emergence of workers for use in bioassays by simulating the environment of the colony. *Apis mellifera* bees used

in the bioassays were fed with candy paste made by mixing 50 g icing sugar with 10 mL of pure honey until it formed a homogeneous mass.

Bacillus thuringiensis: Three strains of this bacterium were used, namely IPS 82, BR 81, and BR 147. These bacteria were obtained from the entomopathogenic collection of the Laboratory of Genetics and Taxonomy of Microorganisms at the Universidade Estadual de Londrina – UEL, identified by the presence of Cry proteins toxic to species of the orders Coleoptera and Diptera. These strains were multiplied and quantified until we obtained the commercial concentration of 3.0×10^8 spores mL^{-1}.

Bioassay 1: Spraying of B. thuringiensis on A. mellifera

The newly emerged *A. mellifera* workers were anesthetized with CO_2 for 120 s, placed in Petri dishes and then sprayed with the treatments using a Pneumatic Sagyma® airbrush coupled to a Fanem® constant pressure pump at 1.2 kgf cm^{-1}. Ten worker bees were then transferred to plastic containers (100 mm × 120 mm), each group of 10 bees was considered a repetition, with a total of six repetitions per treatment. The containers were sealed with voile fabric and food, consisting of candy paste and a piece of cotton wool soaked in distilled water, was placed on top of the fabric.

The control treatment was the spraying of sterile deionized water spray, and treatments based on *B. thuringiensis* were applied at a concentration of 3.0×10^8 spores mL^{-1}. The experiments were conducted in a BOD-type chamber (34 ± 2 °C, RH of 60 ± 5%) and the mortality of the worker bees evaluated at 1; 2; 3; 4; 5; 6; 9; 12; 15; 18; 21; 24; 30; 36; 42; 48; 60; 72; and 96 h after spraying the control agents methodology adapted from (BAPTISTA et al., 2009).

Bioassay 2: Contact of A. mellifera with surface sprayed with B. thuringiensis

Glass Petri dishes (15 cm diameter × 1.5 cm height) were each sprayed with 290 µL of solution containing one of the strains of *B. thuringiensis*. The calculation of volume solution was based on the area of the dish on which the solution is sprayed. A Pneumatic Sagyma® airbrush coupled to a Fanem® with constant pressure pump (1.2 kgf cm^{-1}) was used. Subsequently, these dishes were arranged in a horizontal laminar flow cabinet until the water was completely evaporated. The dishes were then placed in such a way that the gap almost fitted together, to allow room for airflow methodology adapted from (CARVALHO et al., 2009).

Ten *A. mellifera* workers, previously anesthetized with CO_2 for 120 s, were placed inside each dish. Eache dish represented a repetition, with a total of six repetitions per treatment. The control treatment consisted of the spraying of sterile deionized water. After 2 h of contact with the products, the bees were transferred to plastic containers, where they were provided with candy paste and cotton soaked in water. The experimental conditions, evaluated parameters, and data analysis are the same as those described for Bioassay 1.

Bioassay 3: Candy paste containing B. thuringiensis

Ten recently emerged *A. mellifera* workers were anesthetized with CO_2 for 120 s and packed individually in a flat-bottomed glass tube (2.5 × 8.5 cm). The tube was sealed with voile fabric, on which was placed a piece of cotton soaked in distilled water. The food was candy paste containing *B. thuringiensis*. The dose of *B. thuringiensis* was calculated according to the recommended dosage for use in the field. Each group of ten tubes represented a repetition, with a total of six repetitions per treatment. The control bees were supplied with pure candy paste. The experimental conditions, evaluated parameters, and data analysis are the same as those described for Bioassay 1.

Eight workers per treatment group, after its death, were selected for mesenteron examination. For removal of the mesenteron, the body of each bee was divided into fragments to facilitate preparation and ensure the quality of the samples. These samples were fixed in Bouin's solution (250 mL formaldehyde 40% + 50 mL glacial acetic acid PA + 750 mL saturated solution of picric acid 1.4%) for 4 h, washed in 70% alcohol (3 × 15 min) and stored in alcohol 70% until processing.

The samples stored in 70% alcohol were dehydrated by immersion in serial alcohol concentrations, according to an adapted histological method (80% alcohol: 10 min; 90% alcohol: 10 min; 95% alcohol: 10 min; 98% alcohol: 10 min; 100% alcohol: 2 × 30 min), and subsequently cleared by immersion in xylol (alcohol/xylol 1:1 for 30 min; xylol I: 30 min; xylol II: 30 min). The next step was embedding in paraffin wax (xylol/paraffin wax 1:1 for 30 min; paraffin wax I: 180 min; paraffin wax II: 15 min) and blocking in paraffin wax (paraffin wax/beeswax 4:1).

The blocked material was sectioned using a manual rotary microtome into pieces 2 to 7 μm thick, mounted on frosted tip slides (3 × 10 cm) coated with albumin solution, and placed on a hot plate to distend the cuts.

The sections were stained using hematoxylin/eosin (H&E), by take off paraffin (xylol I: 10 min; xylol II: 10 min; 100% alcohol I: 5 min; 100% alcohol II: 5 min) and rehydration (alcohol 90%: 5 min; 80% alcohol: 5 min; running distilled water: 2 min). For staining, the sections were immersed in hematoxylin (40 s), washed under running water (10 min), immersed in eosin (10 s), and again washed in running distilled water (10 s). Sections were dried in an oven at 35 °C for two days, after which they were mounted in Canada Balsam and covered with glass coverslips (2.3 × 3.6 cm) ready for microscopic examination.

Prepared sections were examined using a binocular biological microscope (Zeiss Primo Star) (40 × lens), with a digital camera to capture images. We compared tissue fromthe digestive system of the *A. mellifera* worker bees fed with candy paste containing *B. thuringiensis* with tissue from *A. mellifera* worker bees fed with pure candy paste.

Statistical analyses

The longevity data for *A. mellifera* workers from the three bioassays were submitted to the Shapiro-Wilk normality test, followed by analysis of variance (ANOVA). The average values were compared using the Duncan test at 5% probability and Assistat® software (SILVA; AZEVEDO, 2002).

Results

In the spraying bioassay of *B. thuringiensis* on *A. mellifera* (Bioassay 1), it was observed that only strain IPS 82 caused a reduction in longevity (88.7 h) when compared to the control (104.7 h). The other strains, BR 81 and BR 147 did not cause changes in the longevity of *A. mellifera* worker bees (Table 1).

The workers from the control treatment and from the BR 147 had a survival of 51.7% by the end of 120-h experiment; worker bees from BR 81 (43.4% survival) did not differ from the controls (51.7% survival) after 120 h. After 96 h, worker bees from the IPS 82 treatment group showed 100% mortality, differing from the other treatments (Table 1).

Table 1. Average longevity (h) and survival (%) ± SE of worker bees of the Africanized honey bee, *A. mellifera,* after being subjected to three bioassays with different strains of *B. thuringiensis* (Temperature 34 ± 2 °C, RH 60 ± 5%).

Bioassay	Treatment	Average longevity (h)	Survival after 120 h (%)
Bioassay 1: Spraying	Control	104.7 ± 2.99 a	51.7 ± 0.77 a
	Bt 1 (IPS 82)	88. 7 ± 2.66 b	0 ± 1.84b
	Bt 2 (BR 81)	102.5 ± 2.57 a	43.3 ± 0.80 a
	Bt 3 (BR 147)	105.6 ± 2.70 a	51.7 ± 0.79 a
p value		*p* <0.01	
Bioassay 2: Contact	Control	118.4 ± 0.78 a	93.3 ± 0.12 a
	Bt 1 (IPS 82)	111.6 ± 3.30 a	88.3 ± 0.08 a
	Bt 2 (BR 81)	117.8 ± 1.79 a	96.7 ± 0.40 a
	Bt 3 (BR 147)	101.2 ± 4.19 b	63.3 ± 0.35 b
p value		*p* <0.01	
Bioassay 3: Candy paste incorporated	Control	90.5 ± 4.98 a	52.0 ± 0.24 a
	Bt 1 (IPS 82)	64.5 ± 4.41 b	10.0 ± 0.50 b
	Bt 2 (BR 81)	64.5 ± 5.41 b	18.0 ± 0.36 b
	Bt 3 (BR 147)	60.0 ± 3.72 b	6.0 ± 0.52 b
p value		*p* <0.01	*p* <0.05

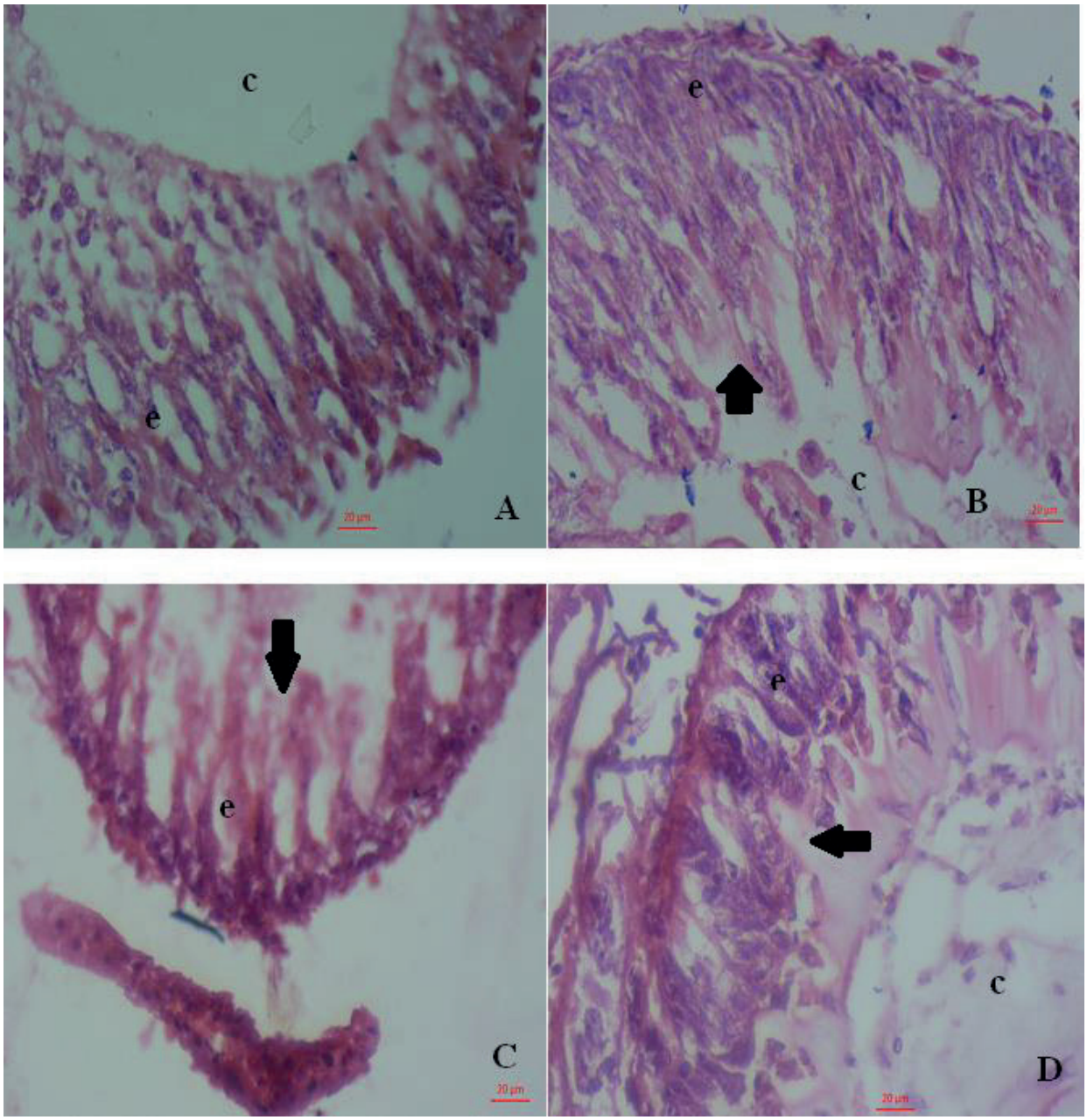
Different letters indicate significant differences between averages of treatments, according to the Duncan test at 95% level of credibility. SE: Standard Error.

In Bioassay 2, when *A. mellifera* bees were in contact with surface sprayed with Bt, only strain BR 147 caused a reduction in longevity of worker bees of *A. mellifera* (101.2 h) compared to the controls (118.4 h). The other strains (IPS 82: 111.6 h and BR 81: 117.8 h) did not present differences in the longevity of the workers bees. At the end of the 120-h experiment, 63.3% of the *A. mellifera* worker bees were alive with strain BR 147, differing from strains IPS 82 (88.3%) and BR 81 (96.7%), and the control (93.3%) (Table 1).

All the investigated strains of *B. thuringiensis*, when incorporated into the food of *A. mellifera* (Bioassay 3), reduced the longevity of the workers (IPS 82: 64.5 h; BR 81: 64.5 h and BR 147: 60 h) in relation to the controls – 90.5 h (Table 1). All three strains investigated reduced the survival of the worker bees. After 120 h, only 10% of the workers were alive with strain IPS 82, while 18% were alive with BR 81, 6% with BR 147 and 52% in the controls (Table 1).

The histology of the mesenteron of the workers of *A. mellifera* fed with candy and the three strains of *B. thuringiensis* exhibited disintegrated midgut with shapeless cells in comparison to the cells of the mesentera of workers fed with pure candy paste (Figures 1A, B, C and D). The overall cell organization, in the form of crypts, is shown in Figure 1A, while in Figures 1B, C, and D this organization is not observable.

Figure 1. Photomicrograph of the midgut of *Apis mellifera* (binocular biological microscope, Zeiss Primo Star, with a digital camera to capture images; 40× magnification). Worker bees fed with: A) Pure candy paste; B) Candy paste + Bt 1 – IPS 82, C) Candy paste + Bt 2 – BR 81, D) Candy paste + Bt 3 – BR 147. e = Epithelial linning; c = Cavity of the midgut.



Discussion

The bioassays "Spraying of *B. thuringiensis* on *A. mellifera*" (Bioassay 1) and "Contact of *A. mellifera* with surface sprayed with *B. thuringiensis* (Bioassay 2) did not affect the longevity of *A. mellifera* worker bees as much as when Bt was incorporated into the candy paste (Bioassay 3). This occurs because *B. thuringiensis* acts when ingested through the crystals that this bacterium produces. Consequently, when bees are fed with candy paste containing strains of bacteria, their longevity is reduced.

However, some strains, such as IPS 82 (used in this study), may reduce the longevity of *A. mellifera* workers. This was also observed when *B. thuringiensis* var. *kurstaki* (commercial name Dipel® PM – concentration of 0.5 g 100 mL^{-1} – commercial dosage) sprayed onto the adult workers caused significant mortality in 52.4% of the bees by the end of a 96-h experiment (BRIGHENTI et al., 2007).

In the bioassay Contact of *A. mellifera* with surface sprayed with *B. thuringiensis*, the *B. thuringiensis* strain BR 147 reduced the longevity of the worker bees. Despite the lack of studies on the effect of contact of *A. mellifera* with the bacteria *B. thuringiensis*, in this study one of the strains investigated had a negative effect. Although *B. thuringiensis* does not act by contact, only by ingestion, contamination may have occurred because social insects, including the young *A. mellifera* bees, exhibit a hygiene habit in which one bee clears the integument from another bee. This can cause them to ingest bacterial spores from their body or from other worker bees, causing changes (BRIGHENTI et al., 2007; TRIPLEHORN; JOHNSON, 2011) and leading to mortality shortly after spraying and/or contact.

Sub-doses of *B. thuringiensis* var. *kurstaki*, strain HD-1, in *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae), negatively influenced the biology of this parasitoid, reducing the percentage and the capabilities of parasitism (BORTOLI et al., 2012). The same occurred with *Telenomus remus* (Hymenoptera: Platygastridae) sprayed with *B. thuringiensis* (commercial product Dipel® PM) (SILVA et al., 2013).

In the bioassay of food, with candy paste containing *B. thuringiensis*, the three strains caused a reduction in the longevity of the bees. It is believed that this reduction occurred due the mode of action of *B. thuringiensis* by ingestion. It features crystals as the active component, and in the digestive tract of the insect, the crystal produced by the bacterium is in an ideal condition (alkaline pH) to be dissolved and absorbed, transforming itself into a toxic substance (60-65 kDa fragments). The epithelial cells of the intestinal villi of the insect recognize the specific receptors that trigger the formation of pores in the membrane of the epithelium, causing extravasation of the digestive liquid, destruction of microvilli membranes, development or overgrowth of epithelial cells, vacuolation of the cytoplasm, and rupture or dissolution of the plasma membrane, leading to paralysis and death of the insect (HABID; ANDRADE, 1998).

Similar to results of the present study, Brighenti et al. (2007) reported that adult workers of *A. mellifera*, fed with *B. thuringiensis* (commercial product Dipel®PM – in the commercial concentration 0.5 g), incorporated into the candy paste, after 96 h had a mortality of 57%. In the first hours it was observed that the bees rejected the food, exhibiting a large loss of liquid feces caused by intestinal disorders, loss of agility, overnight isolation, and overall paralysis before death (BRIGHENTI et al., 2007).

The midgut cells of *A. mellifera* fed with candy paste containing different strains of Bt exhibited loss of destruction (arrow) (Figure 1) since the epithelial cells of the midgut of the bees resembled a crypt, were arranged concentrically, and located almost parallel to each other. The crypt and the epithelial cells feature a radial symmetry, and some cells are still not in contact with the lumen (RAES et al., 1994; CRUZ-LANDIM, 2009)

However, worker bees of *A. mellifera ligustica* and *A. cerana cerana* were not affected when they ingested Cry1Ah toxin (found in transgenic Bt corn) mixed in sugar syrup, and which did not interfere in the consumption of pollen and the mass of the hypopharingeal gland (DAI et al., 2011).

Although no apparent changes were observed in the bees, the Bt Cry spores can trigger physiological changes and differentially change enzymatic activities. This means that the apparent absence of toxicity may mask physiological disruptions that could be harmful to bees, especially in the case

of exposure combined with other environmental stressors or high-dosages (RENZI et al., 2016).

A few minutes after ingestion of *B. thuringiensis*, histopathological changes begin to occur in the microvilli membranes of the midgut and, depending on the insect, extensive disintegration of the epithelial cells of the gut can occur in a few hours (HABID; ANDRADE, 1998) and, as described previously, these conditions can lead to the death of the insects.

The different strains of *B. thuringiensis* produce toxins and substances with specific actions. The most important of these toxins is δ-endotoxin or crystal toxin, which initially targets the epithelium of the midgut, where toxins coming into contact with the epithelium cells cause an osmotic imbalance that leads to a break in feeding, and later causes an intestinal stop. Another toxin, β-exotoxin (thuringiensina) is fatal to insects and causes the death of species of Lepidoptera, Diptera, Coleoptera, Hymenoptera, Isoptera, and Orthoptera (HABID; ANDRADE, 1998).

It was observed that in the food bioassay, the three strains interfered with the longevity of the bees, probably because *B. thuringiensis* acts when it is ingested by insects, increasing mortality. However, as the farmers spray Bt products on the crops, field tests can be realized to assess the longevity of workers of *A. mellifera* and to analyse other environmental factors.

In fied conditions, the bees can come into contact with these products in three different ways in a single foraging: 1) being sprayed with the product, 2) coming into contact with sprayed plants, and 3) ingestion of the bacteria while gathering nectar and pollen. In the field, biotic factors (for example, predators and weeds) and abiotic factors (such as rain, wind, and temperature) interfere with bees coming into contact with the products, and can easily minimize the effects caused by applications of *B. thuringiensis*.

Conclusions

The three strains of *B. thuringiensis* (IPS 82, BR 81, and BR 147) reduced the longevity of workers of the Africanized honey bee, *A. mellifera,* when added to candy paste and caused the disintegration of the epithelial cells of the mesenteron. The strains IPS 82 and BR 147 also reduced the longevity of worker bees in the bioassay of spraying *B. thuringiensis* on *A. mellifera* and in the bioassay of contact of *A. mellifera* with surface sprayed with *B. thuringiensis*, respectively.

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