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Longevity of *Apis mellifera* workers fed on a diet incorporating entomopathogens

Longevidade de operárias de **Apis mellifera** *alimentadas com dieta incorporada com entomopatógenos*

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ABSTRACT: The present study had the objective of evaluating the longevity of *A. mellifera* workers fed on a diet incorporating commercial entomopathogens, *Beauveria bassiana*, and *Bacillus thuringiensis*. It also aimed at verifying possible morphological alterations in the midgut. To this purpose, the entomopathogens used were *B. bassiana* (Product A) (5.0×10^{11} viable conidia.kg^{-1}), *B. thuringiensis* (Product B) (2.5×10^{9} viable spores.g^{-1}), and *B. thuringiensis* (Product C) (1.0×10^{9} viable spores.g^{-1}); and two controls: T1: sterilized distilled water, and T2: sterilized distilled water + Tween 80® (0.01%). For the bioassays, 2 mL of each treatment were incorporated into Candy paste. For each treatment, 80 bees were individually in flat bottom glass tubes (2.5 cm Ø) covered with voile, containing a piece of cotton soaked in water and Candy paste. These tubes were stored in a B.O.D (30 ± 2°C, R.H 70% ± 10%, 12 h), and mortality was evaluated every six hours, for 10 days. Soon after verifying mortality, two bees per treatment were selected for the removal of their midgut. Midgut samples were processed using standard methodology for Scanning Electron Microscopy (SEM). It was verified that products A, B, and C reduced the longevity of bees when compared to T1 and T2 controls. In the qualitative analyses carried out using SEM, it was not possible to observe external or internal morphological alterations to midgut tissues. Although products A, B, and C cause a reduction in longevity, their presence was not verified when tissues were analyzed using SEM.

KEYWORDS: Africanized bee; biological insecticides; selectivity.

RESUMO: No presente trabalho objetivou-se avaliar a longevidade de operárias de *A. mellifera* alimentadas com dieta incorporada com os entomopatógenos comerciais *Beauveria bassiana* e *Bacillus thuringiensis,* e verificar possíveis alterações morfológicas em seu mesêntero. Para isso, os entomopatógenos utilizados foram *B. bassiana* (Produto A) ($5{,}0 \times 10^{11}$ conídios viáveis.kg^{-1}), *B. thuringiensis* (Produto B) ($2{,}5 \times 10^{9}$ esporos viáveis.g^{-1}), *B. thuringiensis* (Produto C) ($1{,}0 \times 10^{9}$ esporos viáveis.g^{-1}); e dois controles: T1: água destilada esterilizada e T2: água destilada esterilizada + Tween 80® (0,01%). Para os bioensaios, 2 mL de cada tratamento foram incorporados à pasta Cândi. Para cada tratamento, 80 abelhas foram acondicionadas, individualmente, em tubos de vidro de fundo chato (2,5 cm Ø), cobertos com *voile*, contendo um pedaço de algodão embebido em água e pasta Cândi. Os tubos contendo as abelhas foram acondicionados em B.O.D (30 ± 2°C, U.R. 70% ± 10%, 12 h), e a mortalidade foi avaliada a cada seis horas, durante 10 dias. Logo após a verificação da mortalidade, foram separadas duas abelhas por tratamento para a retirada do mesêntero. Essas amostras foram processadas em metodologia padrão para Microscopia Eletrônica de Varredura (MEV). Verificou-se que os produtos A, B e C reduziram a longevidade das abelhas quando comparados aos controles T1 e T2. Nas análises qualitativas realizadas com MEV, não foi possível observar alterações morfológicas externas ou internas nos tecidos do mesêntero. Apesar dos produtos A, B e C causarem redução na longevidade, sua presença não foi verificada quando os tecidos foram analisados por MEV.

PALAVRAS-CHAVE: Abelha africanizada, inseticidas biológicos, seletividade.

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INTRODUCTION

Apis mellifera L. bees are classified within the Hymenoptera order, Apidae family, in which approximately twenty thousand different species are known (RAMOS; CARVALHO, 2007). These organisms, especially worker bees, are important to ecosystems, because they are responsible for the pollination of vegetal species (IMPERATRIZ-FONSECA et al., 2012; JACK, 2015; MATUSIEWICZ et al., 2016), besides being producers of honey, propolis, royal jelly, wax, and apitoxin (COSTA-MAIA et al., 2010).

These specimens are present in greater quantity in the colony, and are also responsible for the collection of pollen and nectar, for feeding the queen, cleaning, and defending (GULLAN; CRANSTON, 2009; TAUTZ, 2010). At the time of nectar and pollen collection, worker bees can be exposed to microorganisms, carrying them back to the colony, where infection and dissemination can occur, seen that the colony is a conducive environment to the development of microorganisms. Thus, these factors can contribute to an increased mortality of colonies (D'URSO et al., 2017).

In recent decades, there has been a large-scale decline in the population of bees, known as colony collapse disorder, causing damage to agriculture (MESSAGE et al., 2011; JOHNSON et al., 2009). Various factors may be leading to this phenomenon. Among them, the wide scale usage of synthetic chemical products in pest control is highlighted (COSTA-MAIA et al., 2010; KAPLAN, 2012; RUCKER; THURMAN, 2012), which is the most aggravating factor in Brazil.

Therefore, strategies to reduce or avoid the occurrence of this phenomenon are mandatory, such as, the use of biological control agents, like microbial insect control, which is considered less harmful due to its efficiency at lower concentrations and greater specificity, when compared to synthetic chemical insecticides (GUPTA; DIKSHIT, 2010; D'URSO et al., 2017). Among microbial control agents are entomopathogenic fungi and bacteria (ALVES, 1998), a type of control which has been used more often, especially given the preoccupation with health and environmental protection.

Although the use of biological control with entomopathogens has advantages in relation to synthetic chemicals, further studies must evaluate their possible effects on non-target organisms, and on *A. mellifera* bees in particular. Therefore, the objective of this study was to evaluate the longevity of *A. mellifera* workers fed with a diet incorporating commercial entomopathogens *Beauveria bassiana* (Bals.) Vuill, and *Bacillus thuringiensis* Berliner, and verify possible morphological alterations in their midgut under laboratory conditions.

MATERIALS AND METHOD

The experiments were carried out at Biological Control Laboratory (Laboratório de Controle Biológico) I and II, at Apiculture Teaching and Research Unit (Unidade de Ensino e Pesquisa Apicultura) (UNEPE-APICULTURE) of Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos (UTFPR-DV), and at Laboratory of Electron Microscopy and Microanalysis (Laboratório de Microscopia Eletrônica e Microanálise) (LMEM) of Universidade Estadual de Londrina (UEL).

Obtaining entomopathogens

Entomopathogens are formulated into commercial products: Product A (Cepa PL63), based on the *Beauveria bassiana* entomopathogenic fungus (5.0×10^{11} viable conidia.kg-1); Product B, with a formulation based on *Bacillus thuringiensis, var. kurstaki*, line HD-I, entomopathogenic bacteria (2.5×10^{9} viable spores.g-1), at the concentration recommended by the manufacturer for *Anticarsia gemmatalis* (insect-pest of soybean crops), and Product C, with a formulation based on *B. thuringiensis aizawai* GC-91 entomopathogenic bacteria (1.0×10^{9} viable spores.g-1), at the concentration recommended by the manufacturer for *Anticarsia gemmatalis* (insect-pest of soybean crops), as in Table 1.

Obtaining *A. mellifera* workers

A. mellifera workers were obtained from six honeycombs, from Langstroth hives, for capped brood, from the Experimental Apiary of the Teaching and Research Unit (UNEPE) Apiculture of UTFPR-DV. The honeycombs were allocated in hives chosen based on quality and quantity of the queen's oviposition, and were given an artificial daily diet (isolated soya protein,

Table 1. Treatments and concentrations used in the bioassay.

Treatments	Concentrations
Sterilized distilled water	-
Sterilized distilled water with Tween 80® (0.01%)	10 µL/1000 mL of distilled water
Beauveria bassiana (Product A)	1 g of the product.100 mL^{-1} of sterilized distilled water with Tween 80® (0.01%)
Bacillus thuringiensis (Product B)	0,5 g of the product.100 mL^{-1} of sterilized distilled water
Bacillus thuringiensis (Product C)	0,5 g of the product.100 mL^{-1} of sterilized distilled water

linseed oil, palm oil, beer yeast, sugar, honey, pollen, soya lecithin, and a nucleus of vitamins) until the beginning of oviposition. When the presence of one-day eggs was observed, feeding occurred three times a week, and a 21-day countdown was started (this is normally the time when workers emerge). On the 19th day, the honeycombs were removed from the apiary, wrapped in Kraft paper bags (60 × 70 cm with 50 mm grammage), sealed, perforated, and transported to the Biological Control Laboratory II. The honeycombs were put in a heated B.O.D incubator (30 ± 2°C, R.H of 70 ± 10%, and a 12-hour photophase) to simulate the environment of the hive of origin, until emergence, thereby obtaining workers with a standardized age.

Effects of entomopathogens incorporated into the Candy paste on *A. mellifera* workers

To perform the experiments, the recently emerged *A. mellifera* workers were individually transferred to flat bottom sterilized glass tubes (10 cm long × 2.5 cm Ø), subsequently covered with voile cloth. A piece of cotton soaked in 2 mL of sterilized distilled water, and 5 g of Candy paste (icing sugar and honey) incorporated to the treatments was placed on the cloth. The treatments were: Sterilized distilled water, Sterilized distilled water with Tween 80® (0.01%), *B. bassiana* (Product A), *B. thuringiensis* (Product B), and *B. thuringiensis* (Product C), as described in Table 1.

For all the treatments, 2 mL of control or entomopathogenic solutions were added to 100 g of Candy paste (100 g of icing sugar, and 20 mL of honey) to supply the workers. Each treatment consisted of 80 repetitions, whereby each glass tube containing a bee was considered a repetition. The tubes containing the workers were stored in a heated B.O.D incubator (30 ± 2°C, R.H of 70 ± 10%, and a 12-hour photophase). The workers' longevity was evaluated every six hours, for 10 days.

For the bioassay with entomopathogens incorporated into Candy paste, an analysis of variance ANOVA was carried out, and the means were then compared to each other with Scott-Knott test, at 95% credibility level, on the Assistat program version 7.7 beta® (SILVA, 2014).

Analysis of the midgut of *A. mellifera* using Scanning Electron Microscopy. After verifying bee mortality, two bees per treatment were randomly selected and separated for dissection and removal of the midgut, using metal tweezers, a scalpel, and a Stereoscopic Binocular Microscope [A.cietifica/Edutec (model: 505A/80XB)]. At the time of dissection, the midgut received 1 mL of modified Karnovsky Fixative [Paraformaldahyde 3%, Glutaraldahyde 3% and Phosphate Buffer (PO4 0.1M)] to preserve them from possible decomposition. Midgut samples were set in modified Karnovsky Fixative overnight, and stored in a refrigerator (4°C) at the Biological Control Laboratory II.

Subsequently, samples were taken to LMEM/UEL for washing in phosphate buffer (3 × 15 minutes), fixing in an Osmium Tetroxide 1% (OsO4) solution for 1 hour, and then, another wash in phosphate buffer (3 × 15 minutes). The material was then dehydrated in a battery of alcohol (alcohol 70%: 3 × 10 minutes, alcohol 80%: 3 × 10 minutes, alcohol 90%: 3 × 10 minutes, and alcohol 100%: 4 × 10 minutes), and CO_2 at Critical Point [BAL – TEC (model CPD 030)].

After dehydration, the samples were put on metal supports (stubs) containing silver glue, using a Stereoscopic Microscope, [LEICA model MZ6], and metal tweezers. The stubs with the samples were metalized with gold, using a Metallizer [BAL-TEC (model SCD – 050)], and then observed in a high vacuum of electron beam intensity, under Scanning Electron Microscopy. The images were captured through digital photos, and stored in a computer.

With the images, a qualitative analysis was carried out to verify the presence, or not, of entomopathogens in the samples, compared to the samples from the control.

RESULTS AND DISCUSSION

Effects of the entomopathogens, incorporated into Candy paste, on *A. mellifera* workers. The commercial product formulated based on *B. bassiana* (Product A) reduced the longevity of *A. mellifera* workers (60.45 hours) when compared to controls T1 and T2 (72.97 and 80.77 hours, respectively) (Table 2).

A similar effect was observed in other studies, in which the GHA isolate of *B. bassiana* entomopathogenic fungus, when tested for control of *Varroa destructor* Anderson and Trueman, and for selectivity of *A. mellifera*, in an experiment immersing mites in a solution of spores, caused a 40.83% reduction in the emergence of bees, and a mortality of 59.17%, when the bees came into contact with mites contaminated with the fungus (HAMIDUZZAMAN et al., 2012).

On the other hand, isolates of *B. bassiana*, ARSEF 3769 (ARK), NY (NY, BB008, SCPFRC) and GHA, when tested with the contact method on the surface of canola leaves (*Brassica napus* L.) did not cause mortality in *A. mellifera* workers, and insects infected by the fungus were not observed (AL-MAZRA'AWI et al., 2006). Other biological parameters of *A. mellifera* were evaluated when in contact with *B. bassiana*, of which MEIKLE et al. (2007) verified that *B. bassiana* did not have an effect on the weight of the colony, weight of adult bees, or on honey production. The reduction in longevity of *A. mellifera* workers, after receiving a diet incorporating *B. bassiana*, may be related to the mode of infection, which is by contact. By feeding through the Candy paste, the workers may have entered into contact with conidia of the fungus, which forms a germinative tube when enters into contact with any part of the cuticle of the

insect; the hyphae then crosses the tegument. After that, the fungus multiplies inside the insect, presenting hyphal mass in the hemocoel, initiating the process of colonization (ALVES, 1998; LAZZARINI, 2005).

The germination process may occur from 12 to 18 hours, and penetration of the conidia in the tegument takes approximately 12 hours. The fungus begins to produce and release toxic substances 30 hours after contact, which may cause stress or kill the insect. The insect presents itself as totally colonized 72 hours after inoculation, dying from lack of nutrients and the accumulation of toxic substances (ALVES, 1998). This may have been the cause of reduced longevity in *A. mellifera* workers, after the ingestion of Candy paste incorporated with *B. bassiana* in the present study.

As to bacteria, in this study, the commercial products formulated based on *B. thuringiensis* (products B and C) also reduced the longevity of the *A. mellifera* workers (64.12 and 62.47 hours, respectively) when compared to controls T1 and T2 (Table 2).

It has been verified in other studies that the *B. thuringiensis* entomopathogenic bacteria reduces survival of *A. mellifera* workers when incorporated into Candy paste (10 and 20 g of product/60 g of Candy paste), causing a 100% mortality rate of the workers, 72 hours after ingestion (BRIGHENTI et al., 2007). In the same experiment, the authors verified that the mean longevity of bees was between 2.6 and 3.1 days when submitted to the concentrations of *B. thuringiensis* used (0.25, 0.5, 1, 2.5, 5, 10, and 20 g).

In this study, the two commercial products formulated based on *B. thuringiensis* bacteria caused a reduction in the longevity of the workers, after the ingestion of Candy paste incorporated with the entomopathogen. These workers presented a mean longevity of 64.12 hours (2.67 days), and 62.47 hours (2.60 days) (Products B and C, respectively), similar results to those found by BRIGHENTI et al. (2007). It is possible that the results obtained in this study are related to the way the *B. thuringiensis* control agent acts. By ingesting the bacteria spores, they come into contact with the digestive tract of the insect, which has an alkaline pH (GALLO et al., 2002), and the crystals are thus solubilized, releasing protoxins, which transform into δ-endotoxins in contact with cell proteases (ALVES, 1998; FIUZA, 2009). After, the crystals connect to receptors present in the cell membrane of the epithelium of the insect, causing perforations and then osmotic and ionic imbalance, disintegrating the cells and leading the insect to death by septicemia (ALVES, 1998; POLANCZYK; ALVES, 2003; FIUZA, 2009).

Although this is a valid hypothesis, the action of *B. thuringiensis* is commonly observed in less than 48 hours in other insects, like in lepidoptera (ALVES, 1998), which would be faster than that observed in the present study. DAI et al. (2012) also verified that the Cry1Ah toxin, arising from *B. thuringiensis*, when added to sugar syrup at different concentrations, does not alter survival, longevity, pollen consumption, or weight of the hypopharyngeal gland of *A. mellifera ligustica,* or *Apis cerana cerana* F.

Analysis of the midgut of *A. mellifera* workers using scanning electron microscopy. After qualitative analysis of the images, it could be verified that *B. bassiana* entomopathogenic fungus did not cause external, or internal morphological alterations in the midgut tissues of *A. mellifera* bees, and was not present in the analyzed samples (Fig. 1A). The integrity of the midgut was also observed, with no signs of rupture or damage as a result of the effect of the product, when compared with controls T1 (Fig. 1B), and T2 (Fig. 1C).

In this study, the workers that ingested the Candy paste incorporated with *B. bassiana* possibly ingested the conidia of the fungus. However, these may not have penetrated the cuticle of the insect, and colonization may not have occurred as conidia and hyphae could not be verified in the analyzed midgut samples, which can be seen in Fig. 1A. Although it is not present in the analyzed midgut samples, the *B. bassiana* fungus reduced longevity of the *A. mellifera* workers, probably by producing toxins, such as beauvericin (ALVES, 1998), or the conidia may have caused stress to the bees, reducing their longevity. It was verified that *B. thuringiensis* entomopathogen did not cause external, or internal morphological alterations to the tissues of the midgut of *A. mellifera* workers, and was not present in the analyzed samples, despite reducing the longevity of bees. Integrity of the midgut was also observed,

Table 2. Longevity (in hours) (± E.P.) of *Apis mellifera* workers after provision of Candy paste incorporated with entomopathogens. Temperature 30 ± 2°C, 12-hour photophase, and R.H of 70 ± 10%. Universidade Tecnológica Federal do Paraná, Dois Vizinhos Campus, Paraná State, 2016.

Treatments	Longevity (hours)	Longevity (days)
T1 (Sterilized distilled water)	72.97 ± 3.79 a	3.04
T2 (Sterilized distilled water with Tween 80® (0.01%))	80.77 ± 3.60 a	3.36
Beauveria bassiana (Product A)	60.45 ± 4.02 b	2.51
Bacillus thuringiensis (Product B)	64.12 ± 3.99 b	2.67
Bacillus thuringiensis (Product C)	62.47 ± 3.85 b	2.60
p-value	< 0.01	

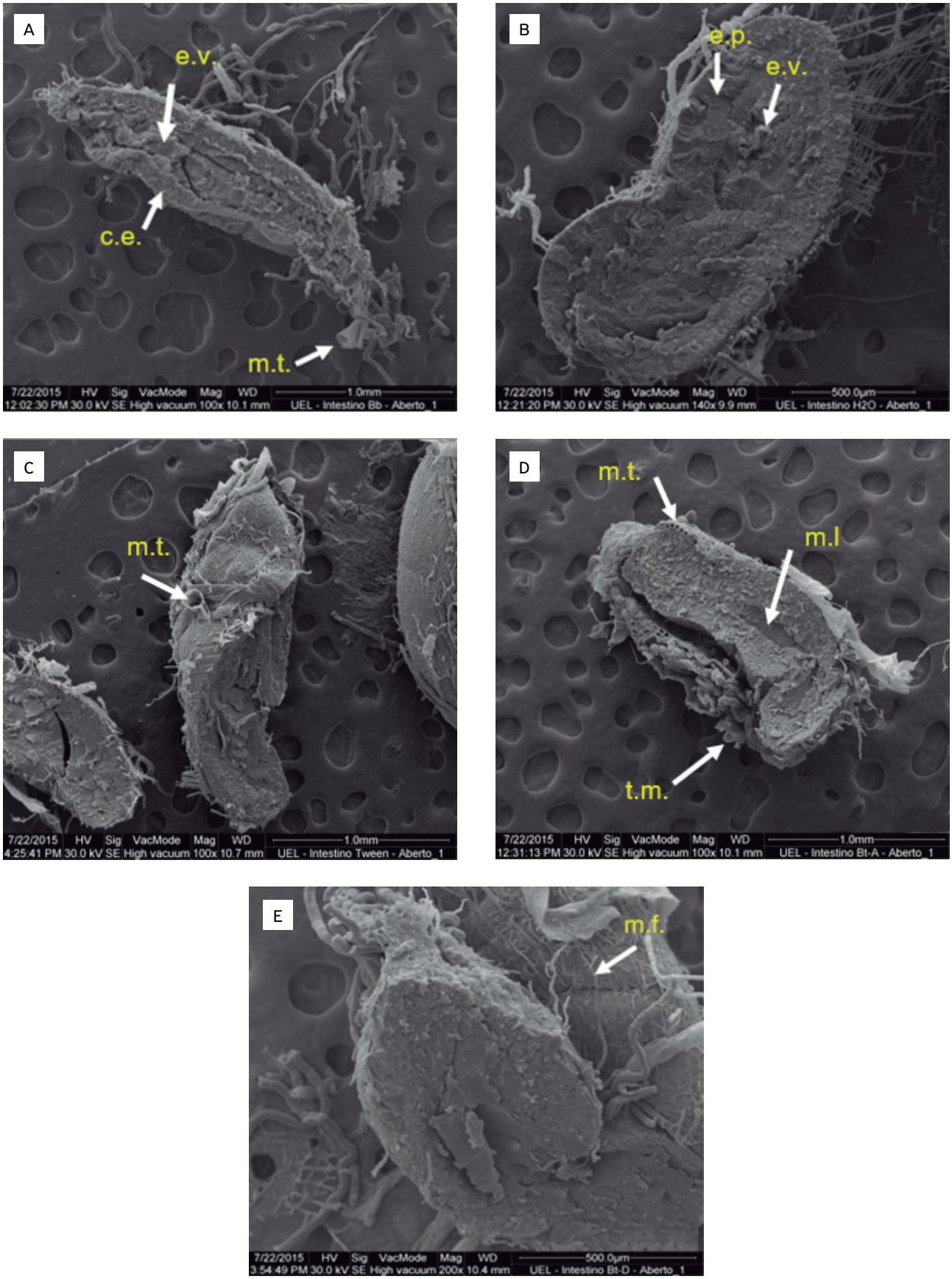

Figure 1. Scanning electron micrograph of the midgut of *A. mellifera* workers, after ingestion of Candy paste incorporated with (A) *Beauveria bassiana* (Product A); (B) Water control (T1); (C) Water control with Tween® 80 (0.01%), (T2); (D) *B. thuringiensis* (product B); (E) *B. thuringiensis* (product C). Midgut with longitudinal cuts. Cells of the epithelium (c.e.), Malpighi tubes (m.t.); epithelium of the ventricle (e.p.); esophagus valve (e.v.); muscular fibers (m.f.); and midgut lumen (m.l.).

with no signs of rupture or damage because of the effect of products B (Fig. 1D), or C (Fig. 1E).

The cry1C and cry2C proteins of *B. thuringiensis*, incorporated into an artificial diet (royal jelly, glucose, fructose, distilled water, and yeast extract) supplied to the larvae of *A. mellifera*, did not provoke damage, visible under optic microscope, to the outer edges of the midgut of this insect (WANG et al., 2015).

Bacillus thuringiensis, at the field denominated concentrations (100.0 $g.hL^{-1}$), low concentration (40.00 $g.hL^{-1}$), and high concentration (24,000.00 $g.hL^{-1}$), provoked irregularities in the epithelium of the midgut of *A. mellifera* workers after 96 hours, whereby at the two highest concentrations these alterations were verified with scanning and transmission electron microscopy after the first 24 hours (D'URSO et al., 2017).

In the present study, the workers that ingested the diet incorporated with entomopathogenic *B. thuringiensis* possibly ingested the spores containing bacteria crystals, but the toxins may not have been activated by the pH of the digestive tract of these *A. mellifera* workers, as the proteases, which make the bacteria crystals toxic, have different activation conditions and may vary in insect species, according to eating habits, type of diet, and physical-chemical conditions of intestine lumen (ALVES, 1998). When the *A. mellifera* workers are fed artificially with honey and sugar, as in the present study, the pH of the digestive tract tends to diminish, as honey has low pH, acidifying the pH of the midgut of the bees (COUTO; COUTO, 2002).

In all the midgut samples analyzed, it is possible to observe the presence of other microorganisms, like fungi and bacteria, which are probably from the insect's own digestive tract or are microorganism decomposers, resulting from the death of bees. In relation to the bacteria observed in the images, it is possible to confirm that they are not *B. thuringiensis* entomopathogenic bacteria, due to the difference in spore size.

The method and the time at which the entomopathogens are applied in the field may cause bees to be exposed to these products, albeit at lower quantities. Therefore, applications at times when the foraging rate is lower, like at the end of the afternoon, may diminish the bees' contact with the products. Another alternative for workers to avoid coming into contact with the entomopathogens is to close the hive during the period of application, performing artificial feeding during this period, or taking the hives to places at least 2.5 km away from where application of the products will be carried out.

Therefore, new studies, with different application methods are needed, so as to evaluate the effect of the entomopathogens tested. Among the different methods, spraying tests, *A. mellifera* contact and field tests can be cited.

CONCLUSIONS

In the tested formulations, *B. bassiana* and *B. thuringiensis* entomopathogens caused a reduction in the longevity of *A. mellifera* workers when incorporated in the bees' diet (Candy paste).

Despite the reduction in longevity, the integrity of the midgut could be observed, with no signs of ruptures caused by *B. bassiana,* or *B. thuringiensis* entomopathogens in the analyzed sample.

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