Revista Brasileira de Entomologia 62 (2018) 23–28

SOCIEDADE BRASILEIRA DE ENTOMOLOGIA FUNDADA EM 1937

REVISTA BRASILEIRA DE
Entomologia
A Journal on Insect Diversity and Evolution

www.rbentomologia.com

Biological Control and Crop Protection

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

Michele Potrich[a,*], Rita T.L. da Silva[a], Fabiana M.C. Maia[b], Everton R. Lozano[a], Robson M. Rossi[c], Fernanda C. Colombo[a], Flavia G. Tedesco[a], Alfredo de Gouvea[a]

[a] *Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos (UTFPR-DV), Laboratório de Controle Biológico, Estrada para Boa Esperança, Paraná, PA, Brazil*
[b] *Universidade Tecnológica Federal do Paraná, Campus Dois Vizinhos (UTFPR-DV), Unidade de Ensino e Pesquisa (UNEPE) Apicultura, Paraná, PA, Brazil*
[c] *Universidade Estadual de Maringá (UEM), Departamento de Estatística, Maringá, PA, Brazil*

ARTICLE INFO

Article history:
Received 27 July 2017
Accepted 4 December 2017
Available online 19 December 2017
Associate Editor: Rodrigo Gonçalves

Keywords:
Bayesian statistics
Entomopathogenic fungi
Entomopathogenic bacteria
Honeybee
Selectivity

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

* Corresponding author.
E-mails: michelepotrich@utpfr.edu.br, profmichele@gmail.com (M. Potrich).

https://doi.org/10.1016/j.rbe.2017.12.002

Beauveria bassiana (Alves, 1998; Shaw et al., 2002; Al Mazra'awi, 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 × 11 × 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.

Table 1
Lethal survival time (mean ± SD a posteriori of the median parameter of the Weibull distribution (1)) of Africanized *Apis mellifera* workers in contact with entomopathogens. Temperature 27 ± 2 °C, 12-h photophase and relative humidity of 60 ± 10%.

Bioassay	Treatment	Survival (h)
Entomopathogens sprayed on *Apis mellifera*	Control[a]	127.6 ± 8.17b
	Metarhizium anisopliae	104.7 ± 5.97c
	Beauveria bassiana	62.6 ± 3.01d
	Bacillus thuringiensis	167.2 ± 7.60a
Entomopathogens on a smooth surface	Control[a]	197.8 ± 4.80a
	Metarhizium anisopliae	128.0 ± 5.80c
	Beauveria bassiana	97.3 ± 1.10d
	Bacillus thuringiensis	144.9 ± 8.00b
Entomopathogens on soy leaves	Control[a]	133.9 ± 6.91a
	Metarhizium anisopliae	134.4 ± 6.90a
	Beauveria bassiana	89.1 ± 3.18b
	Bacillus thuringiensis	136.1 ± 8.03a
Entomopathogens mixed with candy paste	Control[a]	169.8 ± 7.56a
	Metarhizium anisopliae	141.9 ± 9.86b
	Beauveria bassiana	117.3 ± 7.88d
	Bacillus thuringiensis	131.3 ± 7.97c

[a] Sterilized distilled water.
Different letters in the column indicate significant differences between treatment averages, using Bayesian contrasts at a 95% credibility level.

Statistical analyses

Survival analysis for the lifetime (days) of bees during the bioassays was applied, using non-parametric (Kaplan–Meier) and parametric adjustments (Weibull model (1), $f(t)$, $S(t)$ and t_p are, respectively, the probability density function, survival function and p-percentile $t_p = 100p\%$ of the distribution, where $p = 0.5$ is the median). Estimates of these parameters were obtained by Bayesian inference.

$$f(t) = \frac{\gamma}{\alpha^{\gamma}} t^{\gamma-1} \exp\left\{-\left(\frac{t}{\alpha}\right)^{\gamma}\right\}, \; S(t) = \exp\left\{-\left(\frac{t}{\alpha}\right)^{\gamma}\right\} \text{ and } t_p = \alpha[-\log(1-p)]^{1/\gamma} \quad (1)$$

Its notation is $T \sim W(\alpha, \gamma)$, and $\alpha > 0$ and $\gamma > 0$ are the scale and shape parameters, respectively. Assuming the parameterization, such that, $\lambda = \log(1/\alpha^{\gamma})$, making $\beta = e^{\gamma} = 1/\alpha^{\gamma}$ and, consequently, $\alpha = e^{(\log(\beta)/\gamma)}$, non-informative distributions were considered a priori for all parameters, that is, $\lambda \sim N(0, 10^{-6})$ and $\gamma \sim Gama(10^{-3}, 10^{-3})$ (OpenBugs parameterization). The subsequent marginal distributions for the parameters were obtained through the *BRugs* package of the software *R Core Team* (2013). The value "1" and the frequency estimator were considered initial values for γ and λ, respectively. A total of 1,100,000 values were generated in an *MCMC* process, considering a sampling period of 100,000 initial values. The final sample of 11,000 values was formed after applying jumps of 100 in size, in order to eliminate the autocorrelation between values generated in the process. The convergence of the chains was verified through the *R coda* package, using the criterion of Heidelberger and Welch (1983). The parameters of the models, as well as the effects of the contrasts between treatments, were considered significant if their respective intervals, with 95% credibility for the a posteriori means, did not include the value zero.

To perform the histological analysis, the data of the quantitative tests were submitted to an analysis of variance (ANOVA), and the means were compared via the Scott Knott test, with 5% significance in the Assistat software (Silva et al., 2012).

Results

Entomopathogens sprayed on A. mellifera

The entomopathogens *M. anisopliae* and *B. bassiana* reduced the survival of *A. mellifera* workers (Table 1) when sprayed directly on them. *M. anisopliae* reduced *A. mellifera* survival to 104.7 h, and *B. bassiana* to 62.6 h, while the control survival was much greater (127.6 h) (Fig. 1A).

Table 2
Length (μm) (±SE) of Africanized *Apis mellifera* worker midgut (ventricle) cells after feeding with candy paste mixed with the entomopathogens. Temperature 27 ± 2 °C, 12-h photophase and relative humidity of 60 ± 10%.

Treatments	Ventricle cells (μm)
Control[a]	106.7 ± 8.33a
Metharizium anisopliae	92.0 ± 0.17a
Beauveria bassiana	83.2 ± 16.10a
Bacillus thuringiensis	102.3 ± 18.42a
CV%	11.86

[a] Sterilized distilled water.Mean (±SD) followed by the same lowercase letter did not differ statistically from one another by Scott–Knott test ($p < 0.05$).

Entomopathogens on a smooth surface

All the entomopathogens evaluated reduced the survival of *A. mellifera* workers when they came into contact with the smooth surface containing these agents (Table 1). *B. bassiana* was the entomopathogen that most reduced the survival of the workers, in which 50% of the population survived for 97.3 h after the experiment began. *M. anisopliae* reduced survival to 128 h and *B. thuringiensis* to 144.9 h. In the control group, on the other hand, 50% of *A. mellifera* survived for 197.8 h; that is, for 8 days (Fig. 1B).

Entomopathogens on soy leaves

When sprayed on soy leaves, the entomopathogen *B. bassiana* reduced the survival of *A. mellifera* workers (Table 1). In this bioassay, 50% of the workers survived for 89.1 h after the experiment began (Fig. 1C).

Entomopathogens mixed with candy paste

When *B. bassiana* was mixed with the candy paste and offered to the workers as food, it caused a reduction in survival (117.3 h) when compared to other treatments. However, in comparison to other bioassays, this technique least affected the longevity of *A. mellifera* (Table 1 and Fig. 1D).

Histological analysis of the A. mellifera *ventricle*

There were no changes in the ventricular cell length of *A. mellifera* workers fed with candy paste integrated with *B. thuringiensis*, *B. bassiana*, *M. anisopliae* or pure candy paste (Table 2).

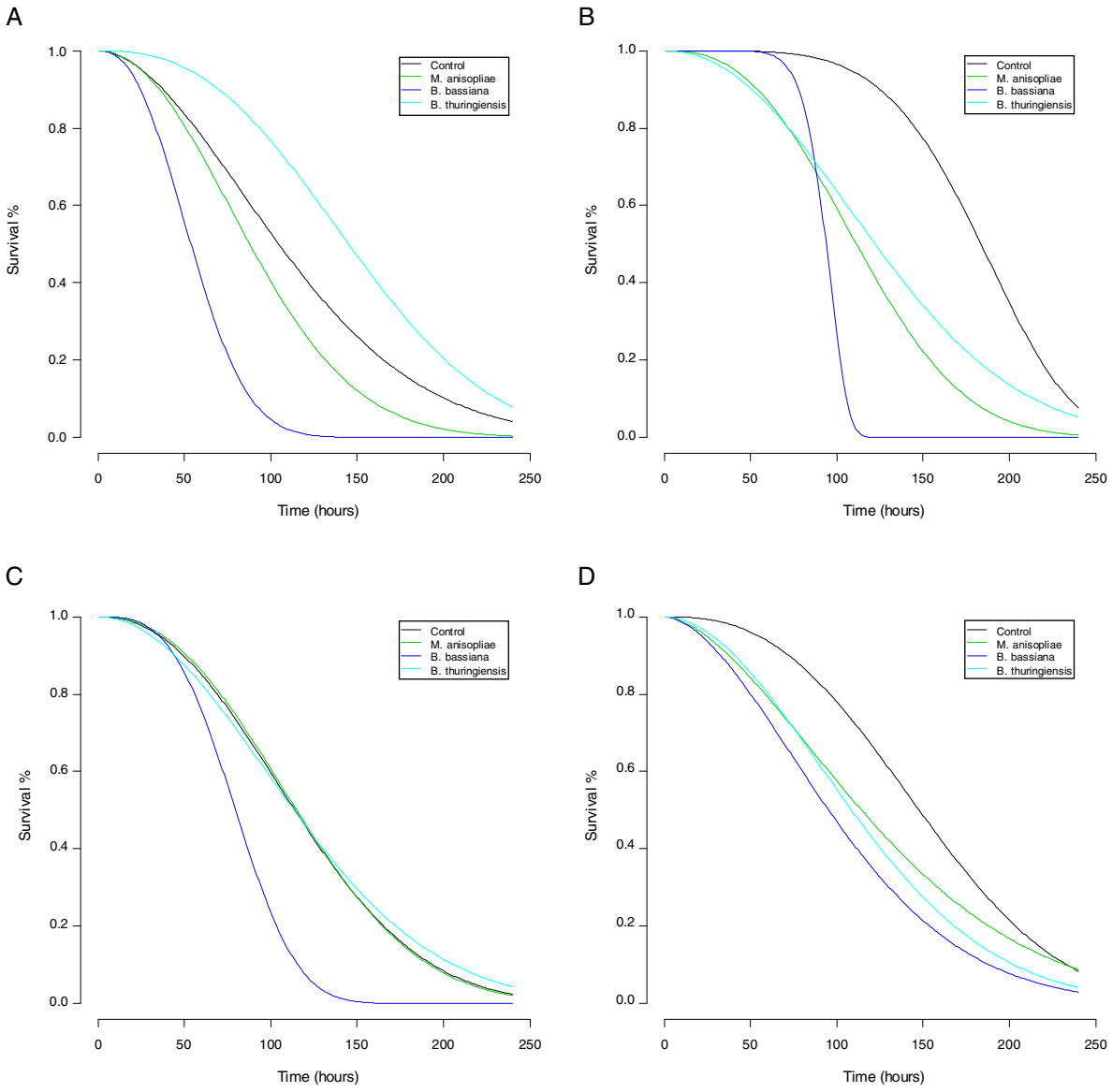

Fig. 1. Survival curve (hours) of Africanized *Apis mellifera* workers. (A) Entomopathogens sprayed on *A. mellifera*, (B) entomopathogens on a smooth surface, (C) entomopathogens on soy leaves and (D) entomopathogens mixed with candy paste. Kaplan–Meier vs Weibull survival test adjusted for time (h). Temperature 27 ± 2 °C, 12-h photophase and relative humidity of 60 ± 10%. Sterilized distilled water.

Discussion

In the bioassays of entomopathogens sprayed on *A. mellifera*, on a smooth surface and on soy leaves, the entomopathogenic fungus *B. bassiana* caused the greatest reduction in survival of Africanized *A. mellifera* workers, especially in the spray bioassay. This is related to the mode of action of *B. bassiana*, which germinates upon contact with insect integument (Alves, 1998). In this case, the direct spray technique allows an even greater concentration of conidia on the body of *A. mellifera*, which can be evidenced by the survival of 50% of the workers for 62.6 h.

The fungus *B. bassiana* 447, when applied topically on Africanized adult *A. mellifera* workers, negatively affected their survival, causing mortality in 50% of the bees (Alves et al., 1996). On the other hand, the isolates of *B. bassiana*, ARSEF 3769 (ARK), NY (NY; BB008, SCPFRC) and GHA, when tested via the contact method on the surface of canola leaves (*Brassica napus* L.), did not cause mortality in *A. mellifera* workers, and no insects infected with the fungus were observed (Al Mazra'awi et al., 2006).

However, the spraying of three other *B. bassiana* isolates (ARK, GHA and LON) (1.0×10^8 conidia mL^{-1}) mixed with corn flour on adult *A. mellifera carnica* workers, also resulted in a reduced survival rate, while the isolate NY did not affect survival (Al Mazra'awi, 2007). Another four isolates of *B. bassiana* (EABb 04/01-Tip, EABb 01/110-Su, Bb-1333, EABb 01/103-Su) sprayed on adult *A. mellifera* workers did not interfere in survival, while isolate Bb-1333 only reduced survival of pupae (García-Fernández et al., 2008).

Although no studies have been reported on the effect of *B. bassiana* on *A. mellifera* when sprayed on a smooth/vitreous surface, some studies have been carried out to verify the effect of this entomopathogen on other hymenopteran insects. These studies showed no interference in the survival of these insects, as verified for *Oomyzus sokolowskii* (Kurdjumov) (Hymenoptera: Eulophidae) (Santos Jr. et al., 2006).

Of the entomopathogens applied on soy leaves, only *B. bassiana* interfered negatively in the survival of *A. mellifera* workers, in which 50% of *A. mellifera* survived for 89.1 h. The contact of the *A. mellifera* workers with *B. bassiana* on the surface of soy leaves, and the

consequent contamination, showed that even when the fungus is on top of a leaf surface, it can adhere to the body of insects. This bioassay reproduces part of what can occur in the field when *A. mellifera* workers are foraging and come into contact with the leaves of plants.

There was a reduction in the survival of *A. mellifera* workers who ingested candy paste incorporated with *B. bassiana*. Despite this, the survival was less affected than workers who came into contact with the product, or by spraying on different treated surfaces. Alves et al. (1996) evaluated the mortality of workers and found that this same fungus (*B. bassiana* isolate 447), when introduced into the diet of *A. mellifera*, caused 76% mortality.

The fungus *M. anisopliae* reduced the survival of *A. mellifera* workers when sprayed directly onto them, when in contact with a smooth surface, and during the candy paste ingestion bioassays. Despite having caused reduction, when compared to the survival of the control bees, no treatment showed a lower survival than that observed for *B. bassiana.*

No studies evaluating *M. anisopliae* on *A. mellifera* when sprayed on a smooth/vitreous surface have been reported, however, some studies have assessed the action of this entomopathogen on parasitoids. As in the example of *O. sokolowskii* (Kurdjumov) (Hymenoptera: Eulophidae), which did not have its survival/longevity altered when it came into contact with plastic cages sprayed (1.0×10^7 conidia mL^{-1}) with *B. bassiana* and *M. anisopliae* (447 and E9, respectively) (Santos Jr. et al., 2006). Furthermore, adult *Cotesia flavipes* parasitoid, exposed to isolates of the fungus *M. anisopliae* (UFGD 05, IBCB 348 and IBCB 425) (1.0×10^7, 0.5×10^8, 1.0×10^8, 0.5×10^9 and 1.0×10^9 conidia mL^{-1}) had little reduction in survival when they came into contact with a treated surface (filter paper), demonstrating low virulence of these isolates for this parasitoid (Hayashida et al., 2012).

Other biological *A. mellifera* parameters were evaluated when in contact with *B. bassiana*, in which Meikle et al. (2007) found that *B. bassiana* had no effect on colony weight, adult bee weight or honey production. Abdelaal and Hany (2013), in studies evaluating *M. anisopliae* (Bioranza®) on biological *A. mellifera* parameters, found that there was no negative effect on their weight gain when sprayed on the workers, and there was no effect on the larval, prepupal, pupal and adult stages. On other hand, Hamiduzzaman et al. (2012) found that the *B. bassiana* isolate GHA caused a reduction in worker emergence and mortality when they came into contact with the fungus-contaminated mite.

It should be noted that the confinement caused by bioassays creates stress that makes the bees more vulnerable to fungi (Al Mazra'awi, 2007). Another factor to be considered is the temperature in the bioassays, which favors infection (Alves, 1998) but is not the ideal temperature for bee development (Al Mazra'awi, 2007). *B. bassiana* and *M. anisopliae* present optimum germination temperatures between 23 and 30 °C (90% moisture) (Alves, 1998). At temperatures out of this range, these fungi are less able to germinate and penetrate the insect, which may occur if the fungi were applied in the field.

In addition to these factors, it should be noted that younger bees have more pronounced grooming and self-grooming behaviors (Brighenti et al., 2007), which may lead to greater contact with the conidia of the fungus, resulting in conidia adhering to the integument of the mouthparts and causing infection.

Contrary to fungal infection mechanisms, *B. thuringiensis* infection occurs through its ingestion (Fiuza, 2009). As such, the spraying of this entomopathogen on insects should have little to no effect, as verified in the spray bioassay on *A. mellifera*. In the present study, *B. thuringiensis* did not reduce the survival of *A. mellifera* workers, only when mixed with candy paste. It was also verified, in other studies, that the entomopathogen *B. thuringiensis* reduces the survival of *A. mellifera* workers when mixed with candy paste (10 and 20 g of the product/60 g of candy paste), causing 100% mortality 72 h after ingestion (Brighenti et al., 2007).

B. thuringiensis acts via ingestion and causes mortality in different insects 48 h after ingestion, on average, but this effect was not observed in the present study, in which 50% of *A. mellifera* workers were still alive 131.3 h after ingesting the candy paste. Dai et al. (2012) also verified that the toxin Cry1Ah, from *B. thuringiensis*, did not alter the survival, longevity, pollen consumption or weight of the hypopharyngeal gland of *A. mellifera ligustica* and *Apis cerana cerana*, when added to sugar syrup in different concentrations. The isolates EA3 and EA26 of *B. thuringiensis*, tested by Alquisira-Ramírez et al., 2014, did not affect the survival of *A. mellifera* when immersed in or ingesting the solution.

The difference found between studies is influenced by 1) different isolates tested, which is important, since new isolates are constantly being released and found in different parts of the world, demonstrating the need for selectivity tests; 2) different concentrations of the fungus used in the bioassays; 3) culture medium in which the fungi were produced, or in the case of commercial fungi, the different adjuvants; 4) subspecies of *A. mellifera* submitted to bioassays; and 5) stage of development and age of the bees used in the tests.

In the analysis of the midgut cells of Africanized *A. mellifera* workers that ingested candy paste containing the entomopathogens, no significant difference was observed between the treatments in the midgut sample analyzed by light microscopy. The midgut is the only organ of the digestive tract of endodermal origin, and is the region of this tube where most of the food digestion and absorption of nutrients takes place. In bees, the midgut is a long and thick cylindrical tube located inside the abdominal cavity. Its wall is formed by the epithelium (constituted of prismatic cells) and by visceral muscular fibers (Cruz-Landim, 2009).

The *B. thuringiensis* proteins, cry1C and cry2C, in an artificial diet (royal jelly, glucose, fructose, distilled water and yeast extract) supplied to the larvae of *A. mellifera*, did not cause damage to the border of the midgut when analyzed via light microscopy (Wang et al., 2015).

B. thuringiensis, at the concentrations denominated as field concentration (100.0 g/hL), low concentration (40.00 g/hL) and high concentration (24,000.00 g/hL) caused irregularities in the midgut epithelium of *A. mellifera* workers after 96 h. These changes were verified after the first 24 h at the two highest concentrations, when analyzed by scanning and transmission electron microscopy (D'Urso et al., 2017).

The method of spray application in the laboratory occurs with the exposure of bees to the products, simulating the contact of these insects at the time of foraging in the field. To avoid contact of the pollinators with the entomopathogens while spraying these products in the field, farmers should, preferably, carry out the applications late in the afternoon, in periods when the foraging rate is lower. It is also necessary to close the hive in order to avoid drift contamination.

In addition, bees can generate more heat in response to an infection in the environment, as seen with *Ascosphaera apis* (Maassen ex Claussen) Olive & Spiltoir in *A. mellifera* (Starks et al., 2000). They can also reduce the impact of pathogens and parasites through hygiene behavior, in which workers identify and remove diseased or infected larvae to avoid or combat these agents (Ibrahim and Spivak, 2006; Richard et al., 2008).

Many studies point out that, even with the changes to *A. mellifera* workers, *B. bassiana* can be used in Integrated Pest Management programs with little to no effect on bees (Alves, 1998; Al Mazra'awi, 2007). In addition, *A. mellifera* has the ability to disseminate *B. bassiana*, which is advantageous to the field (Al Mazra'awi et al., 2006).

The concentrations of entomopathogens applied to Africanized *A. mellifera* in this study are concentrations higher than those recommended by the manufacturers, and the workers are exposed to forced spraying, causing *A. mellifera* to come into contact with these agents, which differs from normal conditions in the field.

Thus, studies of the selectivity of entomopathogens to *A. mellifera* should be continuously carried out, evaluating different species, isolates, concentrations, and temperatures. Further studies should also assess biological, morphological and morphometric parameters. There is also a need for field trials on the production of queens and brood.

Conclusions

B. bassiana reduced the survival of *A. mellifera* workers in the four bioassays tested (spraying on *A. mellifera*, contact on a smooth surface, contact on soy leaves, and mixed with candy paste) in the laboratory.

The entomopathogens *B. bassiana*, *B. thuringiensis* and *M. anisopliae* did not cause morphometric changes in the midgut of *A. mellifera* fed with candy paste.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by the *Fundação Araucária* (project 25.383 – Agreement 229/2013) and Universidade Tecnológica Federal do Paraná (PAPCDT 7/2015).

References

Abdelaal, A.A., Hany, K.A-E., 2013. Efficacy of two fungus-base biopesticidee against the honeybee ectoparasitic mite, *Varroa destructor*. Pakistan J. Biol. Sci. 16, 819–825.

Al Mazra'awi, M.S., Shipp, J.L., Broadbent, A.B., Kevan, P.G., 2006. Dissemination of *Beauveria bassiana* by honey bees (Hymenoptera: Apidae) for control of tarnished plant bug (Hemiptera: Miridae) on canola. Environ. Entomol. 35, 1569–1577.

Al Mazra'awi, M., 2007. Impact of the entomopathogenic fungus *Beauveria bassiana* on the honey bees, *Apis mellifera* (Hymenoptera: Apidae). World J. Agric. Sci. 3, 7–11.

Alquisira-Ramírez, E.V., Paredes-Gonzalez, J.R., Hernández-Velázquez, V.M., Ramírez-Trujillo, J.A., Peña-Chora, G., 2014. In vitro susceptibility of *Varroa destructor* and *Apis mellifera* to native strains of *Bacillus thuringiensis*. Apidologie 45, 707–718.

Alves, S.B., Marchini, L.C., Pereira, R.M., Baumgratz, L.L., 1996. Effects of some insect pathogens on the Africanized honey bee, *Apis mellifera* L. (Hym., Apidae). J. Appl. Ent. 120, 559–564.

Alves, S.B., 1998. Controle Microbiano de Insetos, second ed. Piracicaba, São Paulo.

Baptista, A.P.M., Carvalho, G.A., Carvalho, S.M., Carvalho, C.F., Bueno, J.S.S.F., 2009. Toxicidade de produtos fitossanitários utilizados em citros para *Apis mellifera*. Ciênc. Rural 39, 955–961.

Brighenti, D.M., Carvalho, C.F., Carvalho, G.A., Brighenti, C.R.G., Carvalho, S.M., 2007. Bioatividade do *Bacillus thuringiensis* var. kurstaki (Berliner, 1915) para adultos de *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae). Cienc. Agrotecnol. 31, 279–289.

Codling, G., Naggar, Y.A., Giesy, J.P., Robertson, A.J., 2016. Concentrations of neonicotinoid insecticides in honey, pollen and honey bees (*Apis mellifera* L.) in central Saskatchewan, Canada. Chemosphere 144, 2321–2328.

Couto, R.H.N., Couto, L.A., 2002. Apicultura: Manejo e Produtos, second ed. Jaboticabal, São Paulo.

Cruz-Landim, C.da., 2009. Abelhas: Morfologia e função dos sistemas. Unesp, São Paulo.

Dai, P.L., Zhou, W., Zhang, J., Jiang, W.Y., Wang, Q., Cui, H.J., Sun, J.H., Wu, Y.Y., Zhou, T., 2012. The effects of Bt Cry1Ah toxin on worker honeybees (*Apis mellifera ligustica* and *Apis cerana cerana*). Apidologie 43, 384–391.

D'Urso, V., Mazzeo, G., Vaccalluzzo, V., Sabella, G., Bucchieri, F., Viscuso, R., Vitale, D.G.M., 2017. Observations on midgut of *Apis mellifera* workers (Hymenoptera: Apoidea) under controlled acute exposures to a *Bacillus thuringiensis*-based biopesticide. Apidologie 48, 51–62.

Ferreira, T.P., Oliveira, E.E., Tschoeke, P.H., Pinheiro, R.G., Maia, A.M.S., Aguiar, R.W.S., 2017. Potential use of Negramina (*Siparuna guianensis* Aubl.) essential oil to control wax moths and its selectivity in relation to honey bees. Ind. Crops Prod. 109, 151–157.

Fiuza, L.M., 2009. Mecanismo de ação de *Bacillus thuringiensis*. Biotecnol. Ciência Desenvolv. 38, 32–35.

García-Fernández, P., Santiago-Álvarez, C., Quesada-Moraga, E., 2008. Pathogenicity and thermal biology of mitosporic fungi as potential microbial control agents of *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of honey bee, *Apis mellifera* (Hymenoptera: Apidae). Apidologie 39, 622–673.

Hamiduzzaman, M.M., Sinia, A., Guzman-Novoa, E., Goodwin, P.H., 2012. Entomopathogenic fungi as potential biocontrol agents of the ecto-parasitic mite, *Varroa destructor*, and their effect on the immune response of honey bees (*Apis mellifera* L.). J. Invertebr. Pathol. 111, 237–243.

Hayashida, E.K., Kassab, S.O., Fonseca da, P.R.B., Rossoni, C., Loureiro, E. de S., Amorim, L.G.P., 2012. Efeito dos isolados de *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hyocreales: Clavicipitaceae) sobre parasitoide *Cotesia flavipes* (Cameron, 1891) (Hymenoptera: Braconidae). Nucleus 9, 73–77.

Heidelberger, P., Welch, P., 1983. Simulation run length control in the presence of an initial transient. Oper. Res. 31, 1109–1144.

Ibrahim, A., Spivak, M., 2006. Global meliponiculture: challenges and opportunities. Apidologie 37, 275–292.

Imperatriz-Fonseca, V.L., Gonçalves, L.S., Francoy, T.M., Nunes-Silva, P., 2012. O desaparecimento das abelhas melíferas (*Apis mellifera*) e as perspectivas do uso de abelhas não melíferas na polinização. Doc. (Embrapa Semi-Árido. Online) 249, 210–233.

James, R.R., 2011. Potential of ozone as a fumigant to control pests in honey bee (Hymenoptera: Apidae) hives. J. Econ. Entomol. 104, 353–359.

James, R.R., McGuire, M.R., Leland, J.E., 2012. Susceptibility of adult alfalfa leafcutting bees and honey bees to a microbial control agent, *Beauveria bassiana*. Southwest. Entomol. 37, 13–21.

Kanga, L.H.B., James, R.R., Boucias, D.G., 2002. *Hirsutella thompsonii* and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor*, a honey bee parasite. J. Invertebr. Pathol. 81, 175–184.

Kanga, L.H.B., Jones, W.A., James, R.R., 2003. Field trials using the fungal pathogen, *Metarhizium anisopliae* (Deuteromycetes: Hyphomycetes) to control the ectoparasitic mite, *Varroa destructor* (Acari: Varroidae) in honey bee, *Apis mellifera* (Hymenoptera: Apidae) colonies. J. Econ. Entomol. 96, 1091–1099.

Kanga, L.H.B., Jones, W.A., Gracia, C., 2006. Efficacy of strips coated with *Metarhizium anisopliae* for control of *Varroa destructor* (Acari: Varroidae) in honey bee colonies in Texas and Florida. Exp. Appl. Acarol. 40, 249–258.

Kanga, L.H.B., Adamczyk, J., Patt, J., Gracia, C., Cascino, J., 2010. Development of a user-friendly delivery method for the fungus *Metarhizium anisopliae* to control the ectoparasitic mite *Varroa destructor* in honey bee, *Apis mellifera*, colonies. Exp. Appl. Acarol. 52, 327–342.

Lez, M.D.M., Llado, G., Petro, A.B., Alemany, A., 2014. First field assessment of *Bacillus thuringiensis* subsp. Kurstaki aerial application on the colony performance of *Apis mellifera* L. (Hymenoptera: Apidae). Spanish J. Agric. Res. 12, 405–408.

Lu, C., Warchol, K.M., Callahan, R.A., 2012. In situ replication of honey bee colony collapse disorder. Bull. Insectol. 65, 99–106.

Medici, S.K., Castro, A., Sarlo, E.G., Marioli, J.M., Eguaras, M.J., 2012. The concentration effect of selected acaricides present in beeswax foundation on the survival of *Apis mellifera* colonies. J. Apic. Res. 48, 164–168.

Meikle, W.G., Mercadier, G., Holst, N., Nansen, C., Girod, V., 2007. Duration and spread of an entomopathogenic fungus, *Beauveria bassiana* (Deuteromycota: Hyphomycetes), used to treat varroa mites (Acari: Varroidae) in honey bee (Hymenoptera: Apidae) hives. J. Econ. Entomol. 100, 1–10.

Meikle, W.G., Mercadier, G., Annas, F., Holst, N., 2009. Effects of multiple applications of a *Beauveria* based biopesticide on *Varroa destructor* (Acari: Varroidae) densities in honey bee (Hymenoptera: Apidae) colonies. J. Apic. Res. 48, 220–222.

R Development Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0.

Richard, F.J., Aubert, A., Grozinger, C.M., 2008. Modulation of social interactions by immune stimulation in honey bee, *Apis mellifera*, workers. BCM Biol. 6, 50.

Rucker, R., Thurman, W., 2012. Colony collapse disorder: the market response to the bee disease. PERC Policy Ser., 1–31.

Santos Jr., H.J.G., Marques, E.J., Barros, R., Gondin Jr., M., 2006. Interação de *Metarhizium anisopliae* (Metsch.) Sorok., *Beauveria bassiana* (Bals.) Vuill. e o Parasitóide *Oomyzus sokolowskii* (Kurdjumov) (Hymenoptera: Eulophidae) sobre Larvas da Traça-das-Crucíferas, Plutella xylostella (L.) (Lepidoptera: Plutellidae). Neotrop. Entomol. 35, 241–245.

Serviço Brasileiro de Apoio às Micro e Pequenas Empresas (SEBRAE), 2014. Oportunidades para o mercado de mel, Available at: http://www.sebraemercados.com.br/wp-content/uploads/2015/12/2014_04_09_RT_Jan_Agron_Mel.pdf (accessed 29.0.6.17).

Shaw, K.E., Davidson, G., Clark, S.J., Ball, B.V., Pell, J.K., Chandler, D., Sunderland, K.D., 2002. Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*. Biol. Control. 24, 266–276.

Silva, F., de, A.S.S., Azevedo, C.A.V., 2012. Versão do programa computacional Assistat para o sistema operacional Windows. Rev. Bras. Prod. Agroind. 4, 71–78.

Starks, P.T., Blackie, C.A., Thomas, D., Seeley, P.T., 2000. Fever in honeybee colonies. Naturwissenschaften 87, 229–231.

Wang, Y.Y., Li, Y.H., Huang, Z.Y., Chen, X.P., Romeis, J., Dai, P.L., Peng, Y.F., 2015. Toxicological, biochemical, and histopathological analyses demonstrating that Cry1C and Cry2A are not toxic to larvae of the honeybee, *Apis mellifera*. J. Agric. Food Chem. 63, 6126–6132.

Wolff, L.F., Reis, V.D.A., Santos, R.S.S., 2008. Abelhas melíferas: bioindicadores de qualidade ambiental e de sustentabilidade da agricultura familiar de base ecológica. Embrapa Clima Temperado – (Embrapa Clima Temperado. Documentos, 244).