DOI: 10.1590/1808-1657000052018

Can *Bacillus thuringiensis* affect the biological variables of natural enemies of Lepidoptera?

Bacillus thuringiensis *pode afetar as variáveis biológicas de inimigos naturais de Lepidoptera?*

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ABSTRACT: The entomopathogen *Bacillus thuringiensis* (Bt) is widely used as one of the ingredients in pest control formulations, but researches conducted on its effect on non-target organisms are still in the nascent stage. This investigation aimed to uncover if Bt treated with *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae and pupae could affect the biological variables of *Podisus nigrispinus* (Hemiptera: Pentatomidae) and *Palmistichus elaeisis* (Hymenoptera: Eulophidae), all of which established natural enemies of leaf defoliator caterpillars in the eucalyptus culture. Larvae of *T. molitor* were fed on wheat bran containing different concentrations of *B. thuringiensis* (0.00; 0.25; 0.50; 1.00; 2.00 and 4.00 g Agree/kg bran). When the larvae attained size of about 2 cm, they were used as prey for *P. nigrispinus* (Bioassay I), and their pupae used as hosts for *P. elaeisis* (Bioassay II). Only the biological variables oviposition period and egg numbers by posture of the predator *P. nigrispinus* were altered. The biological variables of *P. elaeisis* were not altered, since it was possible to use these control methods within the integrated pest management.

KEYWORDS: *Podisus nigrispinus*; *Palmistichus elaeisis*; biological insecticide; defoliating caterpillars.

RESUMO: O entomopatógeno *Bacillus thuringiensis* é amplamente empregado no controle de pragas, porém estudos de seu efeito sobre organismos não alvo ainda são incipientes. Com isso, objetivou-se neste trabalho avaliar se larvas de *Tenebrio molitor* (Coleoptera: Tenebrionidae), tratadas com *Bacillus thuringiensis,* podem afetar as variáveis biológicas de *Podisus nigrispinus* (Hemiptera: Pentatomidae) e *Palmistichus elaeisis* (Hymenoptera: Eulophidae), importantes inimigos naturais de lagartas desfolhadoras na cultura do eucalipto. Larvas de *T. molitor* foram alimentadas com farelo de trigo contendo diferentes concentrações de *Bacillus thuringiensis* (0,00; 0,25; 0,50; 1,00; 2,00 e 4,00 g de Agree®/kg farelo). Quando as larvas atingiram em média 2 cm de comprimento, foram usadas como presa para *P. nigrispinus* (bioensaio I) e as pupas como hospedeiro para *P. elaeisis* (bioensaio II). As variáveis biológicas período de oviposição e número de ovos por postura do predador *P. nigrispinus* foram alteradas. Já as variáveis biológicas de *P. elaeisis* não foram modificadas, sendo possível o uso conjunto desses métodos de controle no manejo integrado de pragas.

PALAVRAS-CHAVE: *Podisus nigrispinus*; *Palmistichus elaeisis*; inseticida biológico; lagartas desfolhadoras.

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Received on: 01/12/2018. Accepted on: 10/10/2018

INTRODUCTION

The increase in wood demand in recent decades has led to widespread deforestation of native woodlands. Nowadays, the globe needs to implement massive reforestation, with homogeneous species that are capable of rapid growth, such as those of the genus *Eucalyptus* (FISCHER, 2009).

The eucalyptus tree is an exotic species of the Myrtaceae family. The tree is attacked by insects that migrate from native hosts of the same botanical family, especially the lepidopteran defoliating type (SANTOS et al., 2000). The control of these insects in forest plantations has generally been implemented with the application of synthetic chemicals that are high in toxicity. The reason for it is that they mostly render efficient and immediate results. However, prolonged and excessive use of these products has resulted in harmful consequences to the environment, such as build up of immunity and resistance of the insect pests and environmental contamination, with negative impacts on organisms used in biological control (SOARES et al., 2009; PEREIRA et al., 2016).

Appropriate management, following the principles of social, economic and environmental sustainability, is the basis of the development of inspection instruments known as forest certification. The Forest Stewardship Council (FSC) is one of those certifiers that restrict the use of certain active ingredients in the insecticide (BASSO et al., 2011).

Biological products, on the other hand, have no such restrictions on their use, and are a viable alternative to insect control while limiting the use of chemical insecticides. This practice includes the use of microorganisms, such as *Bacillus thuringiensis* Berliner (Bt) (DIAS et al., 2015; PEREIRA et al., 2016).

Bt entomopathogens are gram-positive, sporogenic, crystalline and facultative anaerobic bacteria, widely used in the control of agricultural and forest pests (ROMEIS et al., 2011). The entomopathogenic activity of this microorganism is due to the presence of genes that express a lethal toxin, usually d-endotoxin, which when ingested leads to the death of the insects (VALLET-GELY *et al.*, 2008).

Podisus nigrispinus Dallas are bedbugs belonging to the Pentatomidae family. This species has a generalist habit and efficient predatory capacity over different agricultural and forest pests, highlighting eucalyptus-defoliating caterpillars (MOHAGHEGH et al., 2001). On the other hand, the parasitoid *Palmistichus elaeisis* Delvare & LaSalle (Hymenoptera: Eulophidae) stands out for its efficiency in parasitism of pupae in defoliating Lepidoptera (BARBOSA et al., 2016; PEREIRA et al., 2008; 2009; RODRÍGUEZ-DIMATÉ et al., 2016). *Palmistichus elaeisis* is a gregarious endoparasitoid with a generalist habit. These conditions characterize them as promising agents that can be efficiently used in the control of lepidopteran defoliators in *Eucalyptus* plantations (PEREIRA et al., 2009; MOHAGHEGH et al., 2001).

Reports showing the side effects that Bt has on non-target organisms have increased in recent years (MAGALHÃES et al., 2015). Most of them involves plants that express the toxin and has shown that the abundance and activity of predators and parasitoids in the field are similar in genetically modified or conventional crops (GLARE; O'CALLAGHAN, 2000). Other reports show that genetically modified insect-resistant plants can affect natural enemies differently (SCHULER et al., 2001).

The objective of this work was to evaluate biological variables of the predator *P. nigrispinus* and parasitoid *P. elaeisis* when consuming larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) treated with different concentrations of Bt formulation.

MATERIALS AND METHODS

The study was carried out in the Biological Control Laboratory (BCL) of the Federal University of the Jequitinhonha and Mucuri Valleys (UFVJM), in Diamantina, Minas Gerais state, Brazil, in an air-conditioned room, with the temperature varying between 24 and 26ºC, relative humidity between 70 and 80% and photoperiod of 12 hours.

Larvae of *T. molitor* were obtained from the rearing kept in the BCL/UFVJM, fed on wheat bran and slices of chayote (*Sechium edule*). About 600 g of larvae of the first stages (2nd and 3rd) were separated into six parts of 100 g each, and placed in plastic trays containing 1 kg of wheat bran. In each of the trays, the following treatments were applied: 0.00; 0.25; 0.50; 1.00; 2.00 and 4.00 g Agree/kg bran. The treatment with 1.00 g corresponded to the industry-recommended concentration for use in the field with one billion viable spores (equivalent to 38.0 g/kg endotoxin — 25,000 µl/mg potency) of *Bacillus thuringiensis aizawai* GC — 91 per g of product. The larvae were kept in these trays until they reached the mean size of 2 cm, to be used as prey (Bioassay I), or till reaching the pupal stage as hosts (Bioassay II).

In the first bioassay, the experimental design was completely randomized, consisting of six treatments and ten replicates. The predator eggs were obtained from the rearing of the BCL/UFVJM collected with dry cotton and placed in Petri dishes (150 x 20 mm). A cotton ball moistened with distilled water was placed in Petri dishes to maintain ambient moisture.

After hatching, the nymphs were transferred to 500-mL plastic pots in which they remained until adulthood. Each replicate (pot) consisted of ten nymphs of the same age, that received a daily quota of distilled water and two larvae of *T. molitor* treated with Bt till they reached adulthood, according to the treatments.

Daily survival and duration evaluations of the immature stages were performed. The reproductive variables were evaluated by randomly selecting one couple per pot, and maintaining ten replications. The periods of pre-oviposition, oviposition and post-oviposition, number of eggs per female and posture, egg viability, number of nymphs per females and longevity of males and females were evaluated.

In the second bioassay, newly formed pupae (24 hours) of *T. molitor* treated with Bt were used as an alternative host for the parasitoid *P. elaeisis*. The experimental design was completely randomized, consisting of the same six treatments and seven replicates.

Pupae of *T. molitor* were individualized in 500-mL plastic pots and exposed to the parasitism of six females of *P. elaeisis* for 48 hours (CAMILO et al., 2016). The numbers of individuals that emerged per pupa, egg-adult period of offspring, sexual ratio, and longevity of males and females were observed.

To evaluate the longevity, a couple from each repetition was used. They were placed in 500-mL plastic pots and fed on a honey droplet on the inner wall of the vial.

The data were submitted to the homoscedasticity and normality tests of the residues. The analysis of variance (ANOVA) was performed, and, in the case of normality, the means were compared by applying the Tukey test ($p \leq 0.05$). In the absence of normal distribution, the Kruskal Wallis test ($p \leq 0.05$) was applied. All the tests were conducted using the software R version 0.99.903 (R CORE TEAM, 2016), ExpDes and Pgirmess package (FERREIRA et al., 2013; GIRAUDOUX, 2016).

RESULTS

The biological variables oviposition period ($p = 0.0028$, $F = 4.1719$; gl = 5) and numbers of laid eggs ($p = 0.0061$; $F = 3.6686$; gl = 5) of the predator *P. nigrispinus* fed by *T. molitor* treated with Bt doses were different. The oviposition period of this predator was higher in the treatment with 0.00 g Agree/kg bran (44 ± 12.87), and the number of eggs per laying was lower when *P. nigrispinus* was submitted to feeding on Bt at the concentration of 0.5 g. The other biological variables did not differ between treatments (Table 1).

The number of *P. elaeisis* parasitoids that emerged from Bt-treated *T. molitor* pupae did not vary ($p = 0.1076$, $F = 1.9646$; gl = 5), with averages of 42.57 ± 31.19 to 89.71 ± 48.11. The values obtained for sex ratio also did not differ between the treatments ($p = 0.05$, gl = 5) and were above 0.91. Male longevity ($p = 0.2788$; $F = 1.317$; gl = X) and females ($p = 0.9977$; $F = 0.0573$; gl = 5) of *P. elaeisis* were unaffected by treatments, as well as the egg-adult cycle ($p = 0.5413$; $F = 0.8234$; gl = 5) of this parasitoid (Table 2).

Table 1. Biological variables (mean ± standard deviation) of *Podisus nigrispinus* (Hemiptera: Pentatomidae) fed on *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae treated with *Bacillus thuringiensis* (23 to 27 °C, 60 to 80% RH and 12-hour photoperiod)

Biological variables	0.00 g	Agree/kg doses of bran				
		0.25 g	0.50 g	1.00 g	2.00 g	4.00 g
Pre-Oviposition (days)[2]	10.5 ± 2.27a	9.5 ± 3.72a	11.7 ± 2.26a	11.1 ± 5.00a	11.5 ± 3.31a	11 ± 1.83a
Oviposition period (days)[1]	44 ± 12.87a	24.66 ± 20.17b	28.4 ± 12.89ab	19.9 ± 9.80b	25.5 ± 10.75b	29.9 ± 9.93ab
Post-Oviposition (days)[2]	1.4 ± 1.35a	1.66 ± 1.51a	4.2 ± 4.98a	8.9 ± 10.2a	7.5 ± 8.63a	1.4 ± 1.07a
Eggs/ females[1]	247.3 ± 73.75a	184.00 ± 99.59a	183.1 ± 63.45a	271.1 ± 130.44a	228.5 ± 70.59a	256.9 ± 63.86a
Eggs/ postures[1]	24.82 ± 4.09ab	23.77 ± 9.07ab	18.94 ± 4.86b	26.79 ± 7.31a	28.40 ± 4.86a	27.19 ± 4.89a
Egg viability[2]	89.76 ± 4.08a	86.74 ± 27.98a	84.72 ± 5.14a	82.07 ± 9.13a	90.37 ± 3.26a	90.89 ± 2.81a
Nymphs by females[1]	223.2 ± 70.86a	158.57 ± 85.29a	154.00 ± 51.97a	227.60 ± 120.65a	206.00 ± 63.49a	233.90 ± 61.45a
Longevity of females[1]	55.90 ± 13.25a	32.70 ± 21.36a	44.30 ± 14.55a	39.90 ± 12.49a	40.70 ± 16.47a	42.30 ± 11.10a
Longevity of males[1]	35.30 ± 12.18a	33.10 ± 21.15a	37.40 ± 15.23a	33.00 ± 17.75a	39.10 ± 18.02a	29.70 ± 11.30a

RH: relative humidity; [1]means followed by the same letter in the line does not differ by Tukey test (p < 0,05); [2]means followed by the same letter in the line does not differ by Kruskal-Wallis test (p < 0,05).

Table 2. Mean ± standard deviation of number of emerged parasitoids, sex ratio, male longevity, female longevity and egg-adult cycle of *Palmistichus elaeisis* offspring (Hymenoptera: Eulophidae) in *Tenebrio molitor* (Coleoptera: Tenebrionidae) pupae treated with *Bacillus thuringiensis* (23 to 27 °C, 60 to 80% RH and 12-hour photoperiod)

Treatments Agree (g)/kg of bran	Biological variables				
	Number of emerged parasitoids[1]	Sex ratio[2]	Longevity of males (days)[1]	Longevity of females (days)[1]	Egg-adult cycle (days)[1]
0.00 g	42.57 ± 31.19a	0.91 ± 0.06a	28.29 ± 16.87a	49.86 ± 15.29a	27.70 ± 2.93a
0.25 g	60.29 ± 30.90a	0.96 ± 0.03a	35.00 ± 21.6a	48.14 ± 21.11a	28.00 ± 2.24a
0.50 g	89.71 ± 48.11a	0.95 ± 0.05a	45.14 ± 16.15a	50.71 ± 14.72a	25.70 ± 2.5a
1.00 g	69.00 ± 26.77a	0.97 ± 0.03a	44.29 ± 15.83a	47.00 ± 12.6a	27.60 ± 2.44a
2.00 g	88.00 ± 31.34a	0.93 ± 0.06a	37.71 ± 13.26a	49.00 ± 12.85a	27.00 ± 3.74a
4.00 g	79.29 ± 32.29a	0.97 ± 0.01a	28.29 ± 17.58a	48.29 ± 7.99a	26.30 ± 0.95a

RH: relative humidity; [1]means followed by the same letter in the line does not differ by Tukey test (p < 0,05); [2]means followed by the same letter in the line does not differ by Kruskal-Wallis test (p < 0,05).

DISCUSSION

The pre-oviposition periods of *P. nigrispinus* females fed on *T. molitor* treated with different Bt doses were close to those observed in the alternative prey *Bombyx mori* L. (Lepidoptera: Bombycidae), 8.1 days (FERNANDES et al., 1996); *T. molitor*, 8.7 days; and *Zophobas confusa* Gebien (Coleoptera: Tenebrionidae), 8.4 days (ZANUNCIO et al., 1996). The post-oviposition periods were relatively low, compared to the oviposition periods, which is desirable in the rearing of *P. nigrispinus* in the laboratory, owing to the reduction in the cost of production. Shorter pre- and post-oviposition periods allow greater energy allocation in the oviposition period (DE BORTOLI et al., 2011), ensuring a better reproductive development of the predator (MENEZES et al., 2014). Despite the differences found in the oviposition periods of *P. nigrispinus* females, these values were higher than those ones found when the predator was fed on *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae) caterpillars (10 days) (VACARI et al., 2007). The period of oviposition of this predator with *B. mori* caterpillars was 23.7 days (FERNANDES et al., 1996), a value close to the one found in this study and, hence, considered satisfactory.

The numbers of eggs per female of *P. nigrispinus* in all treatments were similar to those found in other studies with the alternative prey *T. molitor* (ZANUNCIO et al., 1996; DE BORTOLI et al., 2011). This biological variable was not affected by Bt doses, which was also observed by MAGALHÃES et al. (2015) when *P. nigrispinus* was fed on *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) caterpillars infected with Bt-isolated HD1 — kurstaki (Cry +) subspecies, producer of insecticide crystal.

The number of eggs per *P. nigrispinus* posture was lower in the 0.5 g dose as compared to the other treatments, but it was similar to the value 19.06 ± 0.99 found in the report by OLIVEIRA et al. (2004), in which *P. nigrispinus* were fed on *T. molitor* larvae. The difference observed between the treatments is possible due to the toxic action of the protein produced by the Bt bacterium on metabolic routes, that were different from those observed for Lepidoptera, or by some other components of the product formulated as inerts and adjuvants that may affect the third level trophic (TORRES; RUBERSON, 2008; MENEZES; SOARES, 2016). However, a dose-response relationship between Bt and the number of eggs per *P. nigrispinus* posture was not observed.

The viability of eggs was similar between treatments. These data were similar to the values (81.7 ± 22.1) found by ZANUNCIO et al. (1996) when *P. nigrispinus* were fed to *T. molitor*. Viability is a biological variable that is normally sensitive to feeding immature forms (VACARI et al., 2007). It, however, was not affected by the different doses of Bt formulation in this study.

The number of nymphs per female of *P. nigrispinus* fed on *T. molitor* treated with Bt was close to the results obtained with females of this predator being fed on this alternative prey without any contact with Bt (OLIVEIRA et al., 2004; ESPINDULA et al., 2010). Therefore, this biological variable was not affected by different entomopathogen concentrations.

The similarity of the longevity of *P. nigrispinus* males and females in this study indicates that the different concentrations of Bt did not influence this variable. The longevity of *P. nigrispinus* females was lower than that recorded by OLIVEIRA et al. (2004) for this predator (67.2 days) fed on *T. molitor* larvae. However, it was superior to that reported by ZANUNCIO et al. (2001), who used *T. molitor* pupae in the feeding on *P. nigrispinus*, and found longevity of 35.30 days. The longevity of males was superior to that found by VACARI et al. (2007), who observed the average longevity of 14.87 days in their study when using *D. saccharalis* caterpillars in *P. nigrispinus* feeding.

Food is an important component of the environment. It directly influences the distribution and abundance of insects and affects biological processes such as longevity. The diversification of prey species as food for *P. nigrispinus* can influence their longevity, as verified by OLIVEIRA et al. (2004).

The number of parasitoids of *P. elaeisis* that emerged in the six treatments were similar to those found by ZANUNCIO et al. (2008) (70.07 ± 2.50), in which the alternative host *T. molitor* was parasitized by four females of *P. elaeisis* for 72 hours. This similarity of the values found by these authors demonstrates that the formulation of Bt did not affect the emergence of the parasitoids.

The sex ratio above 0.91 can be considered satisfactory for the use of *P. elaeisis* in biological control programs (ZANUNCIO et al., 2008; PEREIRA et al., 2009). ZANUNCIO et al. (2008) and PEREIRA et al. (2009) considered that high values of sex ratio (> 90) are important in maintaining the population dynamics of the parasitoid, since females are responsible for parasitism and the production of offspring.

The longevity of *P. elaeisis* males and females was greater than 28 days. Similar results were found for *P. elaeisis*, when *T. molitor* pupae were exposed to the parasitism of females of this parasitoid (ZANUNCIO et al., 2008). Thus, the feeding of immature *P. elaeisis* in the Bt-treated pupae did not affect this biological parameter. GENG et al. (2006) also found that the Bt toxin did not affect the longevity of the parasitoid *Trichogramma chilonis* (Ishii) (Hymenoptera: Trichogrammatidae), when adults were fed on pollen from Bt cotton. The production of longer-lived individuals is important in the efficiency of a biological control program. Adults living longer in the field will have greater chances of reproduction and will possess the ability to parasitize more hosts (WILLIAMS; ROANE, 2007).

The egg-adult cycle of the parasitoid *P. elaeisis* in *T. molitor* treated or otherwise with Bt formulation was similar to that found in the pupae of *T. molitor* and other hosts, such as *B. mori*, and *D. saccharalis* (ZANUNCIO et al., 2008; PEREIRA et al., 2009; CHICHERA et al., 2012). The duration of this parasitoid development was considered adequate, since insects that have a shorter lifespan give rise to more generations in a given period (CHICHERA et al., 2012).

CONCLUSIONS

In the present study, just the oviposition period and egg numbers per posture of the predator *P. nigrispinus* varied when they were fed on *T. molitor* treated with the entomopathogen Bt, but no dose-response relationship was detected for these alterations.

The parasitoid *P. elaeisis* did not change in biological variables when developed in hosts previously treated with Bt.

ACKNOWLEDGEMENTS

To the Brazilian National Council for Scientific and Technological Development (*Conselho Nacional de Desenvolvimento Científico e Tecnológico* – CNPq), Coordination for the Improvement of Higher Education Personnel (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* – CAPES) and Foundation for the Support to the Researches in Minas Gerais (*Fundação de Amparo à Pesquisa de Minas Gerais* – FAPEMIG), for financial support.

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