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Mortality of apple leafroller exposed to different *Bacillus thuringiensis* Subspecies in artificial diet, in three assessment periods

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Abstract – Bioinsecticides based on *Bacillus thuringiensis* are the entomopathogen most marketed worldwide. The aim of this study was to evaluate the bioactivity of *kurstaki, aizawai* subspecies and *kurstaki* x *aizawai* hybrid on *B. salubricola* larvae fed on artificial diet in different assessment periods. Mortality was assessed on the seventh, tenth and fifteenth days after treatment. Microplates containing 150 µl of diet in each cell were used. Suspensions containing Bt subspecies was deposited on the surface of the diet, then neonate larvae were released. The mortality assessment performed after fifteen days of treatment was significant for all three subspecies. Bt *kurstaki* was more efficient in all assessment periods, except for fifteen days, which was similar to Bt *aizawai*. Assessments performed up to seven days were more efficient to determine mortality. The three subspecies have influenced the ability for instar change, regardless of concentration. The LC_{50} of Bt *kurstaki* on the seventh and tenth days of assessment was from three to six times smaller than that observed in Bt *aizawai* x *kurstaki*, and Bt *aizawai,* respectively.
Index terms: *Bonagota salubricola,* apple tree, entomopathogen.

Mortalidade de lagarta-enroladeira-da-maçã expostas a diferentes subespécies de *Bacillus thuringiensis* em dieta artificial, em três períodos de avaliação

Resumo - *Bacillus thuringiensis* é o entomopatógeno mais comercializado mundialmente. Objetivou-se com este estudo avaliar a eficiência das subespécies *kurstaki, aizawai* e o hibrido *aizawai* x *kurstaki* no controle de *Bonagota salubricola* (Lepidopetera: Tortricidae) em diferentes períodos de avaliação. Os experimentos foram realizados em dieta artificial. Foram utilizadas microplacas contendo 150 µl de dieta em cada poço. A concentração contendo as subespécies de Bt foi depositada na superfície da dieta, em seguida liberada uma larva neonata, totalizando 24 em cada concentração. A mortalidade foi avaliada no sétimo, décimo e décimo quinto dias após o tratamento. Em todas as concentrações, foi verificado o instar larval. A avaliação de mortalidade realizada após quinze dias do tratamento foi significativa para as três subespécies. Bt *kurstaki* foi mais eficiente em todos os períodos de avaliação, exceto aos quinze dias, período em que foi semelhante a Bt *aizawai*. Avaliações acima de sete dias foram mais adequadas para determinar a mortalidade. As três subespécies influenciaram na capacidade de mudança de instar, indiferentemente da concentração. A CL_{50} de Bt *kurstaki,* aos sete e dez dias de avaliação, foi de três a seis vezes menores que Bt *aizawai* x *kurstaki* e Bt *aizawai*, respectivamente.
Termos para indexação: *Bonagota salubricola*, Macieira, Entomopatógeno.

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Introduction

The entomopathogenic bacterium, *Bacillus thuringiensis* Berliner (Bt) produces crystals of pro-toxins during sporulation (HUNG et al., 2016). Its insecticidal property was recognized in 1911 and its commercial use started in France in 1938 (JOUNG and CÔTÉ, 2000), for the control of *Anagasta kuehniella* (ZELLER) (Lepidoptera: Pyralidae). However, the first formulations with Bt were not successful (JOUNG and CÔTÉ, 2000).

The evolution of fermentation techniques and isolation of new subspecies allowed the emergence of new efficient formulations for the control of lepidoptera containing *kurstaki* (Btk) and *aizawai* (Bta) subspecies. Larval mesentery has ideal pH to promote the action of proteases that activate the toxin, causing the infection of larvae (BULUSHOVA et al., 2011).

In Brazil, Btk is used for the control of *Grapholita molesta* (BUSCK, 1916) (Lepidoptera: Tortricidae), in apple trees (MONTEIRO and SOUZA, 2010), whose action affects other lepidoptera that occur simultaneously such as apple leafroller *Bonagota salubricola* (MEYRICK, 1937) (Lepidoptera: Tortricidae) (MONTEIRO and SOUZA, 2010).

The effect caused by Bt differs according to subspecies, each producing specific pro-toxins that act differently on insects. Formulations containing two Bt subspecies, called *azaiwai x kurstaki* (Btak) transconjugate, were elaborated to increase control efficiency. Another important factor in the use of Bt is the exposure time of larvae to the pathogen, which influences mortality. Schesser et. al (1977) showed that larval mortality may vary according to the assessment period, influencing the result. The assessment period differs among authors, ranging from three (JYOTI and BREWER, 1999), four days (AMARAL et al., 2013), five days (BONCHEVA et al., 2006), seven days (OSTREM et al. 2016) and ten days (SCHESSER and BULLA, 1978).

The aim of this study was to compare the efficiency of three Bt subspecies for the control of *B. salubricola* in artificial diet and in different periods of mortality assessment.

Material and Methods

Experiments were carried out at the Laboratory of Integrated Pest Management (LAMIP), located in the Sector of Agrarian Sciences of the Federal University of Paraná (SCA-UFPR).

Bonagota salubricola. The population was collected in a commercial apple orchard in Porto Amazonas-PR in 2009. Larvae were grown in tubes (80 mm x 25 mm) containing artificial bean-based diet (PARRA et al., 1995). Pupae were kept in greenhouse with photophase of 14 hours, temperature 24 ± 2 ° C, RH of 60 ± 10% until emergence of adults, which were released in cages (35 cm x 35 cm x 35 cm) containing polyethylene strips for oviposition.

Bioassays. Commercial products based on *Bacillus thuringiensis* were from *kurstaki* (Dipel WG, 32 gl^{-1}, Abbott Laboratories, North Chicago, USA), *aizawai* (Xentari WG, 540 $g.kg^{-1}$, Abbott Laboratories, North Chicago, USA) and *azaiwai x kurstaki* (Bt) transconjugate (Agree WP, 500 $g.kg^{-1}$, Certis, Columbia, USA) subspecies. 96-well microplates received 150µl of artificial diet for lepidopterans (Soybean-Wheat, Stonefly Industries, TX). Each well was added of 6 µL of Bt suspension on the diet. Each well received a neonate aged up to three hours after hatching, about 20 minutes after addition of Bt. Three replicates were tested with 24 subjects at each concentration and control. Wells were closed with paraffin waxed paper (Parafilm, Chicago, IL) to prevent larval escape and diet dehydration. Microplates were maintained in BOD (Eletrolab, mod. 112FC, São Paulo), RH 60 ± 10%, temperature of 24 ± 2°C, photophase of 14 hours. Seven concentrations measured in part per million (ppm) of the commercial product (p.c.) were tested and defined from preliminary bioassays and adjusted for each Bt subspecies as follows: Agree - 190.0, 118.8, 74.3, 46 , 4.29, 18.1, 11.3 ppm; Dipel - 248.5, 118.3, 56.4, 26.8, 12.8, 6.1, 2.9 ppm; Xentari - 280.0, 155.6, 86.4, 48.0, 26.7, 14.8, 8.2 ppm.

Assessment. Mortality was considered in three assessment periods (AP): seven (7), ten (10) and fifteen (15) days after application of insecticides. The stages of surviving larvae were monitored to verify the influence of Bt on their development, identifying the cephalic capsules. Larvae that did not react to the touch of a stylet were considered dead.

Mortality data were submitted to Probit analysis to determine LC_{50} (PoloPlus 1.0, Software LeOra, 2003). Mortalities corrected by the Abbott formula (ABBOTT, 1925) in treatments were normalized using the sine-arc function and submitted to factorial analysis, and means were compared by the Tukey test considering significance of $P < 0.05$ (Assistat Software Version Beta 7.6).

Results and discussion

Assessment period. The assessment period of the average mortality of *B. salubricola* larvae influenced the efficiency of the three *B. thuringiensis* subspecies (Table 1) (Btak F = 41.75, p <0.001, df = 2, Btk F = 28.30, 0.001, df = 2; Bta F = 25.31, p <0.001, df = 2). The corrected mortality of *B. salubricola* at the highest concentration tested was directly proportional to AP (Btak $r^2 = 0.97$, Btk $r^2 = 0.70$, Bta $r^2 = 0.80$), ranging from 58.3 to 98.5% respectively at seven and fifteen days (Table 2). The results in *B. salubricola* suggest that assessments performed before seven days may underestimate the efficiency of Bt probably due to the food habit, since larvae of the first three instars consumed little diet, observing galleries of approximately three millimeters in the surface. For large-capacity larvae, evaluations occur earlier (BLANCO et al., 2009) and after seven days (LIAO et al., 2002; YEE et al., 2008); however, Schesser and Bulla (1978) showed differences in mortality rates obtained in different APs.

Development. Monitoring of surviving larvae from the seventh day showed that Bt influences development as a function of subspecies (Figure 1). Analyzing the highest concentration, it was observed that in the seventh day of assessment, most larvae were in the first instar, whereas control larvae were mostly between second and third instar. On average, 70% of larvae in Btak and Bta treatments were found in the second instar on the 10th day, while in Btk, all remained in the first instar, while in the control treatment, larvae occurred in the first four instars, with approximately 60% of them in the third instars. At the lowest concentration, the three Bt subspecies influenced the development of the larvae in relation to the control, although with low mortality rate at seven and ten days. In assessment performed at the fifteenth day, larval escape was observed, reducing the number of samples per treatment (Table 3). Individuals who perforated the parafilm were in the fourth to fifth instars and this generally occurred at concentrations below LC_{50}. This behavior occurred in Btak (11.1%), Btk (8.7%) and Bta (7.8%) in the total of treated individuals. In literature, there are reports of mortality assessments up to ten days after treatment (ER et al., 2007; FRANKENHUYZEN et al., 2008) and there are no references for larval escape.

Table 1. Average mortality (%) of *Bonagota salubricola* larvae fed on artificial diet with different concentrations of three B. thuringiensis subspecies in different assessment periods.

Assessment period (days)	*B. thuringiensis aizawai x kurstaki*[1]	*B. thurinigiensis kurstaki*[2]	*B. thuringiensis aizawai*[3]
7	29.9Bc*	47.1Ab	26.6Bb
10	39.6Bb	54.8Ab	30.1Cb
15	50.6Ba	85.5Aa	85.8Aa

* Means followed by the same lowercase letter in vertical and upper case in horizontal, do not differ statistically by the Tukey test at 5% of probability. [1] Average of concentrations: 190, 118.8, 74.3, 46.4, 29.0, 18.1, 11.3 parts per million (ppm) of the commercial product (p.c.); [2] Average of concentrations: 248.5, 118.3, 56.4, 26.8, 12.8, 6.1, 2.9 ppm of p.c. [3] Average of concentrations: 280.0, 155.6, 86.4, 48.0, 26.7, 14.8, 8.2 ppm of p.c.

Table 2. Corrected mortality (%) of *Bonagota salubricola* larvae fed on artificial diet and exposed to different *Bacillus thuringiensis* subspecies at different assessment periods.

Subspecies	Assessment period (days)	Concentration (ppm p.c)[1]	n[2]	Corrected mortality (%)[3]
B. thuringiensis aizawai x kurstaki	7	190.0	72	59.2Bb*
	10			81.7Ba
	15			94.3Aa
B. thuringiensis kurstaki	7	248.5	72	88.7Ba
	10			92.8Aa
	15			98.5Aa
B. thuringiensis aizawai	7	280.0	72	58.3Bb
	10			66.2Bb
	15			98.5Aa

* Means followed by the same lowercase letter in the subspecies and upper case among species do not differ statistically by the Tukey test at 5% probability; [1] concentration expressed as part per million (ppm) of the commercial product (p.c.). [2] number of assessed individuals. [3] Mortality corrected according to Abbott (1925).

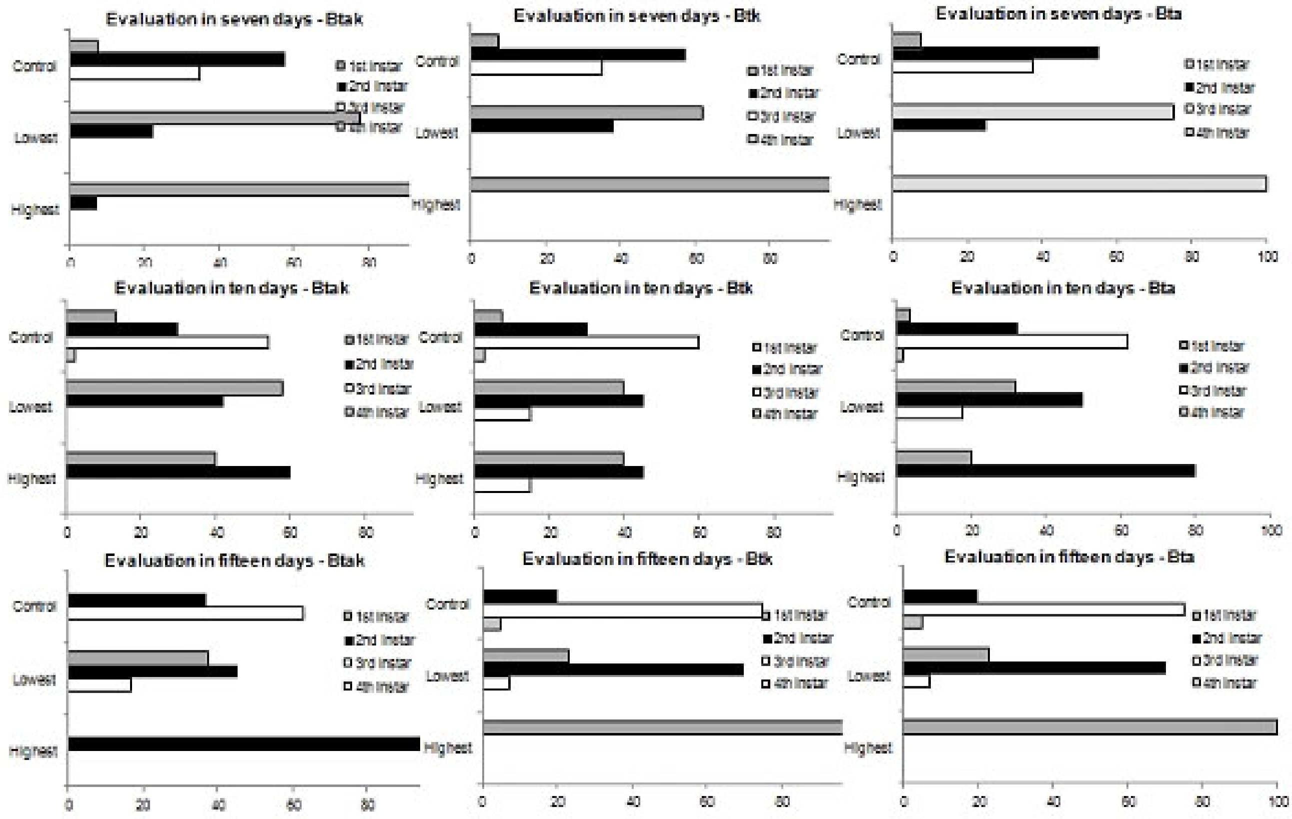

Figure 1. Percentage of surviving *B. salubricola* larvae per instar in three assessment periods (7, 10 and 15 days) exposed to artificial diet containing *B. thuringiensis* subspecies *aizawai* x *kurstaki* (Btak), *kurstaki* (Btk) and *aizawai* (Bta) (highest concentration – Btak= 190.0 ppm, Btk = 248.5 ppm, BTA = 280.0 ppm) (lowest concentration - Btak= 11.3 ppm; Btk= 2.8 ppm; BTA= 8.2 ppm).

Interference in larval development is evident through the stages they were in; however, it was not possible to confirm the death of Bt-affected larvae before 15 days. The results indicate a decrease in larval development with the use of sub-lethal concentrations, and this may be related to the behavior of larvae that after Bt ingestion present food reduction or stopping (CÍCERO et al., 2009), not obtaining the necessary energy for instar change. Other authors mention that there is no instar change in larvae due to starvation observed after Bt infection (Monner et al., 2000; Cicero, 2009). However, the concentrations used were high and caused the rapid larval death. Eizaguirre et al. (2005) in bioassays with *Sesamia nonagrioides* Lef. (Lepidoptera: Noctuidae) found results similar to those of the present work, showing that larvae exposed to sub-lethal concentrations present reduced development.

Bioassay. The results showed that the death caused by *kurstaki* subspecies was statistically significant for the three assessment periods (PA7 F = 109.06, $p < 0.001$, df = 2; PA10 F = 21.25, $p < 0.001$df = 2, PA15 F = 77.05, $p < 0.001$, df = 2) (Table 1), except when compared to Bta at 15 days. In PA7, the $LC_{50}$of Btak and Bta were respectively 3.0 and 5.9 times greater than Btk (Table 4). This trend was also observed in the other two assessment periods. For *B. salubricola*, Btk was more efficient in the control, which may be related to the origin of the population, resistant or susceptible insects exposed to Bt present different mortality rates (Diaz-Gomez et al., 2000). In assessment of leaf injuries caused by *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), Btk was five times more efficient than Bta (GONZÁLEZ-CABRERA et al., 2011), corroborating results obtained in the present work.

Table 3. Number of initial individuals (n), number of dead individuals and percentages of larval escape per concentration in three *Bacillus thuringiensis* subspecies in the 15-day assessment period.

Concentration (µg/g p.c.)	n	Number of Deaths	Larval escape (%)
B. thuringiensis aizawai x kurstaki			
190.0	72	68	0.00
118.8	68	52	2.88
74.3	68	43	2.88
46.4	63	29	6.48
29.0	64	23	5.76
18.1	65	9	5.04
11.3	58	6	10.08
Control	54	3	12.96
B. thuringiensis kurstaki			
248.5	71	70	0.72
118.3	72	66	0.00
56.4	71	59	0.72
26.8	69	56	2.16
12.8	64	53	5.76
6.1	60	44	8.64
2.9	61	39	7.92
Control	57	3	10.80
B. thuringiensis aizawai			
280.0	72	67	0
155.6	72	60	0
86.4	70	52	1.44
48.0	69	47	2.16
26.7	64	45	5.76
14.8	62	42	7.20
8.2	63	38	6.48
Control	59	4	9.36

Table 4. Lethal concentration that kills 50% of the *Bonagota salubricola* population (LC_{50}) and angular coefficient in comparison with *Bacillus thuringiensis aizawai* x *kurstaki, B. thuringiensis aizawai* in relation to *B. thuringiensis kurstaki* in different assessment periods.

Subspecies	Assessment period (days)[1]	LC_{50} µg/g $_{(95\%\ CI)}$	Slope ± SD	X^2	R^2
B. thuringiensis kurstaki	7	37.7 (29.3-48.7)	1.59±0.191	3.4	
	10	24.6 (18.2-32.3)	2.01±0.205	2.31	
	15	1.0 (0.05-3.13)	2.02±0.285	5.48	
B. thuringiensis azaiwai x kurstaki	7	130.6 (104.5-175.1)	1.22±0.144	0.88	3.5
	10	74.9 (63.2-89.2)	1.24±0.121	1.309	3.0
	15	55.6 (45.9-67.2)	0.67±0.121	2.015	55.6
B. thuringiensis azaiwai	7	213.5 (156.0-332.2)	1.16±0.138	0.92	5.7
	10	144.0 (109.5-196.8)	1.42±0.188	1.63	5.9
	15	5.3 (0.25-14.1)	0.64±0.135	5.51	5.3

[1] Days after application in the diet; [2] Ratio calculating the LC_{50} between Bt aizawai x kurstaki - Bt aizawai and Bt kurstaki

Conclusion

Bacillus thuringiensis subsp. *kurstaki* caused higher mortality of *B. salubricola* larvae when compared to the other subspecies. Post-treatment assessment time influenced the percentage of larval mortality and *B. thuringiensis* toxins influenced larval development at non-lethal conc

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