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Activity of plant aqueous extracts on *Bacillus thuringiensis* and their interactions on *Anticarsia gemmatalis* (Lepidoptera: Erebinae)

Atividade de extratos vegetais aquosos sobre *Bacillus thuringiensis* e a interação destes sobre *Anticarsia gemmatalis* (Lepidoptera: Erebinae)

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Abstract

The association of plant extracts and *B. thuringiensis* may be important in situations that control of different insects species is required and/or control of insects in various development stages. However, extracts may have different effects on *B. thuringiensis*, compromising their mode of action. Thus, the objective of this study was to evaluate the activity of aqueous plant extracts on *Bacillus thuringiensis* subsp. *kurstaki* (Btk) and their interactions on *Anticarsia gemmatalis* (Lepidoptera: Erebinae). Cherry plant extracts (*Eugenia uniflora*), pepper (*Capsicum baccatum*), trumpet (*Brugmansia suaveolens*) and grape japan (*Hovenia dulcis*), were used at 5% concentrations. For compatibility studies, the extracts were mixed with Thuricide® (Btk) and the parameters evaluated were Colony Formation Units (CFU mL^{-1}) for spores and, the *A. gemmatalis* mortality for crystals. The cherry extracts, pepper, and japan grape completely inhibited the CFU ml^{-1} of Btk and the cherry extract alone negatively affected the toxicity of Btk crystals, with significantly lower mortality of *A. gemmatalis* (20.40%) than that observed in the control with only Btk (79.44%). In the association between cherry, pepper, and grape japan extracts with *B. thuringiensis,* the extracts exhibited a negative effect in the formation of CFU. The cherry extract demonstrated a negative effect on crystals action.

Key words: Entomopathogenic bacteria. Associated control. Botanical extracts.

Resumo

A associação de extratos vegetais e *B. thuringiensis* pode ser importante em situações que em que se necessite controlar diferentes espécies de insetos-praga e ou insetos em diferentes fases de desenvolvimento. No entanto, os extratos podem apresentar diferentes efeitos sobre *B. thuringiensis,* comprometendo o seu modo de ação. Assim, o objetivo deste trabalho foi avaliar o efeito de extratos vegetais aquosos sobre *Bacillus thuringiensis* subesp. *kurstaki* (Btk) e a interação destes sobre *Anticarsia gemmatalis* (Lepidoptera: Erebinae). Foram utilizados extratos vegetais aquosos de pitanga (*Eugenia*

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uniflora), pimenta (*Capsicum baccatum*), trombeta (*Brugmansia suaveolens*) e uva-do-japão (*Hovenia dulcis*), na concentração de 5%. Para estudar a comaptiilidade a *B. thuringiensis*, os extratos foram misturados com Thuricide® (Btk) e, os parâmetros avaliados foram unidades formadoras de colônia (UFC mL^{-1}) para esporos e, para cristais, a mortalidade de *A. gemmatalis*. Os extratos de pitanga, pimenta e uva-do-japão inibiram totalmente a formação de UFC mL^{-1} de Btk e, apenas o extrato de pitanga afetou negativamente a toxicidade dos cristais de Btk, com redução significativa da mortalidade de *A. gemmatalis* (20,40%), em relação à testemunha Btk (79,44%). Na associação entre os extratos de pitanga, pimenta e uva-do-japão com *B. thuringiensis* estes interferem negativamente na formação de UFC e o extrato de pitanga apresenta efeito negativo para a ação dos cristais.

Palavras-chave: Bactéria entomopatogênica. Controle associado. Extratos botânicos.

Applied biological control with the use of entomopathogenic bacteria, mainly *Bacillus thuringiensis* Berliner (Bacillaceae) and the use of natural products such as plant extracts should be investigated for the control of insect pests, especially in alternative production systems. In general, the mode of action of *Bacillus thuringiensis* is dependent on the action of the spores and crystals in insects gut cells. It takes into account the solubilization steps of the crystal protein, the protease action in the activation of endotoxin and binding of toxins activated to specific receptors in the of insect midgut.

The secondary metabolites present in plants, among their other functions, can act as defensive agents against pathogens and herbivores attack, particularly insects (ISMAN, 2006) and may have acute and chronic insecticide effects, deterrence and repellence action, among others.

Bacillus thuringiensis may be used alone or associated with plant extracts for the control of insect pests (SILVA et al., 2012.). This association can be important, especially in situations that required control of different insects species and/or in various development stages. The association of plant extracts and *B. thuringiensis* may be synergistic, indifferent, or antagonistic. In the case of antagonism, chemicals can negatively affect entomopathogens, inhibiting vegetative growth and sporulation or even causing genetic mutations resulting in virulence decreased of the specific pests (ALVES et al., 1998). In turn, the synergistic interaction can cause a stressor effect on the insect pest, promoting high susceptible, to *B. thuringiensis* resulting in faster action of the pathogen or increasing insect mortality.

The association of the of *B. thuringiensis* with cypress extract, *Taxodium distichum* L. Rich (Taxodiaceae) increased virulence of the bacteria on insects of stored grains (SABBOUR, 2003).

The Myrtaceae and Solanaceae families stand out among the plants with insecticide potential due to the presence of alkaloids and limonoids, respectively, in their compounds, which have been known to have insecticidal effects. There are reports of *Eugenia uniflora* L (Myrtaceae) possessing compounds with insecticidal activity for the diamondback moth *Plutella xylostella* L. (Lep: Plutellidae) (TORRES et al., 2001) and Coleoptera (DEQUECH et al., 2008). In addition, there are records of compounds from Solanaceae species of the genera *Capsicum* with insecticidal effects on Coleoptera (DEQUECH et al., 2008) and Brugmansia genera on *Haematobia irritans* L. (Diptera: Muscidae) (CARRILLO et al., 2011).

Taking this into account and considering that the type and concentration of secondary metabolites may vary according to species and plant tissues used, the form of compounds extraction, the species and the target insect stage, studies aimed at the interaction of these with *B. thuringiensis* are important for the improvement and or development of insect pest control strategies. Thus, the objective of this study was to evaluate the effect of aqueous plant extracts on *Bacillus thuringiensis* subsp.

kurstaki and the interaction of these on *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebinae).

The bacteria used was *Bacillus thuringiensis* subsp. *Kurstaki* HD-1 strain (30-52 × 10^6 viable spores mg^{-1}), formulated in the commercial product Thuricide® WP (32 g kg^1). The insect used as an indicator of the interaction was *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebinae), obtained from those reared at the Biological Control Laboratory of the Universidade Tecnológica Federal do Paraná - Campus Dois Vizinhos (UTFPR-DV).

Obtaining plant extracts: Fruits of *Capsicum baccatum* var. *pendulum* Wild. (Solanaceae) (pepper), leaves of *Eugenia uniflora* L. (Myrtaceae) (Brazilian cherry), pseudofruits of *Hovenia dulcis* Thunb. (Rhamnaceae) (grape japan) and flowers of *Brugmansia suaveolens* Willd. (Solanaceae) (trumpet) were used to obtain the plant extracts. Samples were collected in the morning in Dois Vizinhos country, Paraná State Brazil, located in the third plateau of Paraná, 25° 44′ South latitude and 54° 04′ West longitude, at an altitude of 520 m, with climate type, humid subtropical mesothermal (Cfa), according to the Köppen classification.

The collected material was dried at 40 °C for 48 h and a voucher specimen of each plant was forwarded to UTFPR-DV Herbarium for botanical identification and registration of the voucher copy. After drying, the material was ground in a knife mill (Willye type) to a particle size of up to 0.5 mm. The 5% extracts were obtained by the addition of 5 g of the powder to 100 mL of sterile distilled water and left for 48 h in the dark at a temperature of 25 ± 2 °C. The mixture was then filtered on a double filter paper in a Buckner funnel plugged into a Kitasato coupled to a constant pressure pump of 1.2 kgf cm^{-1}.

Activity of Plant Aqueous Extracts on Spore Viability: A suspension at concentration 4.1 × 10^9 spores mL^{-1} was prepared by adding one gram of the *Bacillus thuringiensis subsp. kurstaki* (commercial product) to 10 mL of sterile distilled water. Thereafter, by serial dilutions a Btk suspension at the concentration of 4.1 × 10^5 spores mL^{-1} was prepared in sterile distilled water. Aliquots of 300 **µ**L of this suspension were added to Erlenmeyer flasks containing 50 mL of 5% plant extract. Four replicates were prepared for each extract in Erlenmeyer flasks (replicate seats) and placed in a horizontal shaker (30 ± 2 °C, 150 rpm for 2 h). The suspension pH was measured before and at the end of the incubation. The mixture from each flask was inoculated at five points in two Petri dishes. Each of the five inoculations in each Petri dish consisted of 5 **µ**L of the mixture on the surface of a nutrient agar culture medium (AN). The plates were exposed in a laminar flux chamber for five minutes to allow evaporation of water excess and subsequently placed in a closed climatic chamber at 30 ± 2 °C for 18 h. These were then quantified for colony forming units (CFU) mL^{-1} per point. The control consisted of Btk in sterile distilled water.

Activity of Aqueous Plant Extracts on Crystals of B. thuringiensis subsp. kurstaki In Vivo: The suspensions with the mixtures of Btk and plant extracts and Btk alone were prepared in Erlenmeyer flasks containing 50 mL of 5% plant extracts and distilled water, respectively. In each flask 0.15 g of commercial product was added; equivalent to an application of 50 g ha^{-1} in 100 L H_2O. This dosage was previously established and promote approximately 80% mortality of*A. gemmatalis* larvae. After addition of Btk, the flasks were placed in a horizontal shaker and pH was determined as described previously. Then 150 µL aliquots of the suspensions were added over 1.5 cm of the side surface of *A. gemmatalis* artificial diet cube, in four Petri dishes per treatment. Each plate (replication) received three diet cubes and 20 second instar caterpillars of *A. gemmatalis*. The plates were then conditioned in a climatized chamber (27 ± 2 °C, RH 70% ± 10% and photoperiod 14 h). The treatments consisted of the extracts, Btk alone, the mixture of extracts, Btk, and sterile distilled water (control). The evaluations were conducted at 24, 48 and 72 h, quantifying the number of dead caterpillars.

In both experiments the data were subjected to the Shapiro-Wilk test for normality and, when necessary, transformed into (x + 0.5) 1/2 for spores and arcsine (arcsine (radix (×/100))) to crystals. Data were submitted to analysis of variance (F test) and the averages compared using the Tukey's test ($p < 0.05$), with the assistance of statistical program Bioestat 5.0®. In the experiment with spores, the data was applied to the equation: for calculating extracts effect on spores, with negative and positive values, respectively, for determining increase or reduction of CFU mL^{-1}, as compared to the control.

It was found that pepper, cherry and grape japan extracts reduced the CFU mL^{-1} by 100%. However, the trumpet extract showed 21.50% reduction in the number of CFU mL^{-1}, which did not differ significantly from the control (Table 1).

Table 1. Mean (± SE) CFU mL^{-1} of *Bacillus thuringiensis* subsp. *kurstaki*, after incubation with sterile distilled water and plant extracts (5%), inoculated in a Petri dish containing nutrient agar culture medium and incubated in a climatic chamber (30 ± 2 °C for 18 h) and initial and final pH values.

Treatments	Mean CFU/mL(× 10^5)	CFU Rel. Test (%)[1]	pH	
			0 h	2 h
Control	3.35 ± 1.91 a	--	7.16	6.85
Trumpet	2.63 ± 0.15 a	- 21.50	4.86	4.76
Pepper	0.00 ± 0.00 b	-100.00	5.16	5.05
Brazilian cherry	0.00 ± 0.00 b	-100.00	4.45	4.32
Grape japan	0.00 ± 0.00 b	-100.00	4.96	5.80
p	0.0004			

Transformed data (x + 0.5) 1/2. Mean (± SE) followed by the same letter in the column do not differ significantly by Tukey's test (P <0.05).[1] Percentage of colony forming units compared to the control.

The negative effects obtained for all extracts, may be due to the acidic pH, since in all the treatments, the pH values were lower than observed in the control, ranging between 4.32 in the Brazilian cherry and 5.80 in grape japan. In a previous study on the germination of Bt spores in soil with different pH, it was observed that the greater the acidity of the soil, the greater was the reduction in the germination of the spores, and below pH 5 germination ceased completely (PETRAS; CASIDA JUNIOR, 1985). Along with the possible effect of pH, the reduction of CFU mL^{-1} could also be related to the compounds in the extracts, which could also cause deleterious effects on the spores. According to Silva et al. (2012) the secondary metabolites found in the extracts can prevent spore germination or destroy the bacterial membrane after germination. This could have been a possibility in this study.

Alkaloids are present as the main secondary metabolites in pepper extract (ALVES et al., 2007). In a study with pepper, *Capsicum baccatum* L. (Solanaceae) the extract was observed to have antibacterial activity for both Gram-negative and Gram-positive bacteria (CARVALHO et al., 2010), corroborating the results obtained in this study as Btk is Gram-positive.

The grape japan presents in its pseudofruits saponins, flavonoids and alkaloids (WANDSCHEER et al., 2011). Flavonoids are capable of inactivating extracellular proteins and cause disruption of the bacterial cell membrane (TSUCHIYA et al., 1996). Thus, it is inferred that in this study, Btk spores germinated in the mix with the extracts, but the bacterial membrane was destroyed because of its contact with them.

As for the Brazilian cherry, the main phytochemicals found in its leaves are flavonoids and tannins (AURICCHIO et al., 2007). The antimicrobial properties of tannins are related to their ability to bind to proteins and enzymes, inactivating them as well as inhibiting oxidative phosphorylation in cellular respiration and causing the complexation of metal ions essential for some microorganisms (SCALBERT, 1991). In this case, besides the action of flavonoids described earlier for bacteria, tannins may have acted on the cells of Btk after germination, preventing the oxidative phosphorylation.

The results observed in this work for the trumpet extract corroborate with Schmidt et al. (2009) who found that the ethanolic and hexanic extracts of this plant did not inhibit the growth of *Bacillus subtilis* in the agar diffusion test. The main compounds found in *B. suaveolens* are tropanic alkaloids (e.g., scopolamine) that act in defense against herbivores (ALVES et al., 2007), and show a hallucinogenic effect on humans. From this it is inferred that the reduction in CFU mL^{-1} observed for the trumpet extract in the cited work is due to the acidic pH effect which reduced the germination percentage of spores.

Regarding the effect of Aqueous Plant Extracts on Crystals of *B. thuringiensis* subsp. *kurstaki* in vivo, *i*t was found that the pepper, grape japan and trumpet extract did not show a negative effect on the toxicity of the crystals, because the cumulative mortality of *A. gemmatalis* for the mixtures did not differ significantly from that of Btk alone. However, the Brazilian cherry extract mixed with Btk inhibited the action of protein crystals, because there was a significant reduction in cumulative mortality of larvae of *A. gemmatalis* (20.40%), compared with Btk alone (79.44%) (Table 2).

The negative effect of Brazilian cherry extract on the activity of the crystal protein may be related to the constituent compounds of this extract, mainly the tannins. According to Lord and Undeen (1990), tannins have the ability to bind to proteins of *B. thuringiensis* especially δ-endotoxins and enzymes, and may cause efficiency loss.

Anticarsia gemmatalis mortality of showed no significant difference in the replicates with only extracts at all time intervals . However, for Btk, the highest percentages of *A. gemmatalis* mortality were observed at 48 h and 72 h, a characteristic of the pathogen mode of action. Similarly, in the mixture of Btk with trumpet and grape japan extracts, highs *A. gemmatalis* mortality percentages was observed at 48 h and 72 h, with no significant difference between them, suggesting that the extracts do not affect the toxicity of the crystals to *A. gemmatalis* (Table 2). In contrast, in the mixture of Brazilian cherry extract with Btk, there was no negative effect of the extract on Btk mode of action, reducing mortality of *A. gemmatalis* (19.08%) at 72 h, differing significantly from Btk alone (41.25%) in the same time period.

The mixture of pepper extract with Btk showed higher mortality of *A. gemmatalis* at 48 h (70.72%), significantly differing from the replicate with Btk alone (35.69%) for the same time period, indicating that the association is positive and increase insect mortality. It is possible that pepper extract has a debilitating effect on the insect, making it more susceptible to pathogen action.

Although plants aqueous extracts analyzed did not exhibited insecticidal effects on *A. gemmatalis* they have shown insecticidal effects as previously mentioned, on other insect species. Thus, the use of aqueous extracts of the plants studied in association with *B. thuringiensis*, except for the Brazilian cherry extract, could act as control agents for different insect species, without interfering with the toxicity of *B. thuringiensis*. Additional studies regarding the effect of spore association and the effect of plant extracts on insect mortality as well as their evaluation in the field, and on other species of insects, are important to validate the association of the control strategies studied.

The Brazilian cherry, pepper, and grape japan extracts have a negative effect on Btk spores, because they inhibit CFU mL^{-1}. In addition, the Brazilian cherry extract has a negative effect on the toxicity of Btk crystals, reducing the mortality of *A. gemmatalis*.

Table 2. Mean Percentage (± SE) mortality of caterpillars, second- instar *Anticarsia gemmatalis*, caused by plant extracts (5%) and mixed with *Bacillus thuringiensis* subsp. *kurstaki* at different times and cumulative mortality after incubation. Determined initial and final pH. Temperature 27 ± 2 ° C, 14 h photoperiod and U.R. 70 ± 10%.

Treatment	**Time h**			**Cumulative mortality**	**p**	**pH**	
	24	**48**	**72**			**0 h**	**2 h**
Control	2.50 ± 2.50 Aa	1.25 ± 1.25 Ab	0.00 ± 0.00 Ab	3.75 ± 3.75 b	0.6100	8.14	8.53
Trumpet	0.00 ± 0.00 Aa	0.00 ± 0.00 Ab	1.32 ± 1.32 Ab	1.32 ± 1.32 b	0.4074	4.56	4.5
Btk[1]	2.50 ± 2.50 Ba	35.69 ± 8.17 Aa	41.25 ± 5.15 Aa	79.44 ± 4.12 a	0.0007	7.12	6.96
Trumpet +Btk	0.00 ± 0.00 Ba	33.09 ± 8.60 Aa	32.50 ± 12.50 Aa	65.59 ± 7.96 a	0.0002	4.6	4.54
p	0.5912	0.0001	0.0001	0.0001			
Control	2.50 ± 2.50 Aa	1.25 ± 1.25 Ab	0.00 ± 0.00 Ac	3.75 ± 3.75 b	0.6100	8.14	8.53
Brazilian cherry	2.50 ± 1.44 Aa	0.00 ± 0.00 Ab	1.25 ± 1.25 Ac	3.75 ± 2.39 b	0.3232	4.83	4.78
Btk	2.50 ± 2.50 Ba	35.69 ± 8.17 Aa	41.25 ± 5.15 Aa	79.44 ± 4.12 a	0.0007	7.12	6.96
Brazilian cherry+Btk	1.32 ± 1.32 Ba	0.00 ± 0.00 Bb	19.08 ± 6.99 Ab	20.40± 7.55 b	0.0024	4.87	4.85
p	0.9582	0.0001	0.0001	0.0001			
Control	2.50 ± 2.50 Aa	1.25 ± 1.25 Ac	0.00 ± 0.00 Ac	3.75 ± 3.75 b	0.6100	8.14	8.53
Pepper	0.00 ± 0.00 Aa	1.25 ± 1.25 Ac	0.00 ± 0.00 Ac	1.25 ± 1.25 b	0.5926	5.36	5.38
Btk	2.50 ± 2.50 Ba	35.69 ± 8.17 Ab	41.25 ± 5.15 Aa	79.44 ± 4.12 a	0.0007	7.12	6.96
Pepper +Btk	3.75 ± 2.39 Ca	70.72 ± 5.55 Aa	19.14 ± 4.62 Bb	93.62 ± 3.80 a	0.0001	5.4	5.4
P	0.6021	0.0001	0.0001	0.0001			
Control	2.50 ± 2.50 Aa	1.25 ± 1.25 Ab	0.00 ± 0.00 Ab	3.75 ± 3.75 b	0.6100	8.14	8.53
Grape japan	0.00 ± 0.00 Aa	0.00 ± 0.00 Ab	0.00 ± 0.00 Ab	0.00 ± 0.00 b	0.8418	5.59	6.01
Btk	2.50 ± 2.50 Ba	35.69 ± 8.17 Aa	41.25 ± 5.15 Aa	79.44 ± 4.12 a	0.0007	7.12	6.96
Grape japan +Btk	1.25 ± 1.25 Ba	38.33 ± 1.67 Aa	32.08 ± 1.25 Aa	71.67 ± 2.04 a	0.0001	5.68	6.02
P	0.7868	0.0001	0.0001	0.0188			

Mean (± SE) followed by the same letter in the column do not differ significantly by Tukey's test (P <0.05). [1]*Bacillus thuringiensis* subsp. *Kurstaki.*

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