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Effects of *Bacillus thuringiensis* subsp. *kurstaki* HD1 spore-crystal mixture on the adults of egg parasitoid *Trichogramma evanescens* (Hymenoptera: Trichogrammatidae)

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In the present study, the potential hazard of *Bacillus thuringiensis* (*Bt*) *kurstaki* HD1 spore-crystal mixture (spore/ δ -endotoxin) on parasitization performance and longevity of female egg parasitoid *Trichogramma evanescens* Westwood was evaluated. For this purpose, *Bt kurstaki* HD1 was incubated at 30 °C in T3 medium at 200 rpm for seven days. Lyophilized spore-crystal mixture (5000 $\mu\text{g mL}^{-1}$) was mixed with 50% honey solution and supplied to 0–24 h old *T. evanescens* adults as a nutrient to ensure the ingestion of the toxins by the parasitoids. The results indicated that spore-crystal mixture of *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*) HD1 did not induce considerable decrease in parasitization performance and longevity of *T. evanescens* adults. Thus, it can be concluded that *Btk* HD1 products can safely be used together with egg parasitoid *T. evanescens* in integrated pest management system to compensate the deficiency of each control tactic alone.

Keywords: spore/ δ -endotoxin; *Bacillus thuringiensis* subsp. *kurstaki* HD1; *Trichogramma evanescens*; longevity; parasitization performance

Introduction

Pest control strategies are among the major parameters for increasing the productivity in agriculture. Chemical pesticides are one of the most effective control methods and hence are used to control many pest insects. The use of chemical pesticides is advantageous, because they do not require information about the ecological origin of the insects and temporarily suppress the pest population. However, they cause serious adverse effects on the nature and also on the human health.[1] Using non-selective insecticides may presumably cause outbreak of insect pests, due to decreasing population of their natural enemies. Also, the widespread use of chemicals gives rise to development of pest resistance, while reducing the natural enemy complexes. Hence, they disrupt the natural ecosystems that often exist between pests and their natural enemies.[2,3] Due to these considerations, many alternative control tactics have been developed. Inundative release of *Trichogramma* spp. and use of microbial insecticides are among the alternatives to chemical control tactics and are common components of integrated pest management systems.[4–8]

Egg parasitoid *Trichogramma* species are well known biological control agents and are widely used commercially in controlling lepidopterous insects pests.[9] Commercial formulations of various *Bacillus thuringiensis*

(*Bt*) strains are also efficiently used as biocontrol agents against many pest insects.[10,11] However, little concern has been given for antagonism between the two control tactics.[12] Although no direct *Bt* poisoning of *Trichogramma* spp has reported, there is still a possibility that they may adversely affect the next generations of native parasitoids.[12] Because of all these reasons, it is obligatory to make transition to the integrated pest management strategies. Scientists from different fields of study have tried to develop environmentally friendly and sustainable control tactics as a component of integrated pest management (IPM) systems. In this respect, Takada et al. [13] concluded that the combination of microbial insecticides and natural enemies give better results than each of the methods used alone. However, *Bt* products should be compatible with the natural enemy populations. That is, before applying these two methods together, the effectiveness of *Bt* products on the natural enemies must be well specified. Thus, safety of microbial insecticides on natural enemies should be an indispensable component of the integrated pest management strategies. In the present study, the side effects of relatively high dose (5000 $\mu\text{g mL}^{-1}$) of *B. thuringiensis kurstaki* HD1 spore/ δ -endotoxin were assessed on the longevity and parasitization performance of egg parasitoid *Trichogramma evanescens* in

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order to determine whether these two strategies can be used together or not.

Materials and methods

B. thuringiensis strain

B. thuringiensis subsp. *kurstaki* HD1 (*Btk*), (Instituto de Biotecnología, Universidad Nacional Autónoma de México) was used in the experimental procedures.

Preparation of spore-crystal mixture, freeze drying and electron microscopy

Btk HD1 was grown in 150 mL T3 medium (3 g triptone, 2 g triptose, 1.5 g yeast extract, 0.005 g MnCl_2 , 6 g NaH_2PO_4 , 7.1 g Na_2HPO_4) and incubated for seven days at 30 °C at 200 rpm to induce sporulation.[14] The bacterial suspension was centrifuged at 4 °C and $15,000 \times g$ for 10 min to obtain spore-crystal mixtures. The pellet was washed twice with sterile distilled water (dH_2O) and centrifuged at $15,000 \times g$ for 10 min. Subsequently, spore-crystal mixture was freeze dried using Labconco–Welch freeze-drier. For electron microscopy, the spore-crystal sample was suspended in dH_2O on a microscope slide and fixed after air drying at room temperature. The sample was sputter coated with 10 nm Au/Pd layer using a SC7620 mini-sputter coater and viewed using a scanning electron microscope (LEO440) at 20 kV beam current.

Determination of δ -endotoxin and colony forming units

The number of spores was estimated by determining the colony forming units (CFUs). One millilitre of *Btk* HD1 culture was incubated at 80 °C for 10 min and dilutions (10^1 , 10^2 , 10^3 , 10^4 , 10^5) were plated on Luria Bertani agar medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 10 g L^{-1} NaCl, 15 g L^{-1} agar). In order to determine the δ -endotoxin concentration, 1 mL of the culture medium was centrifuged for 10 min at $10,000 \times g$ and the pellet was washed twice with 1 mol L^{-1} NaCl and twice with distilled water. The pellet was then suspended in 1000 μL of 50 mmol L^{-1} NaOH (pH 12.5).[7,15] After 3 h incubation at 30 °C, the total protein in the supernatant was measured using the Bradford [16] method. The toxin yield of *Btk* HD1 was calculated by dividing δ -endotoxin ($\mu\text{g mL}^{-1}$) to CFU (spores mL^{-1}).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Protein gel electrophoresis was conducted as described by Sambrook et al. [17] The spore-crystal mixture of *Btk* HD1 was resuspended in equal amount of sample buffer (4 mL 10% SDS, 2 mL glycerol, 1.2 mL of 1 mol L^{-1}

Tris (pH 6.8), 0.01% w/v Bromophenol Blue, 10 mL β -Mercaptoethanol, 2.8 mL dH_2O) and boiled at 95–100 °C for 5–10 min. The samples were then loaded and separated by SDS-PAGE under reducing conditions using a continuous gel containing 12% separating gel and 5% stacking gel. The gel was stained with Coomassie Brilliant Blue R250 and analysed. The used molecular weight marker was SM0431, Fermentas.

Insect cultures

Ephestia kuehniella (Lepidoptera: Pyralidae) were reared on a mixture of wheat flour, wheat bran and glycerol. The insect culture was maintained at constant temperature ($27 \text{ °C} \pm 1 \text{ °C}$), 14L:10D photoperiod and $60\% \pm 5\%$ relative humidity.[18–20] Egg parasitoid *T. evanescens* (Hymenoptera: Trichogrammatidae) was obtained from Adana Agricultural Pest Control Institute and cultured in Biocontrol Laboratory of Biology Department at Erciyes University.

Bioassay

Freshly laid eggs of the host *E. kuehniella* were fixed on 1.5 cm \times 10 cm cardboards using 10% gum arabic. Then the eggs were exposed to a 0–24 h old adult female *T. evanescens* in sterile glass tubes (1.5 cm \times 16 cm). *T. evanescens* adults were supplied daily with 5000 $\mu\text{g mL}^{-1}$ spore-crystal mixture in a final concentration of 50% honey.[21–24] Although low LC_{50} doses are enough to control most lepidopterous pests (465.59 $\mu\text{g g}^{-1}$ for *E. kuehniella*),[7] much higher doses are required for coleopteran pests in storage conditions, as it was indicated in a previous study (5749.50 $\mu\text{g g}^{-1}$ for *B. thuringiensis* subsp. *tenebrionis*).[7] Considering the required doses for all pest populations in storage conditions, the highest amount of spore/ δ -endotoxin concentration was selected to ensure whether *T. evanescens* adults remained active without suffering any side effects. The control group was supplied with sterile distilled water in 50% honey in place of the spore/ δ -endotoxin. Fifty fresh host eggs on cardboards were supplied for every *T. evanescens* female separately and were replaced daily. The parasitized eggs on each of the treated cardboards were counted daily during their lifespan. The longevity of each adult female was also determined in this experiment. For each treatment, 10 *T. evanescens* were used separately to determine the daily egg laying patterns of adult wasps. The treatments were conducted in triplicate.

Statistical analysis

Mean daily parasitization per female of both control and treatment groups were analysed using one-way analyses of variance (ANOVA). Means were separated at the

5% significance level by using Tukey honest significant differences post-test for each group. Females' parasitization rate, as well as control and treated groups' longevity, was compared with independent samples *t-test* for each day.[25]

Results and discussion

Scanning electron microscopy of *Btk* HD1 spore-crystal mixture

Spore-crystal sample was examined under scanning electron microscope, in order to be shown in detail. It was evident that *Btk* HD1 produced bipyramidal, spherical and cubic crystal proteins with different sizes (Figure 1). Morphology of Cry proteins were also detected by some other researchers of *Btk* HD1.[5–7] These types of Cry proteins have specific toxicity against pest insects belonging to Lepidoptera, Diptera and Coleoptera.[26]

Biomass and δ -endotoxin determination

CFU values and δ -endotoxin production of *Btk* HD1 were estimated as $38.67 \pm 0.88 \times 10^5$ spore mL⁻¹ and 620 μ g mL⁻¹ \pm 4.82 μ g mL⁻¹, respectively. Also, the toxin

yield was calculated as 16.03 μ g/10⁵ spores mL⁻¹. Similar results were obtained in a study carried out by Yilmaz et al. [7] Also, Saadaoui et al. [27] estimated the δ -endotoxin production of *Btk* HD1 as 669 μ g mL⁻¹ for 10¹⁰ spore L⁻¹.

SDS-PAGE analysis

Molecular weights of cuboidal, spherical and bipyramidal crystals of *Btk* HD1 were reported as 65, 130 and 130–140 kDa, respectively.[28,29] In the present study, the characteristic banding patterns of Cry1 (130–140 kDa) and Cry2 (65–70 kDa) proteins were confirmed by the SDS-PAGE analysis (Figure 2).

Longevity and parasitization performance

Numerous studies have been documented on the deleterious effects of chemical pesticides on parasitoid wasps and have stressed that the application of chemicals and the release of the parasitoid wasps should not be coincided at the same time period.[13,30–33] On the other hand, some researchers reported that *Bt* products have little or no detrimental effect on *Trichogramma* wasps [22,24,34–37]

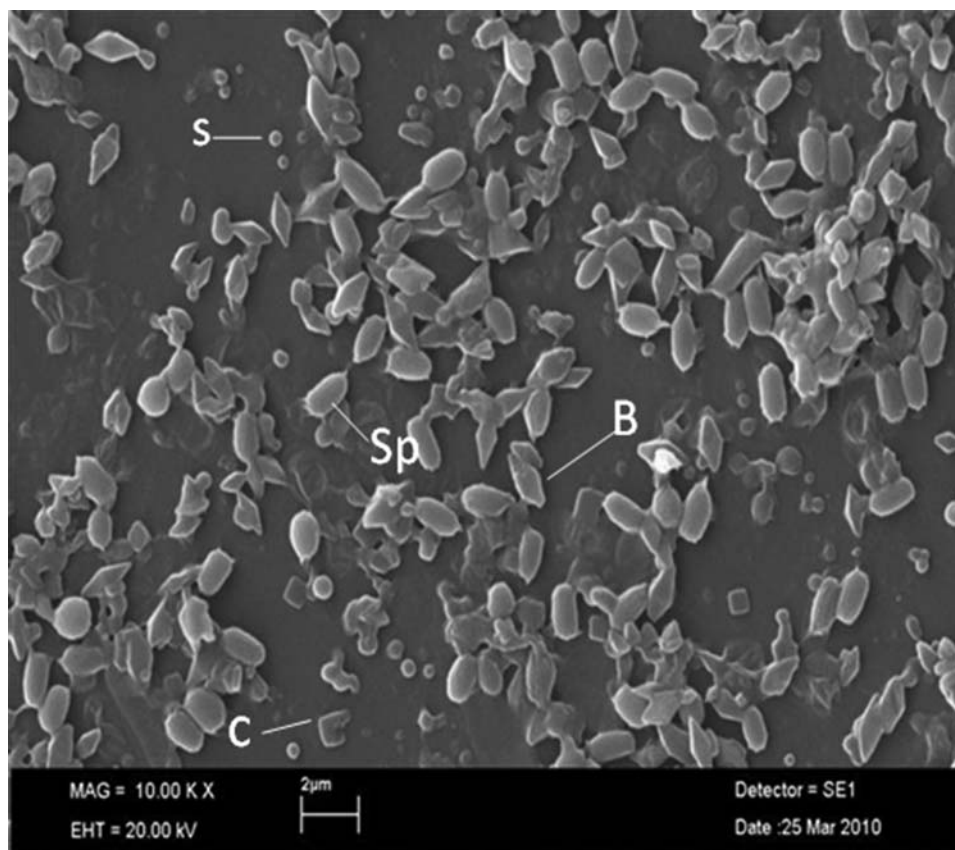


Figure 1. Electron micrograph of *Btk* HD1 spore-crystal mixture. Note: Bipyramidal (B); cubic (C); spherical (S); and spore (Sp).

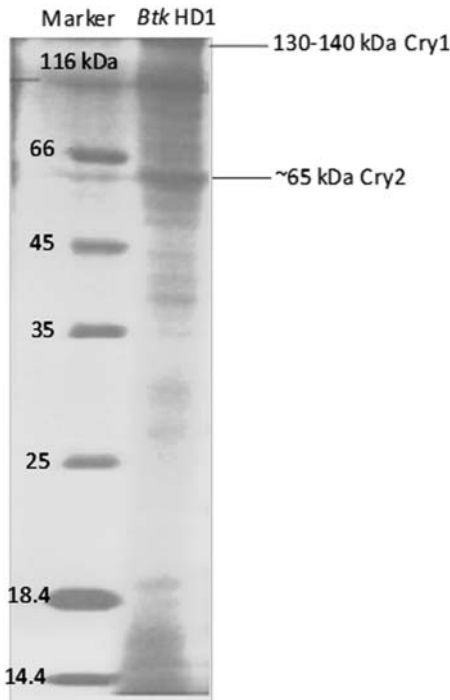


Figure 2. SDS-PAGE (12%) analysis of *Btk* HD1 spore-crystal mixture.
 Note: Molecular weight marker (lane 1) and *Btk* HD1 (lane 2).

and can safely be used simultaneously in the same field. In the present study the average longevity of *T. evanescens* was calculated as 7.3 and 6.3 d for the control and treated groups, respectively ($t = 0.977$; $df = 18$; $P = 0.341$) (Figure 3). Although the present doses were tenfold higher than the doses reported by Salama and Zaki [36] ($500 \mu\text{g mL}^{-1}$), deleterious effects on longevity of *T. evanescens* adults were not observed.

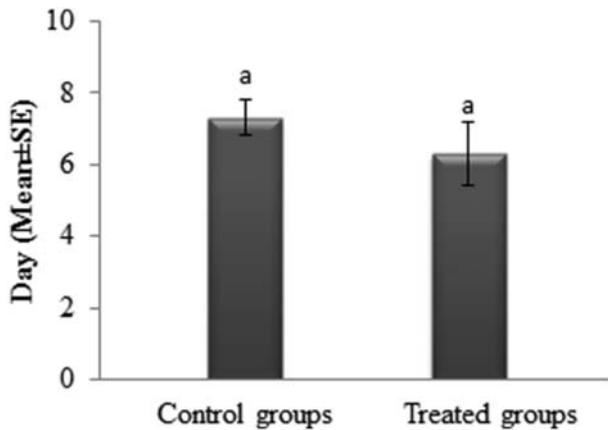


Figure 3. Longevity of *T. evanescens* females after exposition to *Btk* HD1 spore-crystal mixture ($5000 \mu\text{g mL}^{-1}$).
 Note: Means were compared with independent samples *t*-test. Means, followed by the same letter, are not significantly different at $P \leq 0.05$.

Results of the current study indicated that *T. evanescens* female adults parasitized most of the host eggs in the first and second days of the experiment. Daily parasitism per female started to decrease from the third day for both the control and the treated groups (Figure 4). For the control groups: $F = 9.128$; $df = 6$; $P \leq 0.0001$; for *Btk* HD1 treated groups: $F = 8.758$; $df = 6$; $P \leq 0.0001$. For parasitization on the first day: $t = 0.095$; $df = 18$; $P = 0.926$; on the second day: $t = 0.986$; $df = 18$; $P = 0.337$; on the third day: $t = 0.495$; $df = 18$; $P = 0.627$; on the fourth day: $t = 0.594$; $df = 18$; $P = 0.560$; on the fifth day: $t = 0.271$; $df = 18$; $P = 0.789$; on the sixth day: $t = 0.499$; $df = 18$; $P = 0.624$; on the seventh day: $t = 0.356$; $df = 18$; $P = 0.726$.

Researchers tested the parasitization performance and longevity of several other egg parasitoids, such as *Trichogramma ostrinia*, *Trichogramma cacoecia*, *Trichogramma pratissolii*, *Trichogramma pretiosum* and *Trichogramma brassica*, treated with *Bt* and reported no negative effect,[24,34–37] as is the case in the current study. In a similar manner, studies with larval parasitoids *Cotesia plutellae* (Hymenoptera: Braconidae), *Hyposoter exiguae* (Hymenoptera: Ichneumonidae) and *Microplitis croceipes* (Hymenoptera: Braconidae) indicated no decrease in parasitization performance between the control and treated groups.[38–40] In a study carried out by Vaez et al., [12] it was indicated that the application of $9.8 \times 10^5 \text{ IU L}^{-1}$ *Bt* spores to the eggs of the host organism affected negatively the searching efficiency and palpation of *Trichogramma brassicae* adults. On the other hand, Ruiu et al. [23] reported 16.3% mortality rate on housefly pupae parasitoid *Muscidifurax raptor* (Hymenoptera: Pteromalidae) fed with 2×10^9 spores/g of *Btk* strain HD1 in 60% sucrose. Also, Dunbar and Johnson [41] and Salama et al. [42] reported a negative effect of *Bt* treatment on longevity and parasitization performance of various hymenopteran parasitoids.

Conclusions

The results of the present study, conducted on *T. evanescens* after application of relatively high dose ($5000 \mu\text{g mL}^{-1}$) of *Btk* HD1 spore-crystal mixture, indicated that *Bt* products and *Trichogramma* wasps can safely be used in IPM strategies. Thus, the controlling deficiency of the inundative release of parasitoids could desirably be compensated by using *Bt* products. The data obtained from the present study and many other supporting works did not indicate any adverse effects of *Bt* products, when used in combination with *T. evanescens* in controlling important pest species. However, comprehensive preliminary studies should be conducted, considering the reported side effects of *Bt* on other various insect parasitoids, before starting the treatments in the field or storage conditions, for ensuring that *Bt* products do not show adverse effects on

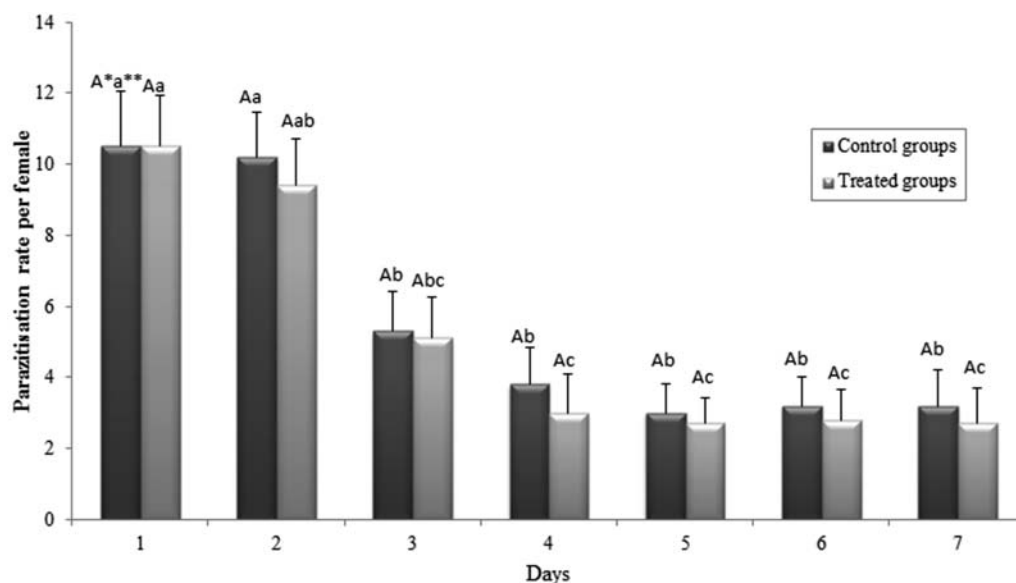


Figure 4. Parasitization of *T. evanescens* per female after exposition to *Btk* HD1 spore-crystal mixture ($5000 \mu\text{g mL}^{-1}$). *Means were compared with independent samples *t*-test for each day. **means were compared with one-way ANOVA test for each group. Means, followed by the same letter are not significantly different at $P \leq 0.05$.

parasitoids and, consequently, for determining the tolerable doses.

Disclosure statement

No potential conflict of interest was reported by the authors.

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