Feeding Rate and Mortality Impact of Crystals and Endospores Versus Crystals – Producing Recombinants of Bacillus thuringiensis Against Cotton Leafworm

Saad Aied AlOtaibi*

Department of Biotechnology, Faculty of Science, Taif University, Kingdom of Saudi Arabia

Abstract: Two *Bacillus* strains belonging to two species; *thuringiensis* and *subtilis* were used in this study. These strains were genetically marking and to be used in conjugation process depending upon the opposite genetic markers to induce recombinants in both strains. This study aimed to evaluate two bioinsecticides induced from recombinants; crystals, crystals + endospores, which derived from two strains of *Bacillus* and four of their transconjugants resulted from conjugation between *Bacillus thuringiensis* and *Bacillus subtilis*, with respect to their toxicity against lepidopterous cotton pest. The results obtained in this study achieved that endospores were effective in reducing feed consumption at the late times of larval age, in contrast, crystals was more effective in reducing feed consumption at the early times of larval age. The results appeared that endospores play a major role in increasing insecticidal activity of bioinsecticides (crystals), this leading to use their in all bioinsecticides. Endospores were effective in reducing the average weight of surviving larvae more than crystals. This indicated that bioinsecticides containing endospores + crystals were more effective than that containing crystals alone. However, most bioinsecticides containing endospores were reduced the average weight of surviving larvae at 48, 72 and 96 hours from treatment. All bioinsecticides containing crystals + endospores were more effective in reducing survival percentage than that containing crystals alone. This achieved that bioinsecticides containing crystals + endospores were more toxic on *S. littoralis* larvae. The mean number of mortality larvae appeared in treatments with crystals + endospores was more than that caused by bioinsecticides containing crystals alone. In most treatments done in this study the insecticidal activity of crystals + endospores caused higher mortality larvae than that caused by bioinsecticides containing crystals alone.

Keywords: *Bacillus thuringiensis*, *Bacillus subtilis*, Mortality percentage, Recombinant bioinsecticides, *Spodoptera littoralis*, Survival.

INTRODUCTION

Insect pest management in agriculture is important to safeguard crop yields and productivity. A large number of chemical insecticides that effectively control insect pests have been proven to be harmful to human health and environment. There is a need to reduce the dependence on pesticides by using safer alternatives to manage insect pests. Many insecticidal proteins and molecules are available in nature, which are effective against agriculturally important pests but innocuous to mammals, beneficial insects and other organisms.

B. thuringiensis was first discovered in 1901 by Japanese biologist Shigetane Ishiwatari. In 1911, *B. thuringiensis* was rediscovered in Germany by Ernst Berliner, who isolated it as the cause of disease called *Schlaffsucht* in flour moth caterpillars. In 1976, Zakharyan reported the presence of a plasmid in a strain of *B. thuringiensis* and suggested the plasmid's involvement in endospore and crystal formation [1, 2]. *B. thuringiensis* is closely related to *B. cereus*, a soil bacterium, and *B. anthracis*, the cause of anthrax. The three organisms differ mainly in their plasmids. Like other members of the genus, all three are aerobes capable of producing endospores [3]. Upon sporulation, *B. thuringiensis* forms crystals of proteinaceous insecticidal δ-endotoxins (called crystal proteins or Cry proteins), which are encoded by *cry* genes. In most strains of *B. thuringiensis*, the *cry* genes are located on the plasmid [4-7].

Cry toxins have specific activities against insect species of the orders Lepidoptera (moths and butterflies), Diptera (flies and mosquitoes), Coleoptera (beetles), Hymenoptera (wasps, bees, ants and sawflies) and nematodes. Thus, *B. thuringiensis* serves as an important reservoir of Cry toxins for production of biological insecticides and insect-resistant genetically modified crops. When insects ingest toxin crystals, the alkaline pH of their digestive tract activates the toxin Cry toxin gets inserted into the insect gut cell membrane, forming a pore. The pore results in cell lysis and eventual death of the insect [8, 9].

Bacillus thuringiensis (*Bt*) is a unique bacterium in that it shares a common place with a number of chemical compounds which are used commercially to control insects important to agriculture and public health. Although other bacteria, including *B. popilliae* and *B. sphaericus*, are used as microbial insecticides,

*Address corresponding to this author at the Department of Biotechnology, Faculty of Science, Taif University, Kingdom of Saudi Arabia; Tel: 00966505727900; Fax: +96627432222;
E-mails: dralotaibaa@yahoo.com, dralotaiba@yahoo.com

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their spectrum of insecticidal activity is quite limited compared to *Bt*. Importantly, *Bt* is safe for humans and is the most widely used environmentally compatible biopesticide worldwide. Furthermore, insecticidal *Bt* genes have been incorporated into several major crops, rendering them insect resistant, and thus providing a model for genetic engineering in agriculture. *B. thuringiensis* is an insecticidal bacterium, marketed worldwide for control of many important plant pests - mainly caterpillars of the Lepidoptera, mosquito larvae and simuliidae black flies that vector river blindness in Africa. *Bt* products represent about 1% of the total 'agrochemical' market (fungicides, herbicides and insecticides) across the world. The commercial *Bt* products are powders containing a mixture of dried spores and toxin crystals. They are applied to leaves or other environments where the insect larvae feed. The vegetative cells contain endospores (phase bright) and crystals of an insecticidal protein toxin (delta endotoxin). Most cells have lysed and released the spores and toxin crystals [10].

Insecticidal proteins present in the soil borne bacterium, *B. thuringiensis*, which has demonstrated its efficacy as a spray formulation in agriculture over the past five decades, have been expressed in many crop species with positive results [11]. *Bt*-transgenic crop species (cotton, corn, rice, tomato and potato) have been commercialized with substantial benefits to the farmers [12]. *Bt* crops were cultivated in an area of 32.1 million hectares out of the global transgenic area of 102.0 million hectares in 2006 [13].

B. thuringiensis is a gram-positive, soil – dwelling, spore-forming, rod-shaped bacteria. *Bacillus thuringiensis* forms a parasporal crystal that is toxic to *Lepidoptera.* The bacteria and protein crystals are marketed as "*Bt*" insecticide, which is used for the biological control of certain garden and crop pests. The endospore of *Bacillus thuringiensis* allows the organism to survive inhospitable conditions in a dormant state. Endospores that contain the protoxin crystal can be applied to fields *via* crop-dusting aircraft. However, in the gut of insects, where the pH is very basic, the protoxin can go into solution. When this happens an insect enzyme splits the molecule. One of the toxin fragments, the delta endotoxin, confers the lethal effect to the insect.The delta endotoxin binds to the epithelial cells lining the gut wall of the insect. By creating holes in the cells, the toxin destroys the functioning of the gut, and causes massive cell death. Another consequence of the destruction is a modification of the pH to a more neutral level that is hospitable for the germination of the endospores. The resuscitation and growth of *Bacillus thuringiensis* within the insect gut kills the larvae [14, 15].

In association with the process of sporulation, some *Bacillus* species form a crystalline protein inclusion called parasporal crystals. The protein crystal and the spore (actually the spore coat) are toxic to lepidopteran insects (certain moths and caterpillars) if ingested. Also, apparently in association with the sporulation process, some bacilli produce clinically - useful antibiotics [11].

This investigation aimed to evaluate insecticidal activity of two *Bt* bioinsecticides; crystals + endospores, crystals, both derived from recombinants of *Bt* transconjugants against larvae of *Spodoptera littoralis*. This was done by subtracted the effect of crystals + endospores from the effect of crystals.

MATERIALS AND METHODS

Microbial Strains

Bacillus thuringiensis serovar *Kurstaki* (NRRL HD-1) and *Bacillus subtilis* (NRRL NRS-744) were obtained from Dr. L.K. Nakamura, U.S. Department of Agriculture, Agricultural Research Service, U.S. Department of Agriculture, Peoria, I Ilinois. The strains were maintained on L.B medium, containing: 1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.5 [16].

Host Plants

Fresh leaves of *Ricinus communis* were collected daily, squares and middle leaves were used for the experiments. Leaves were cleaned and three grams were weighted and placed in clean containers.

Bacillus thuringiensis Formulations Used in the Experiment

Two *B. thuringiensis* preparations were used; crystals + endospores, crystals, in liquid formulations using 200 µl of the suspension. The bioinsecticide were applied on 250 ml bottles as well as mixed with 3 grams of leaves as diet for larvae.

Antibiotic Susceptibility Assays

Antibiotic susceptibility was measured by plate diffusion method, according to Collins and Lyne [17] with cultures grown to logarithmic growth phase in

nutrient broth of LB medium. Bacterial suspension (0.2 ml) was mixed with 10 ml of LB agar medium in petri dishes. Wells (8 mm diameter) were punched in the agar, using a stainless steel borer, and were filled with 0.1 ml of the antibiotic concentration. The plates were incubated overnight at 37°C and the diameter of resulting zones of inhibition was measured, three replicates were used for each bacterial strain, and concentration of antibiotics used [18]. All different antibiotics were used with the concentration of 400 µg/ml, according to Roth and Sonti [19].

rfa Mutation

Strains having the deep rough (*rfa*) character should be tested for crystal violet sensitivity [20]. For the test, nutrient agar plates are seeded with cultures of the strains to be tested and a sterile filter paper disc containing crystal violet is placed on the surface of each seeded plate by pipette 10 µl of a 1 mg/ml solution of crystal violet to the center of sterile filter paper discs (1/4 inch). Invert the plate and incubate at 37°C. After 12 h incubation, a clear zone of inhibition (approximately 14 mm) appears around the disc indicating the presence of the *rfa* mutation which permits large molecules such as crystal violet to enter and kill the bacteria. Wild-type strains or strains containing the *gal* deletion are not inhibited because the crystal violet cannot penetrate the cell.

Conjugation

Nutrient broth cultures, in the late-exponential growth phase, were used. Quantitative spot mating of conjugal transfer was carried out, according to Lessl *et al*. [21], by inoculating 10 µl samples of the donor cultures onto the surface of selective medium, previously seeded with 100 µl of the recipient culture. A single colony of transconjugants was picked up and transferred to LB slant agar medium.

Separation of Crystals and Endospores

Crystals and endospores were collected and purified according to Karamanlidou *et al*. [22]. Bacteria were grown in petri dishes and the spores were collected from nutrient agar washed three times in ice-cold distilled water. Pellets (spores and crystals) were resuspended in small volumes of distilled water. Bacterial cells were lysed to releasing spores and crystals and then collected by centrifugation (10000 x g for 10 min.). Pellets were washed three times with ice-cold distilled waters and final pellets were resuspended in 20 ml of water and stored at -5°C. To purify crystals from spores and cellular debris, samples were sonicated and centrifuged on discontinuous sucrose density gradients (67 to 72 to 79% [wt/vol] sucrose) at 15000 ×g for 2 h. Crystal bands and spore pellets were purified by three centrifugations and washed with distilled water. Final pellets were resuspended in small volumes of distilled water and stored at -5°C.

Bioassay of Toxicity

The toxicity was bioassayed with *Spodoptera littoralis* second instar larvae (mean body weight = 10 mg) according to Klanfon and DeBarjac [23] with some modifications. Bacterial cell component of *B. thuringiensis* was approximately 10^9 crystals and/or spores per milliliter were used with the dilution of 1:1. Larvae of *Spodoptera littoralis* were exposed to the appropriate dose of the component of *B. thuringiensis* using a micropipette to dispense 200 µl of the suspension on three gram of diet surface of *Ricinus communis* [24]. Then this drop was evenly distributed over the diet surface with a sterile glass rod, and the surface was air-dried. Mortality was recorded daily after 24 h for 6 - 7 days. Surviving larvae from each replicate were pooled and weighted daily [25]. To evaluate the toxicity of bioinsecticides containing crystals + endospores in comparison with that containing crystals alone, the effect of crystals was subtracted from the effect of crystals + endospores using the mark of (+) or (-). All bioinsecticides were derived from *B. thuringiensis* and their recombinant transconjugants.

In addition, daily observations were made to count the surviving larvae. Actual values for larval mortality percent were estimated using the following equation;

$$\% \text{ Effect} = (1- Nt / Nc) \times 100$$

Where: Nt and Nc is the number of alive larvae in treatment (*Bt sprayed Ricinus communis*) and check (non *Bt*), respectively.

Evaluate the Effect of Bio-Insecticides

To evaluate these effects, the effect of crystals + endospores was subtracted from the effect of crystals derived from *Bacillus thuringiensis* and their transconjugants. These valuations were included five traits as follows; average of feeding (leaf damage gram/day), average weight of surviving larvae, survival percentage, mean number of mortality larvae and mortality percentage.

RESULTS AND DISCUSSION

Genetic Markers to be Used in Conjugation

Several approaches and techniques have recently been used to manipulate genetically *Bt* strains to improve their insecticidal properties and contribute to their development as biopesticides. Conjugation was first used to construct recombinant strains with new combinations of genes. However, this procedure is only applicable to C'Y~ genes carried by conjugative plasmids and does not allow the association of genes on plasmids belonging to the same incompatibility group. However, despite the demonstrated efficacy of such genetically altered *Bt* products, the efficiency and economic production of *Bt* products could still greatly benefit from the construction of engineered *Bt* strains with a broader activity spectrum and producing larger amounts of each of the crystal, endotoxins in the strain.

Both *Bacillus thuringiensis* and *Bacillus subtilis* used in this study were genetically marked at first to be use the opposite genetic markers in conjugation process. This step was done by tested bacterial strains for resistance or sensitivity to the antibiotic (hiconcil) and drug (Crystal violet) resistance as shown in Table **1**. However, many other antibiotics used did not appear any differences between both strains. Though, *B. thuringiensis* was found to be more resistant to Hiconcil and sensitive to crystal violet. In addition, *B. subtilis* was found to be resistant to crystal violet and sensitive to hiconcil. This have often relied upon resistance as a genetic marker to identify bacterial strains [26]. Crystal violet resistance in *B. subtilis* is also similar to that of hiconcil in *B. thuringiensis* and provides a second potential marker to be use as an opposite genetic marker in conjugation process, as well as, to be use for isolating bacterial transconjugants. The results obtained herein agreed with Stuart *et al.* [27], who examined forty-eight clinical isolates of *Streptococcus suis* for antibiotic sensitivity and the presence of plasmid DNA. It was determined that isolates showed a substantial increase in resistance to erythromycin (ery), clindamycin, and tetracycline (tet). Eleven of the 48 isolates contained plasmid DNA as revealed by DNA isolation and gel electrophoresis. Plasmid DNA from four strains resistant to the above three antibiotics was tested for the ability to transform an antibiotic sensitive recipient.

After the above strains were genetically marking, conjugation process was done between *Bacillus subtilis* (*Hico*$^{-}$ *rfa*$^{+}$) with *Bacillus thuringiensis* serovar *Kurstaki* (*Hico*$^{+}$ *rfa*$^{-}$*)*, depending upon the opposite genetic markers determined in this study. The results obtained herein agreed with that found by Campbell and Reece [28], who reported that genes located on a circular strand of DNA called an R-plasmid may contain several antibiotic - resistant genes. Through a process called conjugation can transfer the antibiotic resistance genes from an R-plasmid to a non-resistant bacterium. This allows a species of bacteria to possess enough genetic variability to adapt changing environment and to compete with its neighbors.

Effect of Recombinant Bioinsecticides on Consumption Rate of Food Leaves

This study, as seen from the results, evaluated insecticidal activity using traits related to toxicity as follows; reducing consumption of fed leaves (gram/day), reducing average weight of surviving larvae (g), reducing survival percentage of neonate larvae, increasing mean number of mortality larvae, as well as, increasing mortality percentage of neonate larvae.

The results summarized in Table **2** appeared that endospores increased insecticidal activity after 24 h of treatment with transconjugant – c *via* reducing the consumption of food leaves. However, after 48h to 144h endospores of transconjugant – A was more effective in reducing the consumption of food leaves. Although, endospores of transconjugants B and C increased insecticidal activity after 96 h of treatment to 144 h *via* reducing the consumption of fed leaves. However, transconjugant D appeared the same trend to reducing the consumption of fed leaves after 120 and 144 h of treatment. The results indicated that endospores was more effective in reducing feed

Table 1: Genetic Markers for Antibiotic and Crystal Violet as Measured by Inhibition Zone

Marking agents	*Bacillus* strains	
	B. thuringiensis	***B. subtilis***
Hiconcil	**+**	**-**
Crystal violet	**-**	**+**

+, - = Presence and absence of inhibition zone, respectively.

Table 2: Evaluation the Effect of Crystals + Endospores Subtracted from the Effect of Crystals in Reducing (-) Average Consumption of Fed Leaves (Leaf Damage, gram/day) by *Spodoptera littoralis* Larvae.

Bioinsecticides	Treatment time (h)					
	24	48	72	96	120	144
B. thuringiensis	+ 0.01	- 0.01	+ 0.03	+ 0.02	- 0.07	0.00
B. subtilis	+ 0.03	+ 0.01	- 0.03	- 0.09	+ 0.11	- 0.02
TA	+ 0.01	- 0.03	- 0.07	- 0.15	- 0.04	- 0.70
TB	0.00	+ 0.10	+ 0.02	- 0.08	- 0.23	- 1.67
TC	- 0.01	+ 0.06	+ 0.05	- 0.05	- 0.19	- 0.02
TD	+ 0.10	+ 0.12	+ 0.01	+ 0.13	- 0.09	- 0.48

Notes: T = Transconjugant, + = Increase the impact of crystals + endospores other than crystals, - = Reducing the impact of crystals + endospores other than crystals.

consumption (leaf damage) at the late times of larval age, in contrast, crystals were more effective in reducing feed consumption at the early times of larval age. The results revealed that endospores play a major role in increasing insecticidal activity of bioinsecticides containing crystals. This has led to use both crystals + endospores in all bioinsecticide preparations, as well as, genetically modified *Bt* strains to induce recombinants of toxic proteins.

The results obtained in this study agreed with Prutz and Dettner [29], who conducted a laboratory scale experiments in order to assess the potential effect of *Bacillus thuringiensis* - corn leaf material on the parasitized herbivore Chilo partellus Swinhoe (Lepidoptera: Crambidae) and on its parasitoid Cotesia flavipes (Cameron) (Hymenoptera: Braconidae). The authors found that food consumption and relative consumption rate of parasitized hosts exposed to *Bt*-corn leaf-material were strongly reduced compared to the control. The number of hosts allowing parasitoid larvae to complete their development was also reduced in the *Bt* group. Moreover, the fresh weight of parasitoid pupae and the dry weight of parasitoid adults was lower than in the control. Only in the *Bt* group, were strong negative correlations found between food intake by the host and the number of parasitoid cocoons. Strong positive correlations were also only found in the *Bt* group, between food intake and parasitoid development time. As effects of *Bt* on the oviposition behaviour of *C. flavipes* could be excluded, differences between the *Bt* group and the control could only be due to the effect of *Bt* toxin on the parasitoid larvae developing inside the host. Whenever food consumption can be measured, the methods used in this study are proposed as a model for future risk assessments on different types of insect-resistant transgenic plants, herbivores, parasitoids, and predators.

On the other hand, Somashekara *et al.* [30] found that food utilization efficiency of *Earias vittella* (Fab) on squares of *Bt* cotton genotypes was greatly reduced compared to non - *Bt* genotypes. Second generation *Bt* cotton genotypes were found to be superior over BG-I hybrids. With statistical advantage of 0.70 less CI in second generation *Bt* hybrids compared to non *Bt*, the food utilization efficacy was found to be significantly reduced, *Bt* hybrids were having 0.61 less CI and found superior over Non *Bt*. Low growth rate was noticed in BG - II (0.10-0.12 mg/day) followed by RCH-2 BG-I (0.18-0.21 mg / day). The same authors was found similar trend followed in efficacy of conversion of ingested food (ECI), approximate digestibility (AD/AE) and efficacy of conversion of digested food ECD (%).

The results obtained herein agreed with Wang *et al.* [31], who tested *Bt* transgenic rice line, i.e. KMD1 containing a synthetic cry 1 Ab gene from *Bacillus thuringiensis* Berliner in the laboratory for evaluating its effects on larval food consumption, growth and survival of striped stem borer (SSB), *Chilo suppressalis* (Walker) under different temperatures. The food consumption and body weight growth, as well as, the survival of both 3$^{rd.}$ and 5$^{th.}$ instar larvae fed on *Bt* rice were significantly reduced, as compared with those on the control. The temperature showed no marked effect on both food consumption and body weight growth of larvae fed on *Bt* rice stems since the onset of 3rd instar, conversely had significant effects as since the initial stage of 5th instar. In contrast, the temperature did not distinctly influence the survival of both 3rd and 5th instar larvae. There was a linearly positive relationship between the corrected mortality of larvae

and the accumulative amount of *Bt* rice tissues ingested by larvae.

In addition, Bienvenu and Chougourou [32] conducted Laboratory tests to determine the effect of two varieties of *Bacillus thuringiensis* (*Bt*) on food consumption and survival of diamondback larvae, *Plutella xylostella* L. Third instar larvae were allowed to feed for 48 h on cabbage disks treated with a series of concentrations (0.006 to 100 µg AI/ml water) of the formulations Dipel 2X (6.4% AI; *Bt* var *kurstaki*) and Xentari (10.3% AI; *Bt* var *aizawai).* Surviving larvae were transferred to untreated leaves to complete their life cycle until pupation or death. After two days of feeding on treated leaves, *Bt* subsp. *aizawai* with LC50 (0.82 µg/ml) was less efficacious than *Bt* subsp. *kurstaki* with LC50 (0.45 µg / ml). Moreover, mortality of larvae on treated leaves increased, whereas food consumption was reduced with increasing concentrations of delta-endoxin. However, consumption of untreated leaves by surviving larvae and duration of larval stage were reduced significantly in both cases (expected 0.006 µg A I/ ml) in comparison to control. Reduction in duration of larval stage by *Bt* var. *kurstaki* was more effective compared to *Bt* var. *aizawai.*

Effect of Recombinant Bioinsecticides on Larval Weight

The results presented in Table **3** demonstrated that endospores were effective in reducing the average weight of surviving larvae more than crystals. This indicated that bioinsecticides containing crystals + endospores were more effective than that containing crystal alone. As shown in this study all bioinsecticides containing endospores were reduced the average weight of surviving larvae after 24, 120 and 144 h of treatment. However, most bioinsecticides containing endospores were reduced the average weight of surviving larvae at 48, 72 and 96 h of treatment.

The present results agreed with Felke *et al*. [33], who studied the effect of transgenic *Bacillus thuringiensis* (*Bt*) maize pollen (event *Bt*-176) on the development and survival of neonate larvae of the Peacock butterfly, Inachis io (L). Their results suggested that the Peacock butterfly may serve as a model organism for assessing potential side effects of new developed transgenic *Bt* crops on non - target butterflies in a GMO environmental risk assessment. This study was done under laboratory conditions by exposing larvae of the Peacock butterfly to various pollen doses of transgenic maize event *Bt* -176 (cv. PACTOL CB) or the conventional isogenic maize (cv. PACTOL) using a no-choice test. Larvae feeding for 48 h on nettle plants (Urtica dioica) that were contaminated with higher pollen concentrations from *Bt*-176 maize (205 and 388 applied pollen.cm^{-2}) suffered a significantly higher mortality rate (68 and 85% respectively) compared to larvae feeding on leaves with no pollen (11%), or feeding on leaves with pollen from conventional maize (6 to 25%). At lower *Bt* maize pollen doses (23 - 104 applied pollen.cm^{-2}), mortality ranged from 11 - 25 % and there were no apparent differences among treatments. The corresponding LC50 and LC90 - values for neonate larvae of the Peacock butterfly were 187 and 448 applied pollen grains cm^{-2} of *Bt*-176, respectively. Weight of larvae surviving consumption of *Bt* - 176 maize pollen declined between 10 and 81% with increased pollen doses (r = -0.95). The highest weight reduction (81%) corresponded to the highest pollen concentration (388 pollen grains applied cm^{-2}).

On the other hand, Jonathan Latham [34] found that larvae which were exposed to higher *Bt* maize pollen

Table 3: Evaluation the Effect of Crystals + Endospores Subtracted from the Effect of Crystals in Reducing (-) Average Weight (g) of *Spodoptera littoralis* Surviving Larvae.

Bioinsecticides	Treatment time (h)					
	24	48	72	96	120	144
B. thuringiensis	- 0.012	- 0.033	- 0.038	- 0.107	- 0.193	- 0.086
B. subtilis	- 0.017	- 0.035	- 0.058	- 0.175	- 0.104	- 0.251
TA	- 0.012	- 0.031	- 0.030	- 0.157	- 0.139	- 0.849
TB	- 0.011	- 0.032	+ 0.024	- 0.226	- 0.429	- 1.764
TC	- 0.013	- 0.029	+ 0.027	- 0.106	- 0.189	- 0.081
TD	- 0.012	+ 0.021	+ 0.055	+ 0.057	- 0.272	- 0.651

Notes: T = Transconjugant, + = Increase the impact of crystals + endospores other than crystals, - = Reducing the impact of crystals + endospores other than crystals.

densities consumed more pollen had a lower survival rate. The LD50 with regard to larvae surviving to adulthood was 13.72 pollen grains consumed by first - instar larvae. Uptake of *Bt* maize pollen led to a reduced plant consumption, to a lower body weight, and to a longer development time of larvae. Effects on pupal weight and duration of the pupal period were present but less pronounced and smaller than effects on larvae. Larvae having consumed *Bt* - maize pollen as first instars had a lower body weight as adult females and smaller forewings as adult males. The authors concluded that possible effects of *Bt* maize on European butterflies and moths must be evaluated more rigorously before *Bt* maize should be cultivated over large areas.

Survival of *S. littoralis* Larvae Feeding on Leaves Sprayed with Recombinant Bioinsecticides

As shown from the results presented in Table **4**, all bioinsecticides containing crystals + endospores were more toxic effective in reducing survival percentage than that containing crystals. In addition, transconjugants B, C and D appeared more reduction in survival percentage than their parental strains at 24, 48 and 72 h from treatment. However, transconjugant C appeared the same trend at 96 and 120 h from treatment. Meanwhile *B. thuringiensis* was more toxic than *B. subtilis* because it was caused more reduction in survival percentage than that caused by *B. subtilis*. Insect - protected plants *via* spraying *Bt* formulations are among the products of modern biotechnology that have proven to be effective in controlling insect pests and consequently, reducing pesticide usage, increasing yield, and reducing mycotoxin contamination of corn kernels.

The study clearly demonstrated the efficacy of crystals + endospores present in *Bt* formulation in controlling insects. Insects may increase their chance of survival after exposure to *Bt* by temporarily reducing their feeding rate until the *Bt* residues have declined, or by actively abandoning the feeding site. It is possible that larvae can survive for a sufficient period to avoid ingesting a lethal dose. The larvae were able to recover by feeding on the *Bt* - free diet.

These results suggest that there is potential to improve the control of cotton and vegetables leaf worm. There may be benefits by using crystals + endospores, as well as, adding feeding stimulants (e.g. Pheast) to *Bt* sprays to increase effectiveness. For example, feeding raspberry leaves treated with *Bt* + Pheast to larval *Choristoneura rosaceana* resulted in a 93% greater mortality than that observed in larvae feeding on *Bt* alone [35]. Additional control techniques, such as mating disruption, may allow more reliable control of *C. jactatana* and reduce the industry reliance on *Bt* [35].

Mortality Percentage of *S. littoralis* Larvae Feeding on Leaves Sprayed with Recombinant Bioinsecticides

Data summarized in Table **5** indicated that bioinsecticides containing crystals + endospores were more toxic on *S. littoralis* larvae. This insecticidal activity of crystals + endospores appeared by increasing the mean number of mortality larvae than that caused by bioinsecticide containing crystals. In addition, bioinsecticides B, C and D were more toxic against *S. littoralis* larvae than their parental strains at 24, 48 and 72 h from treatment. This referred to the higher number of mortality larvae induced than that resulted by their parental strains.

Table 4: Evaluation the Effect of Crystals + Endospores Subtracted from the Effect of Crystals in Reducing (-) Survival Percentage of *Spodoptera littoralis* Neonate Larvae.

Bioinsecticides	Treatment time (h)					
	24	48	72	96	120	144
B. thuringiensis	- 8.33	- 20.83	- 20.83	- 33.33	- 29.17	- 45.84
B. subtilis	- 12.50	- 16.67	- 20.83	- 12.50	- 20.83	- 16.66
TA	- 8.33	- 4.16	00.00	- 8.34	- 33.33	- 33.34
TB	- 90.83	- 37.50	- 37.50	- 25.00	00.00	- 4.16
TC	- 16.67	- 29.17	- 33.31	- 41.67	- 45.83	- 41.67
TD	- 33.33	- 41.67	- 41.67	- 39.50	- 20.83	- 12.50

Notes: T = Transconjugant, + = Increase the impact of crystals + endospores other than crystals, - = Reducing the impact of crystals + endospores other than crystals.

Table 5: Evaluation the Effect of Crystals + Endospores Subtracted from the Effect of Crystals in Increasing (+) the Mean Number of Mortality Larvae of *Spodoptera littoralis*.

Bioinsecticides	Treatment time (h)					
	24	48	72	96	120	144
B. thuringiensis	+ 0.67	+ 1.67	+ 1.67	+ 2.67	+ 2.33	+ 4.00
B. subtilis	+ 1.00	+ 1.33	+ 1.67	+ 1.00	+ 1.67	+ 1.34
TA	+ 0.67	+ 0.34	0.00	+ 0.67	+ 2.67	+ 2.66
TB	+ 1.67	+ 3.00	+ 3.00	+ 2.00	0.00	+ 0.34
TC	+ 1.33	+ 2.33	+ 2.66	+ 3.33	+ 3.67	+ 3.33
TD	+ 2.67	+ 3.33	+ 3.33	+ 3.33	+ 1.67	+ 1.00

Notes: T = Transconjugant, + = Increase the impact of crystals + endospores other than crystals, - = Reducing the impact of crystals + endospores other than crystals.

The results obtained in this study agreed with Navon *et al*. [36] who determined the potency of two new *B. thuringiensis (Bt)* preparations (coded ABG 6104 and ABG 6105) and of Dipel (*B. thuringiensis* var. *kurstaki*) against *Spodoptera littoralis* (Boisd) 5th-instar larvae on a calcium-alginate diet. With this bioassay, the new *Bt* products were more toxic than twice as potent as Dipel. They were also 2-3 times more active than Dipel on alfalfa and cotton leaves in the laboratory. When applied in an alfalfa field at the rate of 312 mg/m^2, ABG 6104 and ABG 6105 caused 40% mortality of 5th - instar larvae and reduced the weight of the survivors to 30 – 40 % of the control, only half of this activity was obtained with Dipel. On cotton, the activity of all *Bt* products was low. Neonate *S. littoralis* larvae were effectively controlled on avocado seedlings; however, there was high mortality also in the untreated controls. All three *Bt* preparations had a similar effect on *Boarmia (Ascotis) selenaria* on avocado leaves in the laboratory.

The results presented in Table **6** appeared that recombinant bioinsecticides C and D increased mortality percentage than that resulted from their parents at 24, 48, 72 and 96 h from treatment. However, the same trend was also appeared by recombinant bioinsecticide C at 120 and 144h, as well as, recombinant bioinsecticide B, which appeared the same trend at 24, 48, 72 h. In most treatments done in this study the insecticidal activity of crystals + endospores caused higher mortality larvae than that caused by bioinsecticides containing crystals alone.

The present results also agreed with Hibbard *et al*. [37], who evaluated mortality of the western corn rootworm, *Diabrotica virgifera virgifera LeConte*, larvae due to feeding on maize, *Zea mays* L., expressing *Bacillus thuringiensis* Berliner (*Bt*) in five Missouri sites in 2007, 2008 and 2009. They found that mortality due to 5307, MIR604, and 5307 × MIR604 was 99.79, 97.83, and 99.91%, respectively. There was an 8.0-d delay in time to 50% beetle emergence from 5307 compared with isoline maize, which was significantly later than to the other three maize lines. The average delay to 50% emergence from MIR604 and 5307 x MIR604 averaged 4.1 and 4.6 d, respectively later than

Table 6: Evaluation the Effect of Crystals + Endospores Subtracted from the Effect of Crystals in Increasing (+) Mortality Percentage of *Spodoptera littoralis* Neonate Larvae.

Bioinsecticides	Treatment time (h)					
	24	48	72	96	120	144
B. thuringiensis	+ 8.33	+ 20.83	+ 20.83	+ 33.33	+ 29.17	+ 45.83
B. subtilis	+ 12.50	+ 16.67	+ 20.83	+ 12.50	+ 20.83	+ 16.66
TA	+ 8.33	+ 4.16	00.00	+ 8.34	+ 33.33	+ 33.34
TB	+ 20.83	+ 37.50	+ 37.50	+ 25.00	- 10.00	+ 4.16
TC	+ 16.67	+ 29.17	+ 33.34	+ 41.67	+ 45.83	+ 41.67
TD	+ 33.33	+ 41.67	+ 41.67	+ 41.67	+ 20.83	+ 12.50

Notes: T = Transconjugant, + = Increase the impact of crystals + endospores other than crystals, - = Reducing the impact of crystals + endospores other than crystals.

50% emergence from isoline maize. Female beetles had a significant delay in time to 50% emergence compared with male beetles from all treatments with the exception of 5307 x MIR604.

The results also agreed with Guo *et al.* [38] who used transgenic *Bt* cotton line GK12 to test the toxicity of *Bt* cotton leaves, flowers, buds and bolls to the non - target pest, common cutworm *Spodoptera litura* (Fabricius), with its parental non-*Bt* cotton line Simian - 3 as control. They measured survival rates on 2nd, 4th, 6th and 10th day of the newly-hatched larvae when they were fed with above cotton organs of the two cotton lines respectively. Both cotton lines and cotton organs had significant influences on larval survival. But there was no interaction between cotton lines and cotton organs. Larval survival of *S. litura* didn't differ significantly within 10d when they fed on newly stretched cotton leaves (1st from the top) of either cotton line. Neither did on leaves in the middle part ($3^{rd.}$ and $6^{th.}$ from the top) or on cotton buds of tested cotton lines. Feeding on matured cotton leaves ($14^{th.}$ from the top) of GK12, larval survival was significantly lower than control from 4d to 10d. Feeding on cotton flowers, larval survival only differed within 4d between the two cotton lines. Larvae fed on cotton bolls had lower survival on GK12 than on its relative control, but the difference was not significant.

In addition, Barwale *et al.* [39] found that *Bt* cotton hybrids exhibited excellent control of American Boll worm and reduced the use of insecticides leading to create ecofriendly environment without compromising on profitable yield. As compared to insecticide mediated control of bollworms, *Bt* cotton technology does not harm non-target beneficial insects; besides reduction in production cost, increase in profit, reduced farming risk and improved economic outlook for cotton are the highlights of this novel technology. Use of this technology is also helpful in improving wild life population, reduced run-off of insecticides, reduced air pollution and improved safety to farm workers and neighborhood.

FUTURE ASPECTS AND CONCLUSION

Although Biotechnology is by all means the most controversial agricultural technology innovation due to the uncertainty and concerns raised by its biosafety and environmental impacts; but the rise of modern biotechnologies and life science bring many surprises, change the paradigms of the society and revolutionize our daily lives. Against the many exciting successful examples of biotechnology, it is important that all technologies, bio and non-bio, are to serve the ultimate objective of improving the overall welfare of human beings and the nature. Thus crop biotechnology is no exception. It is the foundation of people's livelihood. Hence the very high using rate of *Bt* formulations especially in Asian countries by farmers reflects the fact that biotech crops have consistently performed well and delivered significant economic, environmental, health and social benefits to both small and large farmers.

In conclusion, this work demonstrated that recombinant bioinsecticide containing crystals + endospores reduced the rate of consumption, reduced body weight of surviving larvae, reduced the mean number of surviving larvae and increased accumulated mortality percentage if compared with the effect of bioinsecticides containing crystals alone. This work presents an intriguing model for the recommended components of bioinsecticides which play a major role against cotton leaf worm. Bioinsecticide containing crystals + endospores rendered effective control of *S. litura* on cotton when compared to standard checks and can fit ideally in biocontrol strategies as a potential component thereby reducing dependence on conventional insecticides.

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