The potency of the vegetative insecticidal protein (Vip3A), in comparison with crystalline (Cry) toxins of *Bacillus thuriengensis* against *Spodoptera littoralis* (Boisd.)

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ABSTRACT

The susceptibility measurements were performed on larvae of the cotton leafworm, *Spodoptera littoralis* with two *Bacillus thuriengensis* authentic strains; *Bt* subsp. *kurstaki* (HD-1) and *Bt* subsp *entomocidus* (60.5). The protoxin (≈ 130 kDa), the active toxin (≈ 66 kDa) in the cell pellet after sonication and the vegetative insecticidal protein (≈ 88 kDa) were confirmed by SDS-PAGE. The vegetative insecticidal protein (Vip3A) can be detected in the growth media. LC_{50} values were computed with respect to the Lc-p lines statistical parameters (slope & Chi^2). All parameters were determined at different exposure periods. In general, the HD-1-Vip3A toxin is more toxic against *Spodoptera* larval instars than the tested Cry toxins (*entomocidus* (60.5) and *kuristaki* HD-1). The toxicity is dependant on the larval instar age.

Keywords: Vegetative insecticidal protein, *Spodoptera littoralis*, *Bacillus thuriengensis*.

INTRODUCTION

The wide use of synthetic compounds creates an excessively high pressure on the environment and destabilizes the existing ecological balance. Chemical pesticides generally affect beneficial insects as well as pest species and insects tend to acquire resistance to the chemicals so that new pest problems rapidly developed. Moreover, notwithstanding the huge expenditures (more than ten billion dollars annually) on production of synthetic chemicals, the annual loss reaches 37% of the total crop and its cost is estimated at 300 billion dollars; insect pests are responsible for 13% of the loss (Thomas, 1999). Although more specific and less stable protectants are continuously developed to reduce the ecological hazard created by using them, the consumption of synthetic chemicals has started to decrease. Hence, it is necessary to design new technologies in order to reduce the use of synthetic compounds without increasing the loss of crops or to even decrease the loss.

It is generally accepted that *Bt* is a harmless organism and that it does not pose any threat to the environment, operators, or consumers. As the public and environmentalists become more aware of the side effects of some synthetic pesticides, *Bt*'s safety is expected to form a cornerstone of the anticipated market increase of *Bt*-based bio-insecticides (Lambert and Peferoen, 1992).

Bacillus thuringiensis is a gram-positive, rod-shaped, aerobic bacterium measuring from 2- 5-μm long by 1-μm wide. Most strains are motile, moving by peritrichous flagellae. Biochemically, several strains of *B. thuringiensis* resemble *B. cereus*, but the presence of entomocidal crystals (parasporal bodies) is accepted as evidence that *B. thuringiensis* as a separate species (Lennox and Duke, 1997).

Bacillus thuringiensis toxins are a diverse group of proteins with activity against insects of different orders (Lepidoptera, Coleoptera, Diptera) and also against other invertebrates such as nematodes. Their primary action is to lyse midgut epithelial cells by inserting into the target membrane and forming pores. Among this group of proteins, members of the 3-Domain Cry family are used worldwide for insect control, and their mode of action has been characterized in

some detail. Phylogenetic analyses established that the diversity of the 3-Domain Cry family evolved by the independent evolution of the three domains and by swapping of domain III among toxins. Like other pore-forming toxins (PFT) that affect mammals, *Bt* toxins interact with specific receptors located on the host cell surface and are activated by host proteases following receptor binding resulting in the formation of a pre-pore oligomeric structure that is insertion competent (Bravo *et al.*, 2007).

The crystal is composed of a protein of dimer approximately 230 kilo-Daltons (kDa). This is composed of two major components, each about 130-140 kDa M.W. (Aronson *et al.*, 1986). The protein is hydrolyzed by proteolytic enzymes in the alkaline midgut of susceptible larvae to yield active toxins of 55-58 kDa (Oppert, 1999).

Recently, a new family of insecticidal proteins produced during its vegetative stags of growth (vegetative insecticidal proteins "Vips") has been identified. The Vip3A possesses insecticidal activity against a wide spectrum of lepidopteran insects and displays acute bioactivity towards the black cutworm (BCW), fall armyworm, and cotton leafworm, Vip3A affords 260 fold-higher insecticidal activities than some Cry1A proteins, which have been reported to be active against the BCW. The Vip bears no similarity to delta-endotoxin, and it encodes 88kDa protein that is secreted into the supernatant fluid of *Bt* culture. Histopathological observations indicated that Vip3A ingested by susceptible insect caused gut paralysis and complete lysis on epithelial cells resulting in larval death. This suggests that the midgut epithelial cells of susceptible insects are the primary target for the Vip3A insecticidal protein (Milne et al. 2008). Furthermore, it has a lack cross-resistance with *B. thuringiensis* crystal (Cry) toxins (Fang *et al.*, 2007).

The present study was aimed to compare the potency of the vegetative insecticidal protein (Vip 3A) with the crystalline (Cry toxins) of *Bacillus thureingensis* against *Spodoptera littoralis* demands and to shed some light and strengthen our activities in adopting biopesticides.

MATERIALS AND METHODS

Insect:

Larvae of the cotton leafworm, *S. littoralis* (Boisd.) were reared on castor oil leaves, *Ricinus communis* L., according to eldefrawi et al., 1964. The strain was obtained from the Agriculture Genetic Engineer Research Institute (AGERI) of Agriculture Research Center, Ministry of Agriculture.

Bt strains:

Two authentic strains of *Bt* were tested. *Bt* subsp. *kurstaki* (HD-1) & *Bt* subsp. *entomocidus* (60.5) were obtained from AGERI.

Bacteriological media:

All media were made up in glass deionized distilled water and sterilized by autoclaving (AGDW) at 15 Ib/sq. In for 20 minutes.

Luria-bertani (LB) media:

Tryptone 10 gm, Yeast extract 5 gm, and NaCl 5 gm were dissolved in one liter (AGDW) adjust to pH 7.2, then autoclaved and stored at 4 °C. Solidified media contains the same constituents in addition to 2% agar, autoclaved and poured in agar plates.

TP media:

Solution I: Tryptone 10 gm, Soluble starch 10 gm, Yeast extract 2 gm, Glycerol 5 gm, KH_2PO_4 3.4 gm, and K_2HPO_4 4.35 gm were dissolved in one liter (AGDW).

A) Salt solution stock: one liter contains (24.6 gm $MgSO_4$, 0.4 gm $MnSO_4$, 2.8 gm $ZnSO_4$ and 4 gm $FeSO_4$), 5 ml were add up to one liter of solution I.

B) $CaCl_2$ solution stock: one liter contains (36.6 gm), 5 ml were added to one liter solution I. The pH of the media was adjusted to 7.2, autoclaved and stored at 4 °C.

Terrific broth media:

Tryptone 12 gm, Yeast extract 24 gm, Glycerol 4 gm, KH_2PO_4 2.31 gm, and K_2HPO_4 12.54 gm, were dissolved in one liter (AGDW), and the pH value adjusted to 7.2.

Spores and δ-endotoxin preparation:

Bt inoculum of both authentic strains *Bt kurstaki* HD-1 and *entomocidus* 60.5 were seeded into LB under sterile conditions, spread onto solidified LB media and incubated for 24-48 hr. Streaking for single colony was performed. Single colonies were inoculated into TP broth for sporulation (Travers *et al.*, 1987) in batches of media in 1L flasks and incubated at 30 °C with shaking at 200 rpm for 72-96 hr. Cultures were harvested after 72 hr and were checked by light microscopy for liberation of spores and crystals. After complete cell lysis was achieved, the cultures were harvested by centrifugation at 5500 rpm for 30 min at 4 °C using IEC-CRU 5000 cooling centrifuge.

The pellets were washed with NaCl/EDTA solution (1M NaCl-10 mM EDTA), three times in sterilized ice-cold AGDW containing 1mM phenyl methyl sulphonyl fluoride (PMSF) as a general proteases inhibitor and suspended in AGDW and then sonicated for 4min (5 sec, intervals) to release crystals from intact bacteria, using heat systems sonicator (set at 70% duty and 40% out put) and kept frozen at -20 °C. The protein concentration in the crystal preparations were estimated after the crystals were dissolved in 100 mM NaOH.

Dried powders containing δ-endotoxin were prepared by the lactose-acetone co-precipitation method (Dulmage, *et al.*, 1970; Robacker, *et al.*, 1996) via centrifugation of the pellets slurry with addition of 1/10 of its original volume of 5% lactose solution followed by gradual addition of high purity acetone (4-6%) with continuous stirring. Solution is centrifuged at 3500 rpm for 15 min at 4 °C. The pellet (δ-endotoxin preparation) is washed once with acetone and 3 times deionized H_2O, resuspended in deionized water and finally lyophilized using Edwards' lyophilizer and stored frozen, under nitrogen.

Preparation of the vegetative insecticidal protein (Vip3A):

Insecticidal protein was partially purified from supernatant of *Bt kurstaki* HD-1 strain, which proved to produce Vip3A (Guttmann and Ellar, 2000), according to Estruch *et al.* (1996).

Culture was grown for 24 hour at 30 °C in Terrific media. Supernatant was collected by centrifugation at 5000 rpm for 20 min using IEC-CRU 5000 cooling centrifuge. Proteins presented in the supernatant were precipitated with saturated solution of 70% ammonium sulfate (salting out).

The precipitated protein was harvested by centrifugation (5000 r.p.m, for 20 min.). The pellet was resuspended in minimum volume of 20 mM tris-HCl buffer, pH 7.5, and dialyzed overnight against deionized water (4 liter with 4 changes), lyophilized and kept frozen until use. The purity and molecular weight of the toxin have been confirmed by electrophoresis (SDS-PAGE).

Electrophoresis (SDS-PAGE):

Toxins identification has been carried out using SDS-PAGE technique according to (Laemmli, 1970). Aliquots (5 -20 µl based on protein conc.) of toxin preparation were heated (100 °C, 10 min) with equal volume of 2X sample buffer (2% SDS, 10% glycerol, 3M tris-HCl, pH 8.8, 5 % ß-mercaptoethanol (ß-Me) and 0.002% bromophenol blue) and submitted to 10% SDS-PAGE with an acrylamide / N,N methylene bio acrylamide ratio of 100 : 1 (Thomas and Ellar 1983).

Bio-assay of *Bt* toxins:

Bio-assay procedures were carried out according to Dulmage (1973). *Spodoptera* larval instars (2^{nd}, 3^{rd} and 4^{th}) were used for all assays in 3 replicates. Leaf-dip technique was used, for assessing toxicity (Shelton, *et al*., 1993) and spores and insecticidal crystalline proteins (ICPs) (Tang, *et al*., 1996), since leaf-dipping technique simulates natural conditions more closely than the diet incorporation method.

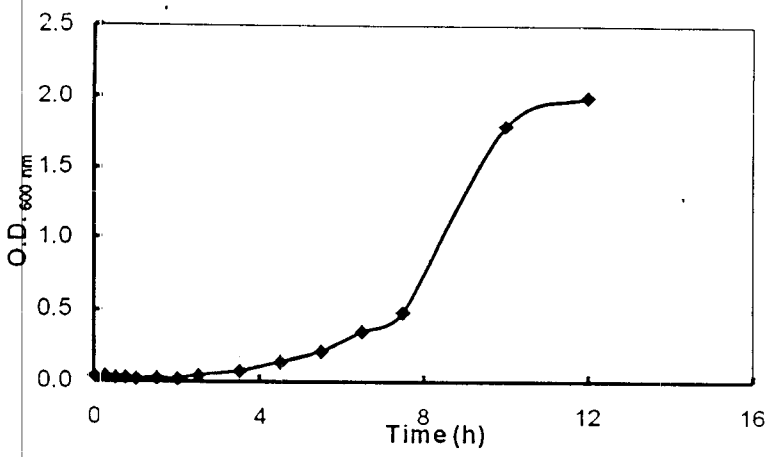
Each toxin was diluted in GDW that contain triton X-100 as detergent, and mixed with a vortex mixer for 1 min. Castor oil leaves were cut into discs (2 cm^2) (Mansour *et al*., 1966). Each disc was dipped into the test solution for 10s (Aboul-Ela *et al*., 1993), to ensure an even leaf surface coverage, held vertically to allow excess solution to drip off, and placed on a rack to dry. After 2 hr drying time, leaves were offered to the larvae previously starved for (2-4 hr) and left under controlled conditions (27 ± 2 °C) for 2 days. Each treatment in 3 replicates, 10 larvae in each plastic cup covered cheese cloth and tied with rubber bands. Survivors were transferred with fresh untreated castor oil leaves to clean cups and kept under the same conditions. Mortality counts were recorded daily for 7 days for crystalin toxins and 4days for Vip 3A toxin. Percent mortality was calculated for each concentration and corrected for natural mortality according to Abbott equation (Abbott, 1925). The mortality data were subjected to Probit analysis (Finney, 1952).

RESULTS

Concentration-mortality response of the *Spodoptera* larval instars to *Bt- kurstaki* (HD-1) and *entomocidus* (60.5):

The susceptibility measurements were performed on larvae of the cotton leafworm with two *Bt* authentic strains; *Bt kurstaki* (HD-1) and *Bt entomocidus* (60.5). Selecting these two strains depends on the literature where *S. littoralis* larvae are susceptible to CrylC, and Cry1E. It is worth to note that, CrylC toxin is produced by *B. thuringiensis* vars. *entomocidus*, *aizawai* (Sneh *et al*., 1981; Visser *et al*., 1988). CrylE is produced by *B. thuringiensis* vars. *kenyae* and *dendrolimus* (Van Rie *et al*., 1990) and The HD-1 strain of *B. thuringiensis* subsp. *kurstaki* is

widely used in commercial products for control of lepidopteran pests in agriculture and forestry



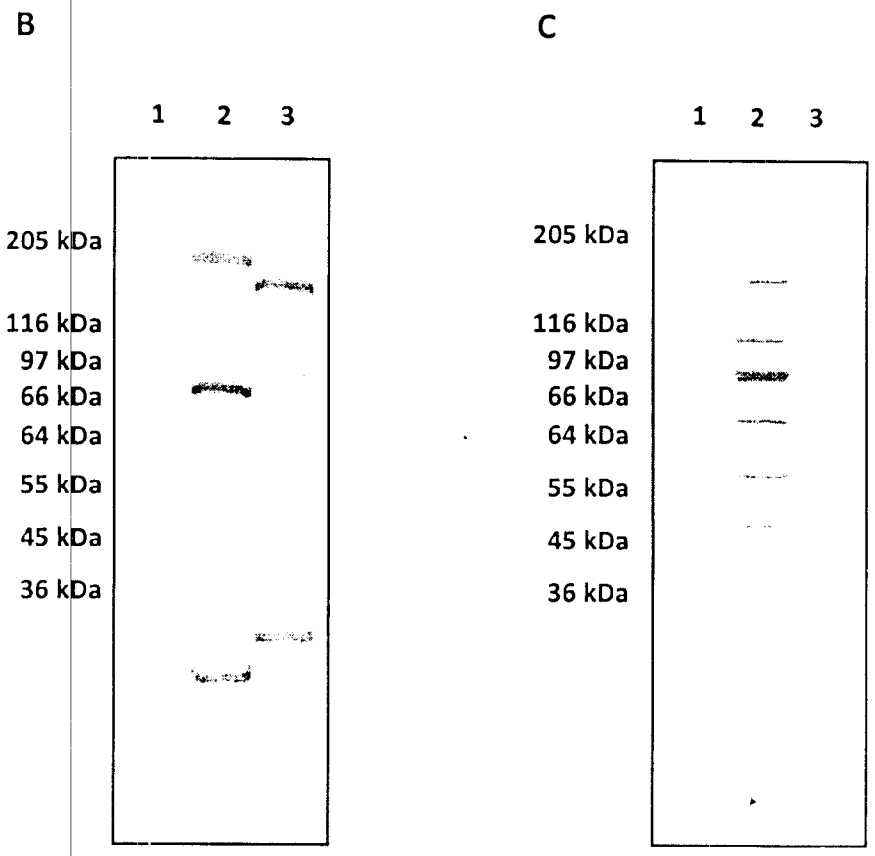


Fig. (1): SDS-PAGE separation crude *Bt*-toxins against the molecular weight markers. Aliquots of the insecticidal active protein were electrophoresed on 10% polyacrylamide gel. (A) Growth curve of shake flask culture "time of hours after inoculation. (B) Expression of cry toxins from *Bt kurstaki* and *entomocidus* (60.5) (lane 1, molecular weight markers; lane 2, *kurstaki* HD-1 and lane 3, *entomocidus* (60.5). (C) Expression of Vip3A from *Bt kurstaki* HD-1 (lane 1, molecular

weight markers; lane 2, supernatant after dialysis and lane 3, supernatant before ammonium sulfate dialysis).

(Donovan *et al.*, 2001) beside this subsp. secreted the vegetative insecticidal protein (Estruch *et al.*, 1996) which displays toxicity to a broad range of lepidopteran pests (Milne et al., 2008).

The initiation of sporulation was determined by direct microscopic observation and by analyzing cell pellets for the presence of δ-endotoxins. Cryl type proteins could be detected almost at the late stage of the stationary phase, during and after sporulation. Dried lyophilized powders containing δ-endotoxins were prepared. With known protein concentration, toxin powder was suspended in deionized water for the toxicity experiments. Samples from the culture of the TB media were taken throughout its growth curve "sporulation Phase". The *Bt* insecticidal proteins of two strains can be detected in the growth media, the protoxin (≈ 130 kDa) and the active toxin (≈66 kDa) was confirmed by SDS-PAGE in the cell pellet after sonication (Fig. 1.B).

Results of the toxicity of the two strains are presented in Table (1) for *kurstaki* HD-1 and Table (2) for *entomocidus* (60.5) against *Spodoptera* larval instars. Median lethal concentration values (LC_{50}) were computed with respect to the LC-p lines statistical parameters (slope & Chi^2). These were determined at different exposure periods. The toxicity of tested *Bt* toxins were decreased with the advancement of larval instar. Whereas *kurstaki* HD1 Cry toxin was more toxic against the 2nd instar larvae than 3rd and 4th instars by 1.1 and 13.6 fold, respectively. *Entomocidus* was more toxic against the 2nd instar than the 3rd and 4th instars by 1.6 and 11.2 after 7 days of exposure, respectively. The LC_{50} values of *Bt Kurstaki* were 581.3, 635.7 and 7911.2 ppm against the 2nd, 3rd and 4th instar larvae after 7 days of exposure, respectively. On the other hand the LC_{50} values of *Bt entomocidus* (60.5) were 542.6, 886.6 and 6047.3 ppm against the 2nd, 3rd and 4th instar larvae after 7 days of exposure, respectively. In general, the toxicity of *Bt entomocidus* (60.5) against the larval instars of *S. littoralis* larvae was higher than the toxicity of *Bt kurstarki*.

Insecticidal activity of *Bt*-Vip3A protein against *Spodoptera* larval instars:

Expression of the Vip3A insecticidal protein (partially purified) was analyzed by SDS-PAGE. Samples from Terrific broth culture were taken throughout its growth curve and sporulation (Fig. 1.A). The partially purified Vip3A insecticidal protein can be detected in non concentrated supernatants in this culture during logarithmic phase as early as 12 – 15 hrs after initiating the culture. It reached its maximum level during early stages of stationary phase and remained at high levels during and after sporulation.

Vip preparation was assayed for insecticidal activity against *Spodoptera* larvae. Where supernatant from the strain *kurstaki* HD-1 was partially purified, lyophilized and kept at –20°C. The reconstituted protein was active against *Spodoptera* larvae, and the active component was characterized where it confirmed by SDS-PAGE after and before dialysis (Fig. 1.C).

Several supernatants from this strain were active against different *Spodoptera* larval instars. Subsequent purification from the partially purified preparation was identified as a major protein with an estimated molecular mass of 88 KDa. Obviously there is a correlation with the *Spodoptera* larvae mortality. Stages of *Spodoptera* larval mortality were determined as a result of treatment with varied concentrations of Vip3A. Concentration-depended response to this preparation against the *Spodoptera* larval instars at different exposure times are shown in Table (3). Vip3A which had been identified were more toxic against the 2nd instar than the 3rd and 4th

instars by 2.5 and 7.3 fold after 4 days of exposure, respectively. After 4 days of exposure, the LC_{50} values of Vip3A were 453.18, 1118.99 and 3322.475 ppm against the 2^{nd}, 3^{rd} and 4^{th}, respectively.

Table (1): LC_{50}, slope and Chi^2 values of *Bt kurstaki* HD-1 Cry-toxin against *S. littoralis* larvae at different exposure time.

Days after exposure	Larval instars											
	2nd				3rd				4th			
	LC_{50} (ppm)		Slope	Chi^2	LC_{50} (ppm)		Slope	Chi^2	LC_{50} (ppm)		Slope	Chi^2
	Upper	Lower			Upper	Lower			Upper	Lower		
3	4210.4		1.3±7.7E-02	1.662	7045.7		1.3±5.1E-02	2.772	--		--	--
	7165.0	2485.5			11532.2	4321.5			--	--		
4	1876.8		1.3±6.7E-02	0.892	3625.4		0.9±3.7E-02	0.951	--		--	--
	2754.2	1280.1			6105.33	2160.37			--	--		
5	1318.4		1.4±4.3E-02	1.634	2261.6		0.9±3.6E-02	0.602	16275.5		1.1±6.0E-02	0.905
	1862.8	933.8			3710.1	1379.4			30580.1	8707.6		
6	947.9		1.4±0.0	1.389	1382.4		1.1±3.9E-02	1.007	11507.0		1.1±5.2E-02	0.888
	1323.2	678.9			2117.4	900.9			20279.4	6558.2		
7	581.2		1.4±4.5E-02	0.577	635.7		1.0±4.1E-02	0.899	7911.2		0.9±4.6E-02	0.777
	834.5	404.2			1166.2	344.0			13556.6	4633.3		

Table (2): LC_{50}, slope and Chi^2 values of *Bt entomocidus* (60.5) Cry-toxin against *S. littoralis* larvae at different exposure times.

Days after exposure	Larval instars											
	2nd				3rd				4th			
	LC_{50} (ppm)		Slope	Chi^2	LC_{50} (ppm)		Slope	Chi^2	LC_{50} (ppm)		Slope	Chi^2
	Upper	Lower			Upper	Lower			Upper	Lower		
3	2890.8		1.7±6.6E-02	0.566	5355.5		1.5±5.5E-02	1.790	--		--	--
	4070.2	2056.7			7793.8	3687.0			--	--		
4	1369.9		1.6±4.9E-02	0.738	3345.6		1.3±4.3E-02	0.645	63268.2		1.2±0.31 1011.8	0.372
	1855.6	1011.8			4888.6	2292.8			408231.9	1855.6		
5	899.5		1.6±4.8E-02	0.849	1900.1		1.3±4.2E-02	0.206	13132.3		1.5±7.0E-02 664.3	0.166
	1217.8	664.3			2729.5	1322.3			20546.3	1217.8		
6	720.4		1.5±4.8E-02	0.604	1397.0		1.3±4.3E-02	0.317	9021.1		1.3±5.5E-02 526.4	1.269
	985.1	526.4			2019.0	965.3			13672.9	985.1		
7	542.6		1.5±4.7E-02	0.626	886.6		1.2±4.3E-02	0.409	6047.3		1.3±5.1E-02	1.226
	768.1	382.6			1379.2	568.0			8681.6	4216.4		

Table (3): LC_{50}, slope and Chi^2 values of *Bt kurstaki* HD-1 Vip3A-toxin against *S. littoralis* larvae at different exposure times.

Days after exposure	Larval instars											
	2nd				3rd				4th			
	LC_{50} (ppm)		slope	Chi^2	LC_{50} (ppm)		slope	Chi^2	LC_{50} (ppm)		slope	Chi^2
	Upper	Lower			Upper	Lower			Upper	Lower		
1	5883.5		1.3±0.2	0.647	--		--	--	--		--	--
	11541.0	3015.6			--	--			--	--		
2	2638		1.0±3.9E-02	0.986	3028.9		1.3±4.9E-02	1.308	14908.8		2.6±1.4	5.903E-02
	4580.1	1528.8			4385.0	2094.7			34565.8	6443.7		
3	1215.4		1.2±4.0E-02	2.112	1742.2		1.2±4.5E-02	0.369	5426.4		1.6±0.1	0.163
	1785.8	833.3			2532.4	1198			7791.7	3784.3		
4	453.2		1.2±4.2E-02	2.354	1119.0		1.3±0.1	0.988	3322.5		1.4±5.3E-02	0.769
	702.4	291.2			1620.1	771.5			4705.4	2348.8		

In general, the HD-1-Vip3A toxin is more toxic against *Spodoptera* larval instars than the tested Cry toxins (*entomocidus* (60.5) and *kurstaki* HD-1) approximately by 3 and 4.1 fold respectively for 2nd instar and by 3 and 3.2 fold respectively for 3rd instar and by 19 fold compared with *entomocidus* (60.5) for 4th instar after 4 days of exposure while *kurstaki* HD-1 didn't exhibit any toxicity against 4th instar at the same time.

DISCUSSION

Pesticides are unusual among environmental pollutants in that they are used deliberately for the purpose of killing some form of life. The ideal situation, of course, is that pesticides be highly selective, destroying target organisms while leaving nontarget organisms unharmed. In reality, most pesticides are not so selective. In considering the use of pesticides, the benefits must be weight against the risk to human health and environmental quality. Among the benefits of pesticides are control of vector borne disease, increased agriculture productivity, and control of urban pests. A major risk is environmental contamination, especially translocation within the environment where pesticides may enter both food chains and natural water systems. Factors to be considered in this regard are persistence in the environment and potential for bioaccumulation judged by the most precise and accurate analytical procedures.

In many countries in the world, agriculture plant protection relies heavily on pesticides. While providing effecting control, chemical pesticides have produced major well known problems: health hazards to humans and animals, destruction of natural biotic control agents and increase resistance of major insect species and subsequently, the steady increase in dosages required to achieve reasonable control. Ultimately, as we mentioned earlier, these lead to many environmental problems, which required a very clever approach in pesticide management. In spite of a more than 15–20 fold increase in pesticide use since 1945 (Thomas, 1999), crop losses due to pests varies and it reaches a nontolerable percentages in different crops in several parts of our world. This situation accelerates the intensive movement towards more sound control methods by intervention of the advancements in discoveries of the nonchemical alternatives.

Among the most promising alternatives are bacterial insecticides and insecticidal transgenic plants based on δ-endotoxin proteins of the spore-forming bacterium *Bt*. Recently, this bacterium was classified as the main class of biopesticides. Biopesticides, including microbial pesticides (in addition, to *Bt*), entomopathogenic nematodes, baculoviruses, plant derived pesticides, and insect pheromones, as alternatives to chemical pesticides and as key components of integrated pest management (IPM) systems (Madelin, 1963). However, the reality is that biopesticides currently represent only a relatively small fraction of the world pesticide market. At the present time, various academic and economic forecasting services estimated the growth rate of biopesticides at 10 – 15 % per annum in contrast to 20 % for chemical pesticides (Menn and Hall, 1999).

The best known group of biopesticides, the *Bts*, are produced by fermentation but yield insecticidal crystal proteins rather than discrete chemicals, such as the spinosyns (from *Sacsharopoly spora* species) and avermectins (from *Streptomyces* species as macrocylic lactones). By selection of natural isolates from the thousands known, as well as those derived from plasmid conjugation and recombinant DNA technology, it has become feasible to tailor make *Bts* that are targeted against specific insect pests (Trumble *et al.*, 1994). Although identifying the limitations of field persistence, we have faced in our laboratory in the early and mid sixties, and incomplete coverage of target surfaces, our research group at University of Alexandria project, as positive outlook for this vast array of toxins as attractive as alternatives to

existing products. The success depended and will in the future on developments in improved formulation and an improved understanding of dose-transfer processes.

In contrast to the discrete chemicals from other known biopesticides, the *Bts* produce crystal proteins. Sporulating cells of *B. thruringeinsis* synthesize parasporal inclusions comprised of one or more insecticidal proteins, referred to commonly as δ-endotoxins or insecticidal crystal proteins (ICPs). These proteins fall into tow unrelated groups, crystalline proteins (Cry) and cytolytic proteins (Cyt) (Hofte and Whiteley, 1989). In a susceptible host, the intoxication pathways are similar for all Cry toxins, requiring ingestion, solubilization, and enzymatic activation by midgut proteases (Knowles and Ellar, 1987). Activated toxin molecules bind to glycoprotein receptors on the midgut epithelium microvillar membrane and form pores or lesions leading to osmotic swelling, cell lysis, and damage to the midgut-hemocoel barrier, resulting in death (Knowles and Ellar, 1987; Sangadala *et al.*, 1994). There are also, many hypotheses to explain the real mode of action of these toxins. Cyt toxins also cause midgut lysis, although their primary affinity appears to be for lipids in the microvillar membrane (Li *et al.*, 1996; Thomas and Ellar, 1983).

The toxicity of *Bt* crystal inclusions follows, after ingestion by insect, a complex process including multiple steps. These include the (i) solubilization of crystal to release the Cry proteins in their protoxin forms, (ii) activation of the protoxins by midgut proteases to their active form, (iii) binding of the toxin to a midgut receptor, and (iv) pore formation. Insects that become resistant to *Bt* do so by altering one or more steps of this process. Resistance to *Bt* was first reported in *Plodia interpunctella* (McGaughey, 1985), and it was subsequently described in other insect species that have developed resistance to one or more Cry proteins. Thus, resistance to *Bt* was found in field populations of *Plutella xylostella* and in laboratory-selected strains of *Heliothis virescens*, *Spodoptera exigua*, *Trichoplusia ni* and other insect species (Van Rie and Ferré, 2000).

Isolates of *Bt* toxic to lepidopteran insects have been known for almost 100 years and have been in commercial use for more than four decades. These bacterial insecticides, with appropriate formulations, and insecticidal transgenic plants are considered by many entomologists and growers to be selective, environmentally compatible technologies, especially in comparison to broad-spectrum chemical insecticides.

Numerous investigations have been published on the use of *Bt* for the control of lepidopteran insects, but the variability in results obtained in the field, and from that of the susceptible strains, has not been adequately explained. The literature on the susceptibility of various *Spodoptera* spp. in Egypt and elsewhere are very confusing, but they generally showed a weak sensitivity in varying degrees to different *Bt* strains (Cry toxins) (Gonvindarajan *et al.*, 1975). Several explanations were cut forward attributing this weak sensitivity to the presence of antibacterial substances in the gut, low pH, low proteolytic activity and low phenol and ascorbic acid content in the gut (Narayanan *et al.*, 1976). These phenomena have been explored in details later but between early and advanced larval instars in the same strain. Where, part of the present studies was carried out in order to provide quantitative and conclusive data regarding the susceptibility of the cotton leafworm to δ-endotoxins of different *Bt* subspecies and trials for comparison between different larval instars (Keller *et al.*, 1995).

The toxicity of *entomocidus* (60.5) Cry-toxin was more than that of *kuristaki*-HD-1 Cry-toxin against all tested larval instars (Table 1 and 2). These data show some harmony with the

others previously reported (Hofte and Whitely, 1989; Keller *et al.*, 1995) indicating that *S. littoralis* larvae are susceptible to CrylC, and Cry1E. CrylC toxin is produced by *B. thuringiensis* vars. *entomocidus*, *aizawai* (Sneh *et al.*, 1981; Visser *et al.*, 1988). CrylE is produced by *B. thuringiensis* vars. *kenyae* and *dendrolimus* (Van Rie *et al.*, 1990). Sneh *et al.* (1981) reported that among crystals of 14 *Bt* strains, the crystal of *Bt* subsp. *entomocidus* are the most potent against *S. littoralis* since there is strong correlation between toxicity of Cry1C derived from *Bt* subsp. *entomocidus* and binding to brush border membrane vesicles of *S. littoralis*. In another situation, larvae of *Spodoptera* species are tolerant to most of the Cry proteins. More over, a screening trial of the larvicidal activity of more than 900 strains of *Bt* against *S. littoralis* showed that only a few belonging mainly to subspecies *aizawai*, *kenyae* and *entomocidus* exhibited high level of toxicity (Kalfon and DeNarjac, 1985; Sneh *et al.*, 1981). In another study, there is an evidence that mixtures of insecticidal proteins (HD1; a mixture of Cry IA(a), IA(b), IIA and IIB) may be more toxic than the individual proteins (Broza *et al.*, 1984). This confirms the high potency of *Bt* var *entomocidus* on the *S. littoralis* larvae than *Bt* var *kuristaki* HD-1 Cry toxin.

During spore formation *Bt* synthesize large amount of certain proteins which coalesce to form inclusions termed crystal proteins. *Bt* cultures containing crystal proteins are often highly toxic to lepidopteran insect larvae. Purified crystal protein alone is often toxic and the addition of *Bt* spores to purified crystal protein may increase insecticidal activity, a phenomena referred to as spore effect. It has been recognized for sometime that *Bt* spores can contribute to the killing of insect larvae (Somerville *et al.*, 1970). For example, the toxicity of purified crystal protein was increased 10-fold against *Plutella xylostellla* (Liu *et al.*, 1998), 10-fold for *Spodoptera exigua* (Moar *et al.*, 1995), 35-fold against *Plodia interpunctella* (Johnson *et al.*, 1998), and 1000-fold against *Galleria mollonella* (Li *et al.*, 1987) by the addition of purified *Bt* spores. The spore effect has been attributed, in part, to the presence of crystal toxin-like proteins within the *Bt* spore coat (Du and Nickerson, 1996; Johnson *et al.*, 1998).

In addition, there are another *Bt* insecticidal proteins expressed during *Bt* growth stage, have been identified, the vegetative insecticidal proteins (Estruch *et al.*, 1996; Estruch and Yu, 2001; Selvapandiyan *et al.*, 2001; Yu et al., 1997). The *Bt vip3A* gene (vegetative insecticidal protein) encodes a protein of 88 KDa (Vip3A) that is secreted into the extracellular environment by *Bt* cells during vegetative growth and during stationary phase (Estruch *et al.*, 1996). Vip3A represents a new type of insecticidal protein because it is secreted as a soluble protein, rather than forming crystal inclusion inside the *Bt* mother cell, and because Vip3A does not share sequence homology with *Bt* crystal proteins (Donovan *et al.*, 2001). Interestingly, Vip1A shows sequence similarity to the protective antigen of the tripartite *Bacillus anthracis* toxin (Petosa *et al.*, 1997). The Vips have shown a broad insecticidal spectrum, including activity toward a wide variety of lepidopteran and also coleopteran pests (Estruch *et al.*, 1996; Estruch and Yu, 2001; Selvapandiyan *et al.*, 2001; Yu *et al.*, 1997). Furthermore, it has a lack cross-resistance with *B. thuringiensis* Cry toxins (Fang *et al.*, 2007). Of the various Vip proteins that have been reported, the Vip3Aa has been characterized most extensively. Vip3Aa kills susceptible larvae by binding to specific receptors on midgut cell membranes (Lee *et al.*, 2006), resulting in pore formation (Lee *et al.*, 2003) and cell lysis (Yu *et al.*, 1997). At least 27 vip3Aa gene sequences have been reported (Milne *et al.*, 2008).

Bt kurstaki HD-1 was grown to late vegetative phase in Terrific broth media and proteins in the culture supernatants harvested by ammonium sulfate precipitation. Supernatant proteins were analyzed by SDS-PAGE which showed that HD-1 produced apparently identical arrays of

soluble proteins. Vip3A-sized protein is produced by HD-1 (Fig. 4.3A and C). Donovan *et al.* (2001) found that Vip3A protein which was produced by HD-1 and was not produced by HD-1Δ*vip3A* (the deleted version of *vip3A*) where the Δ*vip3A* allele consists of an internal deletion within the coding region of *vip3A*. The evidence supports that the differences in the insecticidal activities of HD-1 and HD-1Δ*vip3A* culture should be due to the Vip3A protein. The latest protein exhibited LC_{50} values of 453.18, 1118.99, and 3322.48 ppm for 2nd, 3rd, and 4th *S. littoralis* instar larvae, respectively. Thus, Vip3A or a related protein may be a component of the lepidopteran toxicity of a variety of *Bt* strains, although this remain to need more studies.

Yu *et al.* (1997) also reported that The Vip3A protein is a member of a newly discovered class of vegetative insecticidal proteins with activity against a broad spectrum of lepidopteran insects. Histopathological observations indicate that Vip3A ingestion by susceptible insects such as the black cutworm (*Agrotis ipsilon*) and fall armyworm (*S. frugiperda*) causes gut paralysis at concentrations as low as 4 ng/cm^2 of diet and complete lysis of gut epithelium cells resulting in larval death at concentrations above 40 ng/cm^2. The European corn borer (*Ostrinia nubilalis*), a nonsusceptible insect, does not develop any pathology upon ingesting Vip3A. While proteolytic processing of the Vip3A protein by midgut fluids obtained from susceptible and nonsusceptible insects is comparable, *in vivo* immunolocalization studies show that Vip3A binding is restricted to gut cells of susceptible insects. Therefore, the insect host range for Vip3A seems to be determined by its ability to bind gut cells. These results indicate that midgut epithelium cells of susceptible insects are the primary target for the Vip3A insecticidal protein and that their subsequent lysis is the primary mechanism of lethality. Disruption of gut cells appears to be the strategy adopted by the most effective insecticidal proteins.

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الملخص العربى

فعالية السم الخضرى Vip3A مقارنة بالسموم المتبلورة Crys لبكتيريا الباسيللس ثيورنجنسيس على دودة ورق القطن

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أجريت قياسات الحساسية على يرقات دودة ورق القطن لسلالتين من بكتيريا الباسليس ثيورنجنسيس Kuristaki و Entomocidus, وأن السم الأولى≈130 كيلو دالتون والسم النشط≈60.5 فى الراسب وبعد التعرض للموجات فوق الصوتية والسم الخضرى≈88 كيلو دالتون أمكن التعرف عليهم فى بيئة النمو والتأكد منهم عن طريق الفصل الكهربى للبروتين. قيم الـ LC_{50} تم حسابها من خلال Lc-p lines وإستخراج القيم الإحصائية (Slope & Chi^2) وأن كل القيم والمعايير تم تقديرها عند فترات تعرض مختلفة. عموما السم الخضرى Vip3A أكثر فعالية على دودة ورق القطن من السموم المتبلورة Crys الناتجة من السلالتين وأن السمية معتمدة على العمر اليرقى.