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Characterization of *cry2A*-type Gene(s) from Pakistani Isolates of *Bacillus thuringiensis* Toxic to Lepidopteran and Dipteran Insects

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Abstract.- *Bacillus thuringiensis* (*Bt*) δ-endotoxin crystal (Cry) proteins are known to have toxicity against a variety of insects and have been exploited to control insect pests through transgenic plants and biopesticides. Genotyping of 56 local *Bt* isolates revealed 11 strains harboring *cry2* gene(s). LC_{50} values of these *cry2* positive strains ranged from 62.5 µg/ml to 775 µg/ml for *Helicoverpa armigera* and 124 µg/g to 1172 µg/g for *Musca domestica*. Subtyping of these isolates showed multiple *cry2A*-type genes in most of the isolates. Primer pairs were designed to amplify the full length *cry 2A* genes. The PCR products (1.9 kb) were cloned in pTZ57R/T vector, sequenced and submitted to EMBL DNA database. Eight new toxin genes *viz., cry2Ab11, cry2Ac7, cry2Ac8, cry2Ac9, cry2Ac11, cry2Ac12, cry2Ad3,* and c*ry2Ad4,* have been added to the *Bt* toxins database. These *Bt* isolates and Cry2A-type toxins can provide better control of insects either in the form of *Bt*-formulation or when the *cry2A*-type genes are expressed in transgenic plants.

Keywords: *cry2Ab* gene, *cry2Ac* gene, *cry2Ad* gene, *Helicoverpa armigera, Musca domestica, Bt* genes, *Bacillus thuringiensis,* biopesticide.

INTRODUCTION

Bacillus thuringiensis (Bt) is an aerobic, Gram-positive, endospore-forming soil bacterium. It produces a parasporal inclusion bodies called insecticidal crystal protein (Cry and Cyt proteins) or δ-endotoxin during sporulation. These proteins are toxic to dipteran, lepidopteran and coleopteran insect larvae (Johnson *et al*., 1998). They are also toxic to some hymenopteran, homopteran and mallophaga insects, as well as to many nematodes, flat worms, and Sarcomastigophora (Horak *et al*., 1996). Despite the actual or presumed presence of various pathogenicity factors, *Bt* does not have a significant history of mammalian pathogenicity (deMaagd *et al*., 2001).

More than 250 Cry proteins based on *cry* gene nucleotide sequences and amino acid homologies have been described (Ben-Dov *et al*., 1997; Crickmore *et al*., 1998). The genes encoding Cry proteins are found (often clustered) on transmissible plasmids and flanking transposable elements, which explains their easy spread within the species (Ben-Dov *et al*., 1996, 2001; Schnepf *et al*., 1998). Conjugation between different strains has been observed in soil environment as well as within the insects (Thomas *et al*., 2001). Individual Cry toxins have a defined spectrum of insecticidal activity, usually restricted to a few species within one particular order of insects. A few toxins have an activity spectrum that spans two or three insect orders – most notably Cry1Ba, which is active against larvae of moths, flies and beetles (Zhong *et al*., 2000), and Cry2Aa which is toxic to dipteran as well as lepidopteran insects (Winder and Whiteley, 1989).

cry2A gene encodes approximately 65-kDa protein, which forms cuboidal crystals (Winder and Whiteley, 1989; Yamamoto and McLaughlin, 1981). Three *cry2A* genes *viz.*, *cry2Aa* (Winder and Whiteley, 1989; Donovan *et al*., 1988), *cry2Ab* (Winder and Whiteley, 1989; Dankocsik *et al*., 1990), *cry2Ac* (Wu *et al*., 1991), *cry2Ad*, *cry2Ae* and *cry2Af* have been reported. Cry2Aa is toxic to lepidopteran and dipteran larvae, while Cry2Ab and Cry2Ac are toxic only to lepidopteran species. *cry2Aa* and *cry2Ac* genes have a common characteristic in that these are placed at third position in a three-gene operon. However, the crystallization of cry2Aa protein requires the second gene, *orf2,* in the *cry2Aa* operon (Crickmore and Ellar, 1992), while the two *orf*s upstream of *cry2Ac* gene do not have any role in the formation of Cry2Ac inclusions (Wu *et al*., 1991). On the other hand, *cry2Ab* gene is cryptic (Winder and Whiteley,

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1989; Dankocsik *et al*., 1990; Crickmore *et al*., 1994). Dankocsik *et al.* (1990) found Cry2Ab protein to be highly toxic to *Lymantria dispar, Heliothis virescens* and *Trichoplusia ni,* but was not toxic to *Aedes aegypti.* Kota *et al*. (1999) have demonstrated that over-expression of the *Bt* Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and *Bt*-resistant *Heliothis virescens*. Not much is known about other Cry2A–type toxins regarding their insecticidal activities.

Besides their long-term use as a biological insecticide in the form of sprays of spore–crystal mixtures, individual Cry toxins have been expressed in transgenic plants to render crops resistant to insect pests. Since Cry1-type toxins have extensively been used in transgenic plants there are reports that insects have developed resistance against some of these toxins. Akhurst *et al*. (2003) have reported resistance of pests against *Bt*-cotton expressing Cry1Ac. In Pakistan Maqbool *et al*. (1998) had generated transgenic rice *indica* expressing *cry2A* gene which was reported to be effective against two major rice pests in the Indian subcontinent - yellow stem borer and the rice leaf folder. Later Zaidi (2005) produced a transgenic tobacco plant, *Nicotiana tabacum* with *cry2A* to protect it against *Heliothis virescens*.

However, information about the distribution of *cry* genes is still limited and does not cover many distinct geographic areas. There is, therefore, a need to search for novel and more potent strains with new pathogenic spectra and wider host ranges, especially in parts of the world that have not yet been adequately sampled. Pakistan constitutes one such area which needs to be explored for crystalliferous strains with wider host ranges in this region. The present report characterizes *cry2A*-type gene(s) from these strains, and evaluates the toxicity of Cry2A proteins against the target insects, *Helicoverpa armigera* and *Musca domestica.*

MATERIALS AND METHODS

Bacterial isolates

Fifty six local *Bacillus thuringiensis* (*Bt*) isolates were used in this study, of which fifty were obtained from the bacterial culture isolated and stocked in the School of Biological Sciences, University of the Punjab, Lahore (SBS *Bt*1-50) by the authors, while six were obtained from second author's collection stocked in Cell and Molecular Biology Lab. (CMBL), Department of Zoology, University of the Punjab, Lahore (CMBL *Bt*1-6). Positive control HD29 was a generous gift from Professor David J. Ellar.

Biochemical characterization and ribotyping of the isolates

The *Bt* isolates were characterized by Gram staining, endospore position and various biochemical tests such as gas and acetoin production from glucose, phenylalanine deamination, nitrate reduction, tyrosine decomposition, starch hydrolysis etc. according to Bergey's Manual of Determinative Bacteriology (Bergey, 1974). Ribotyping was done to finally confirm the *Bt* species. The full length 16S rDNA (1692 bp) gene was amplified on Applied BioSystems 2720 thermal cycler using PCR reaction mixture (50µl) containing *Taq* buffer 1x, $MgCl_2$ 1.5 mM, dNTP's 200 µM, each of the following primers described by Sacchi *et al*. (2002) 50 pmol, DNA 0.5 µg, and *Taq* DNA polymerase 2.5 Units.

67F 5' TGAAAACTGAACGAAACAAAC 3' and
1671R 5' CTCTCAAAACTGAACAAAACGAAA 3'

The reaction cycle consisted of pre-PCR heating at 94ºC for 5 min, final extension at 72ºC for 10 min, and 30 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 45 sec, extension at 72ºC for 2 min. The genes were cloned in pTZ57R/T vector. Confirmation of clones was done by digesting with *Eco*RI and *Hin*dIII enzymes which would yield two fragments (*i.e.,* 1 kb and 0.7 kb) of the 1.69kb insert. Both strands of PCR product were sequenced and submitted to the EMBL DNA database (http://www.ebi.ac.uk/embl/Submission/webin.html).

The 16SrDNA nucleotide sequences of the *Bt* isolates were compared with the reference sequence (AY 138920) reported by Sacchi *et al.* (2002) and the others in the literature (database) and percent homologies determined.

Genotyping of Bt *isolates for* cry *gene and subtypes of* cry2A *genes*

For genotyping of *Bt* isolates for *cry 1*, *cry2* and *cry 4* genes, the following universal set of primers as described by Ben-Dov *et al*. (1997) were used.

cry1
Un1(d), 5'-CATGATTCATGCGGCAGATAAAC-3'
Un1(r), 5'-TTGTGACACTTCTGCTTCCCATT-3'

cry2
Un2(d), 5'-GTTATTCTTAATGCAGATGAATGGG-3'
Un2(r), 5'-CGGATAAAATAATCTGGGAAATAGT-3'

cry4
Un4(d), 5'-GCATATGATGTAGCGAAACAAGCC-3'
Un4(r), 5'-GCGTGACATACCCATTTCCAGGTCC-3'

The *cry1Ac* detection and subtyping of *cry2A*-type genes was done using following primer pairs designed by Alberola *et al.* (1999).

1AcF 5'GTATGCTTCTGTAACCCCGATTCACCTC3'
1AcR5'CCTGCAGTCCCACTAAAATTTCTAACACCTACTA3

2AaF5'GGATATTGAGTGAATTATGGGGGATA3'
2AaR5'CCGCTATAATTAACCCTGGCACTATTCAATGA3

2AbF 5'CACAGCAGACCCAATCATTTACTTCACAAGA3'
2AbR 5'CTGTAAAAGCACCACTCCTTAACCCTAAA3'

2AcF5'GGAGTGTCATCTAGCCGCATAGGTCAAG3',
2AcR 5'ACCATAATATTCATAAGCTCAAATTGTGGATTG3'

2AdF 5'ATGAATACTGTATTGAATAACGGAAG3'
2AdR 5'CCTTAATAAAGTGGTGGAAGATTAG3'

The PCR reaction mixture (50µl) and the reaction cycles were the same as used above.

Total DNA was isolated according to Kronstad *et al*. (1983). Briefly cells grown in 500 ml of Spizizen medium [$(NH_4)_2SO_4$ 2 g, KH_2PO_4 6 g, sodium citrate.$2H_2O$ 1 g, $MgSO_4.7H_2O$ 0.2 g, glucose 0.5%, $K_2HPO_4.3H_2O$ 18.3 g, tryptone 20 g, yeast extract 5 g dissolved per liter of water] in a 2-liter flask with shaking at 37°C were harvested at an optical density of 0.7 at 600 nm. The cultures were harvested by centrifugation at 6,000 rpm (4,355 rcf) for 10 min at 4°C in a Beckman centrifuge, washed with 100 ml of a solution containing 100 mM NaCl, 10 mM Tris pH 7.9 and 10 mM EDTA. The bacterial pellet was resuspended in 5 ml of a solution containing 150 mM NaCl and 100 mM EDTA at pH 7.9. Lysozyme was added to give a final concentration of 0.25 mg/ml, and the preparation was incubated at 37°C for 20 min. To lyse the cells, 6.25 ml of a third solution (100 mM Tris pH 7.9, 100 mM NaCl, 2% SDS) was added. The preparation was mixed gently by inverting the tube four or five times and incubated at 60°C for 30 - 45 min until clear. The lysate was then extracted four times with phenol-chloroform (1:1) which had been equilibrated with the above Tris-NaCl-SDS solution. The aqueous phase was extracted each time with a wide-bore pipette. After the final extraction, cold ethanol was added and the DNA was spooled out with a glass rod. The DNA was then rinsed with 70% ethanol, dissolved in TE buffer (10 mM Tris pH 7.9, 1 mM EDTA) and stored in aliquots at -20°C.

Oligonucleotides were synthesized by Gene Link, USA. Restriction enzymes and materials used in gene cloning were obtained from Fermentas Life Sciences, EU.

Amplification and sequencing of cry2A full length gene and its subtypes

To amplify full length *cry2A* gene from local *Bt* isolates, the following primers were specially designed from pre-existing sequences of *cry2A* genes from DNA databases.

Cry2AcT F 5'ATGAATACTGTATTGAATAACGGAAG3' and
Cry2AcT R 5' CCTTAATAAAGTGGTGGAAGATTAG3'

Gene(s) of interest were amplified through PCR. The PCR reaction mixture and the reaction cycle were the same as explained above.

Gene clean and T/A cloning were done according to manufacturer's instructions (Fermentas Life Sciences, EU). DNA sequencing was performed on Beckman-Coulter CEQ 8000 DNA sequencer according to manufacturer's instructions.

cry *gene analysis and Clustal analysis*

Sequences of known Cry2A toxins were retrieved from *Bt* toxin database (http://www.lifesci.susx.ac.uk/home/Neil_Crickmor

e/Bt/). The amino acid sequences of toxins were aligned to see the level of homologies using ClustalW programme from DNA Databank of Japan (http://www.ddbj.nig.ac.jp/clustalW).

The toxin sequences were aligned using CLUSTAL X ver. 1.83 software (Thompson *et al*., 1997) and phylogenetic tree was produced using TreeView programme.

Bioassays using Bt *spore suspension*

For determining toxicity of *Bt* isolates, a 10% suspension of the *Bt* spores was prepared in the autoclaved distilled water and used against neonate larvae of *Helicoverpa armigera* and *Musca domestica*. For preparation of *Bt* spores, *Bt* strains were grown on nutrient agar plates at 30ºC for 96 hours, scrapped off in 0.5 M NaCl with the help of glass spreader, centrifuged at 6,000 rpm (4,468 rcf) for 10 min at 4ºC and the pellet was dried in a desiccator at 37ºC.

Bioassays with Helicoverpa armigera

Various volumes such as 50, 100, 200, 300 and 500 µl of Bt spore suspension (10%) were mixed in 3 ml artificial diet prepared according to Ahmed and McCaffery (1991) [90 g chick pea powder blended in 300 ml of distilled water for 7-10 min followed by addition of 3.75 ml of 10 % formaldehyde, 7.5 ml of 20 % choline chloride, vitamin mixture (ascorbic acid 2 g, sorbic acid 0.7 g, thiamin-HCl 0.1 g, folic acid 0.1 g, cholesterin 0.6 g, methyl 4-hydroxy benzoate 0.7 g), 12 g baking yeast , 10 g dissolved agar in 250 ml of distilled water and 1 ml wheat germ oil in succession and the mixture blended for 2-3 minutes after each addition] and left at room temperature for some time to adsorb the suspension. In negative control, spore suspension was not added. For egg laying, 3-6 pairs of moths were placed in a big jar inverted on a vial containing cotton immersed in 70% sucrose solution. Eggs were collected in plastic bags filled with air and incubated at 24±2°C. Neonate larvae, which hatched in 24 hours, were used in the bioassays. Eight neonate larvae were placed on the diet surface to allow them to feed *ad libitum*. The vials were covered with aluminum foil and incubated at 24±2ºC. Mortality was recorded after 72 hours. All experiments were done in triplicates.

Bioassays with Musca domestica

Different volumes such as 100, 200, 300, 500, 750, and 1000 µl of Bt spore suspension were mixed in artificial diet for *Musca domestica* (housefly) prepared according to Shakoori and Butt (1980) and left at room temperature for some time to adsorb the suspension. In negative control, spore suspension was not added. Sterile tissue papers were added to the opposite side of artificial diet slant in the bottles in such a way that the base of the tissue paper was moist. Fifty eggs of housefly were placed on the moist tissue paper on the surface of diet, in a jar covered with three layered cheese cloth and placed at 26±2ºC. The larvae hatched in 8 – 16 hours. Mortality was recorded by counting the number of alive flies in the jar, which took about after 10 – 13 days. All experiments were done in triplicates.

For egg laying, a paste of skimmed milk powder and sucrose (2:1) was made in autoclaved distilled water and placed in a glass container. On one side, moist sterile tissue paper was placed. About fifty flies (male and female almost 1:2) were shifted in the container, covered with three layered cheese cloth and placed at 26±2ºC for 8-12 hours. Off white, cylindrical eggs were collected from moist layers of the tissue paper and used in the bioassays.

RESULTS

Characterization and ribotyping of Bt *isolates*

Colonies of all the isolates showed typical *Bt* like appearance *viz.,* off-white, dry, smooth, rich and round with the exception of CMBL-*Bt*1 which made very small, colorless, mucoid colonies and SBS-*Bt*2 making gelatinous colonies. The full length 16S rDNA of 1,692 nucleotide base pairs has 69bp flanking region each at 5' and 3' end. The complete 16S rRNA gene sequences of local isolates and that of HD29 were aligned using ClustalW programme and nucleotide changes were encountered which are shown in Table I.

The reference sequence (accession number AY138290) differs from the HD29 sequence (accession number AM779003) at base position 72, where G has been replaced with T, and at positions 461 and 1345, where HD29 has T instead of C. In all the *Bt* isolates the various base positions where

deviations have been detected are shown in the Table I.

Genotyping of local Bt *isolates for cry genes*

All these isolates had multiple *cry* genes (Table II). PCR with universal set of primers indicated occurrence of *cry2* gene in 11 out of 56 local *Bt* isolates, showing thereby, 20% incidence. All CMBL *Bt* isolates had *cry1, cry2* and *cry4* genes, whereas all SBS *Bt* isolates had *cry2* genes but no *cry1* gene. Further sub-typing with gene specific primers for *cry2Aa, cry2Ab, cry2Ac* and *cry2Ad* revealed presence of multiple *cry2A* genes in most of the cases (Table II). Amongst CMBL strains, *cry2Ac* was found in 100% isolates, followed by *cry2Ab* (80%), *cry2Ad* (40%) and *cry2Aa* (20%) genes, whereas among SBS strains *cry2Ab* and *cry2Ac* genes were equally distributed, each found in 2 (SBS *Bt*1, SBS *Bt*6) out of 6 (33.33%) isolates, while *cry2Aa* gene was found in 1 (SBS *Bt*2) out of 6 (16.6%) isolates. None of the SBS isolates harbored *cry2Ad* gene.

cry2Ac gene was the most abundant, present in 7 (63.63%) isolates, followed by *cry2Ab* (54.54%), *cry2Ad* (18.18%) and *cry2Aa* (18.18%) genes (Table II). HD29 strain of *Bt* serovar *galleriae* harboring *cry2Ab* and *cry2Ac* genes was used as positive control.

Sequencing of cry2A*-type genes*

Eight full length *cry2A*-type genes *viz.*, *cry2Ab11* (1902 bp), *cry2Ac7* (1872 bp), *cry2Ac8* (1872 bp), *cry2Ac9* (1872 bp), *cry2Ac11* (1872 bp), *cry2Ac12* (1872 bp), *cry2Ad3* (1902 bp) and *cry2Ad4* (1902 bp) were amplified (Fig. 1) and cloned. The confirmation of clones was demonstrated by double digestion of clones with *Eco*RI and *Hin*dIII, which yielded two bands of 2kb (insert) and 3kb (vector) (Fig. 2). Seven of these genes *viz.* c*ry2Ac7, cry2Ac8, cry2Ac9, cry2Ac12, cry2Ab11, cry2Ad3, cry2Ad4* were isolated from local isolates (CMBL *Bt*1, SBS *Bt*1, CMBL *Bt*1, CMBL *Bt*2, CMBL *Bt*3, CMBL *Bt*5, and CMBL *Bt*2, respectively), whereas c*ry2Ac11* was isolated from standard *Bt* strain HD29. The sequences of the full length toxin genes were submitted to EMBL DNA database (http://www.lifesci.susx.ac.uk/home/Neil_Crickmore/Bt/) under the accession numbers AM691748 (*cry2Ab11*), AM292031 (*cry2Ac7*), AM421903 (*cry2Ac8*), AM421904 (*cry2Ac9*),

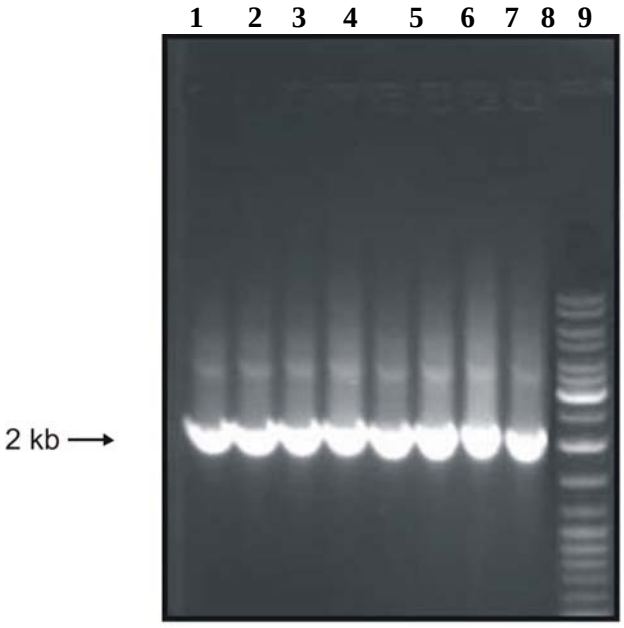

Fig. 1. PCR amplification of *cry2A* gene (2 kb) from strains HD29 (lanes 1 and 2), CMBL *Bt*1 (lane 3), CMBL *Bt*2 (lane 4), CMBL *Bt*3 (lane 5), CMBL *Bt*5 (lane 6), SBS *Bt*1 (lanes 7 and 8), and DNA ladder (lane 9).

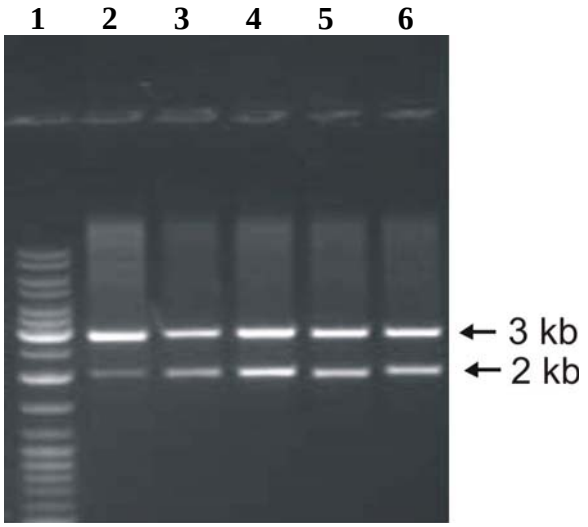

Fig. 2. DNA fragments of 2kb (insert) and 3kb (vector) produced by double restriction of T/A cloned *cry2A* gene with *Eco*RI and *Hin*dIII from CMBL *Bt*1 (lane 2), CMBL *Bt*2 (lane 3), CMBL *Bt*3 (lane 4), CMBL *Bt*5 (lane 5), SBS *Bt*1 (lane 6), and DNA ladder (lane 1).

Table I.- Base changes encountered in 16S rDNA sequences of locally isolated *Bt* isolates, CMBL *Bt*1-5, SBS *Bt*1-6, HD29 (accession number AM779003) and a reference sequence with accession number AY138290.

Sr. No.	Base Position	CMBL-*Bt*					SBS-*Bt*						Reference sequence
		1	2	3	4	5	1	2	3	4	5	6	HD29	
1	69	A	A	A	A	A	A	A	A	A	G	A	A	A
2	72	G	G	G	G	G	G	G	G	G	T	G	G	T
3	181	A	A	G	A	A	A	A	A	A	A	A	A	A
4	182	C	C	C	T	C	C	C	C	T	T	C	C	C
5	192	C	C	C	T	C	T	C	C	C	T	C	T	Y
6	208	G	G	G	G	G	G	G	G	G	A	G	G	G
7	260	T	A	T	T	T	T	T	T	T	T	T	T	T
8	461	C	C	C	C	C	C	C	C	C	C	C	T	C
9	476	T	T	T	T	T	T	T	T	T	T	C	T	T
10	701	T	T	T	T	T	T	C	T	T	T	T	T	T
11	714	G	G	G	G	G	G	G	G	G	A	G	G	G
12	768	A	A	G	A	A	A	A	A	A	A	A	A	A
13	994	C	C	C	C	C	T	C	C	C	C	C	C	C
14	1015	C	C	C	C	C	C	C	C	C	A	C	A	A
15	1034	C	C	C	C	C	C	T	C	C	C	C	C	C
16	1084	-	-	-	-	-	-	-	T	-	-	-	-	-
17	1147	T	T	T	T	T	T	T	T	T	A	T	A	A
18	1302	C	T	C	C	C	C	C	C	C	C	C	C	C
19	1345	C	C	C	C	C	C	C	C	C	C	C	T	C
20	1420	G	A	A	A	A	A	A	A	A	A	A	A	A
21	1464	T	T	-	T	T	-	T	-	T	T	T	T	T
22	1496	T	T	T	T	T	T	C	T	T	T	T	T	T
23	1529	A	A	A	A	A	A	A	G	A	A	A	A	A

Accession numbers of 16S rDNA sequences CMBL *Bt*1, AM292029; CMBL *Bt*2, AM778995; CMBL *Bt*3, AM778996; CMBL *Bt*4, AM778997; CMBL *Bt*5, AM292032; SBS *Bt*1, AM778998; SBS *Bt*2, AM292033; SBS *Bt*3, AM778999; SBS *Bt*4, AM779000; SBS *Bt*5, AM779001; SBS *Bt*6, AM779002

Table II.- *cry* gene profiles of locally isolated *Bt* strains harboring *cry2* gene.

Sr. No.	Catalogue No.	PCR based *cry* gene detection							
		cry1	*cry1Ac*	*cry2*	*cry2A*				*cry4*
					a	*b*	*c*	*d*	
1	HD29	+	-	+	-	+	+	-	-
2	CMBL *Bt*1	+	-	+	+	+	+	-	+
3	CMBL *Bt*2	+	-	+	-	+	+	+	+
4	CMBL *Bt*3	+	-	+	-	+	+	-	+
5	CMBL *Bt*4	+	-	+	-	-	+	-	+
6	CMBL *Bt*5	+	-	+	-	+	+	+	+
7	SBS *Bt*1	-	-	+	-	+	+	-	-
8	SBS *Bt*2	-	-	+	+	-	-	-	+
9	SBS *Bt*3	-	-	+	-	-	-	-	-
10	SBS *Bt*4	-	-	+	-	-	-	-	+
11	SBS *Bt*5	-	-	+	-	-	-	-	+
12	SBS *Bt*6	-	-	+	-	+	+	-	+

AM689531 (*cry2Ac11*), AM689532 (*cry2Ac12*), AM268418 (*cry2Ad3*) and AM490199 (*cry2Ad4*).

A consensus tree based on full length sequences of amino acids of all Cry2-type toxins was generated from 100 bootstraps (Fig. 3). The tree was rooted against an amino acid sequence of type

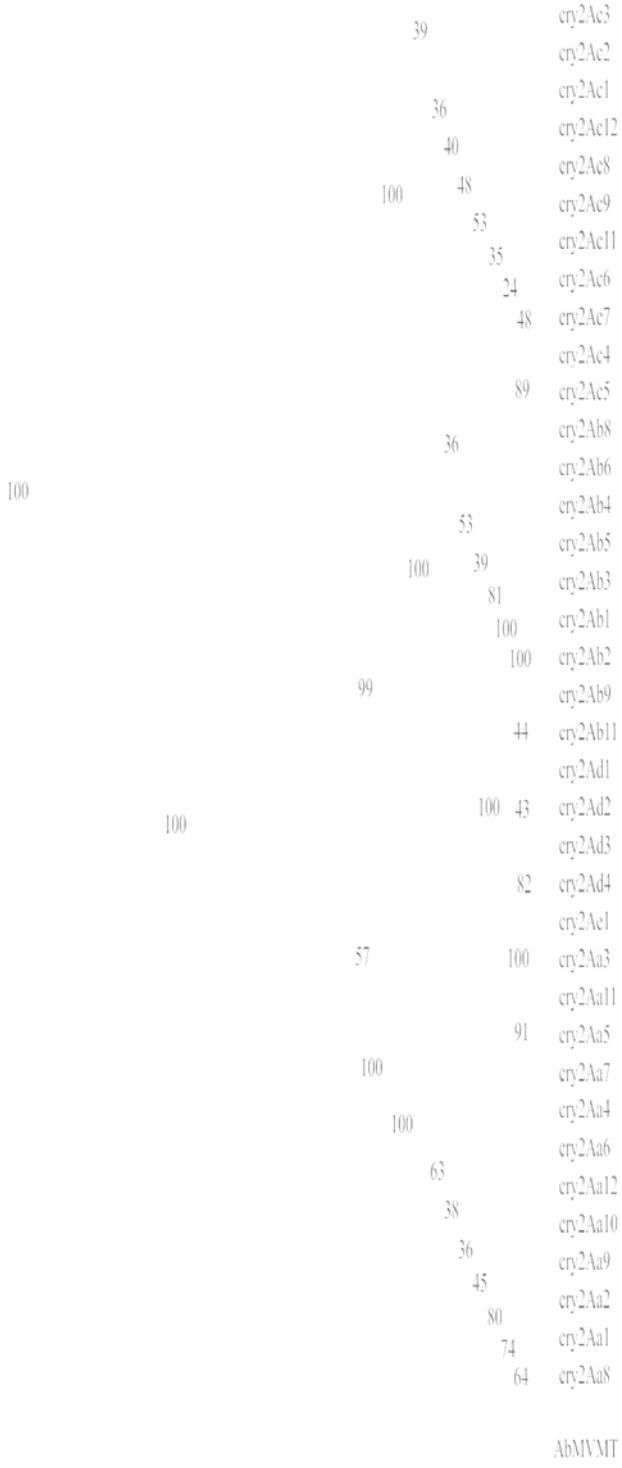

Fig. 3. Phylogram demonstrating amino acid sequence identity among Cry 2A-type toxins. Toxins reported in the present study are highlighted. The Tree is rooted against AbMVMT sequence.

toxins of Cry2-type toxins (Cry2Aa, Cry2Ab, Cry2Ac, Cry2Ad and Cry2Ae) clustered separately in the tree. Cry2Ac produced two subgroups. Cry2Ac1, Cry2Ac2, Cry2Ac3, Cry2Ac6, Cry2Ac7, Cry2Ac8, Cry2Ac9, Cry2Ac11 and Cry2Ac12 produced a subcluster distinct from Cry2Ac4 and AbMVMT (a distinct viral sequence). The sequences of the present study are shown in rectangular boxes (Fig. 3). It is evident that the sub Cry2Ac5. Cry2Ac1 and Cry2Ac3 are isolates from USA, Cry2Ac2, Cry2Ac4, Cry2Ac5 and Cry2Ac6 are isolates from China, while Cry2Ac7, Cry2Ac8, Cry2Ac9 and Cry2Ac12 are isolates from Pakistan (present study data in rectangular boxes). Cry2Ac4 and Cry2Ac5 are isolates from China but distinct from other Chinese Cry2Ac2 and Cry2Ac6 which may be due to earlier divergence during the course of evolution.

Cry2Ab bifurcate in two groups originating from USA (Cry2Ab1 and Cry2Ab2) and from China (Cry2Ab3, 4, 5, 6 and Cry2Ad8) produce a distinct group from Cry2Ab 9 (China origin) and Cry2Ab11 (Pakistan origin).

Cry2Ad has two subclusters, one cluster with Cry2Ad1 (Korean origin) and Cry2Ad2 (China origin) toxins, while other cluster has Cry2Ad3 and Cry2Ad4 (Pakistan origin). The only available amino acid sequence of Cry2Ae occupies an independent position in the tree. All Cry2Aa type toxins cluster together.

It is evident from the tree that each of the Cry2 toxins of present study has close amino acid sequence identities and grouped together. While Cry2 toxin isolated from China showed variation and produce two clusters. Cry2Ac2 and Cry2Ac6 formed a separate cluster from Cry2Ac4 and Cry2Ac5. Similarly Cry2Ab3, 4, 5, 6 and Cry2Ab8 produce a different group from Cry2Ab9.

Tables III and IV refer to amino acid variations encountered in Domain I, II and III of Cry2Ac and Cry2Ad toxins, respectively. On the whole, amino acids are varied on 45 different positions in Cry2Ac-type toxins and on 11 different positions in Cry2Ad-type toxins. These variations are extended over all the three domains of the toxins in both cases. Interestingly in conserved Block 1 of all Cry2Ac as well as Cry2Ad toxins, Leu (hydrophobic) at 183 position replaces Met. In all

Table III.- Differences in amino acid composition of subtypes of Cry2Ac toxins from local *Bt* isolates.

Sr. No.	Amino acid position	Cry2Ac										
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**	**12**
1	3	T	T	T	**S**	**N**	T	T	T	T	T	T
2	7	N	N	N	**S**	**S**	N	N	N	N	N	N
3	42	K	K	K	K	**R**	K	K	K	K	K	K
	Domain I											
4	84	S	S	S	S	S	S	**G**	S	S	S	S
5	137	P	P	P	P	**L**	P	P	P	P	P	P
6	156	L	L	L	L	L	L	L	L	L	**S**	L
7	167	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	**R**
8	183	**F**	L	L	L	L	L	L	L	L	L	L
9	184	**N**	H	H	H	H	H	H	H	H	H	H
10	190	**G**	D	D	D	D	D	D	D	D	D	D
11	214	**K**	N	N	N	N	N	N	N	N	N	N
12	216	**H**	T	T	T	T	T	T	T	T	T	T
13	218	D	D	D	**N**	D	D	D	D	D	D	D
14	225	N	N	N	N	N	N	N	**D**	N	N	N
15	226	**P**	T	T	T	T	T	T	T	T	T	T
16	236	**H**	T	T	T	T	T	T	T	T	T	T
17	239	**P**	H	H	H	H	H	H	H	H	H	H
	Domain II											
18	279	G	G	G	G	G	G	G	G	G	G	**S**
19	326	**-**	G	G	G	G	G	G	G	G	G	G
20	327	**V**	S	S	S	S	S	S	S	S	S	S
21	328	**Y**	T	T	T	T	T	T	T	T	T	T
22	329	**H**	T	T	T	T	T	T	T	T	T	T
23	330	**N**	T	T	T	T	T	T	T	T	T	T
24	331	**S**	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
25	335	F	F	F	F	F	F	F	F	F	F	**V**
26	440	H	H	H	**Y**	H	H	H	H	H	H	H
27	442	N	N	N	N	N	N	N	N	N	**I**	N
28	457	T	**P**	T	T	T	T	T	T	T	T	T
29	467	D	**A**	D	D	D	D	D	D	D	D	D
	Domain III											
30	521	**P**	T	T	T	T	T	T	T	T	T	T
31	525	Y	Y	Y	Y	**H**	Y	Y	Y	Y	Y	Y
32	603	G	G	**D**	G	G	G	G	G	G	G	G
33	609	L	L	L	L	L	L	L	L	**F**	L	L
34	614	F	F	**L**	F	F	F	F	F	F	F	F
35	615	V	V	**F**	V	V	V	V	V	V	V	V
36	616	P	P	**Q**	P	P	P	P	P	P	P	P
37	617	T	T	**L**	T	T	T	T	T	T	T	T
38	618	N	N	**I**	N	N	N	N	N	N	N	N
39	619	L	L	**F**	**I**	**I**	L	L	L	L	L	L
40	620	P	P	**H**	P	P	P	P	P	P	P	P
41	621	P	P	**H**	P	P	P	P	P	P	P	P
42	622	L	L	**F**	L	L	L	L	L	L	L	L
43	623	Y	Y	**I**	Y	Y	Y	Y	Y	Y	Y	Y
44	624	-	-	**K**	-	-	-	-	-	-	-	-
45	625	-	-	**V**	-	-	-	-	-	-	-	-

NCBI accession numbers of Cry2Ac1, X57252; 2, AY007687; 3, AAQ52385; 4, DQ361267; 5, DQ341379; 6, DQ359137; 7, AM292031; 8, AM421903; 9, AM421904; 11, AM689531; 12, AM689532.

* Bold letters show most different amino acids.

Table IV.- Differences in amino acid contents of Cry2Ad toxins of local *Bt* isolates.

Sr. No.	Amino acid position	Cry2Ad1 (AF200816)*	Cry2Ad2 (DQ358053)	Cry2Ad3 (AM268418)	Cry2Ad4 (AM490199)
1	3	S	S	**T****	**T**
2	7	S	S	N	**N**
3	10	**T**	N	N	N
4	45	N	N	**I**	N
	Domain I				
5	100	F	F	**L**	F
	Domain II				
6	354	S	**P**	S	S
7	370	S	S	**P**	S
8	376	S	S	**G**	S
9	400	T	T	**A**	T
	Domain III				
10	629	**I**	L	L	L
11	630	**S**	P	P	P

* NCBI accession numbers.
** Bold letters show most different amino acids.

Table V.- Expected and experimental toxicity of local *Bt* isolates harboring *cry2* gene against lepidopteran and dipteran larvae.

Sr. No.	Strain	Expected toxicity against	Toxicity assays with			
			H. armigera		*M. domestica*	
			LC_{50} (µg/ml)	Relevance factor (R^2)	LC_{50} (µg/g)	Relevance factor (R^2)
1	HD29	Lepidoptera, Diptera	62.5	0.3164	479	0.9433
2	CMBL *Bt*1	Lepidoptera, Diptera	327	0.7879	692	0.9549
3	CMBL *Bt*2	Lepidoptera, Diptera	62.5	0.8739	430	0.9305
4	CMBL *Bt*3	Lepidoptera, Diptera	62.5	0.9907	281	0.9409
5	CMBL *Bt*4	Lepidoptera, Diptera	62.5	-	621	0.9648
6	CMBL *Bt*5	Lepidoptera, Diptera	62.5	0.976	1172	0.9758
7	SBS *Bt*1	Lepidoptera	147	0.6885	124	0.8746
8	SBS *Bt*2	Lepidoptera, Diptera	775	0.9461	124	0.9122
9	SBS *Bt*3	Lepidoptera, Diptera	688	0.8063	407	0.9366
10	SBS *Bt*4	Lepidoptera, Diptera	585	0.951	124	0.9651
11	SBS *Bt*5	Lepidoptera, Diptera	82	0.7406	124	0.9417
12	SBS *Bt*6	Lepidoptera, Diptera	287	0.7467	560	0.8436

Cry2Ad toxins, Glu196Asp were observed in conserved Block 1, whereas Leu624Phe was observed in all possible variants of Block 5, as classified by Schnepf *et al.* (1998). In all Cry2Ac toxins however, Glu608Asp was consistently present in all the possible variants of Block 5 region. Each of Cry2Ac7, Cry2Ac8, Cry2Ac9, Cry2Ac11 and Cry2Ac12 contain at least one unique variation in amino acid sequence when analyzed against all other Cry2Ac-type toxin sequences *i.e.,* Cry2Ac7 Gly84Ser, Cry2Ac8 Asp225Asn, Cry2Ac9 Phe609Leu, Cry2Ac11 Ser156Leu and Ile442Asn, Cry2Ac12 Arg167Gln, Ser279Glu and Tyr335Phe (Table III). As even a single amino acid change can dramatically reduce stability of Cry proteins and hence can affect the toxicity of the toxin. Further

study is needed to analyze amino acid variation(s) which are crucial in assigning toxicity spectra to Cry2Ac-type toxins.

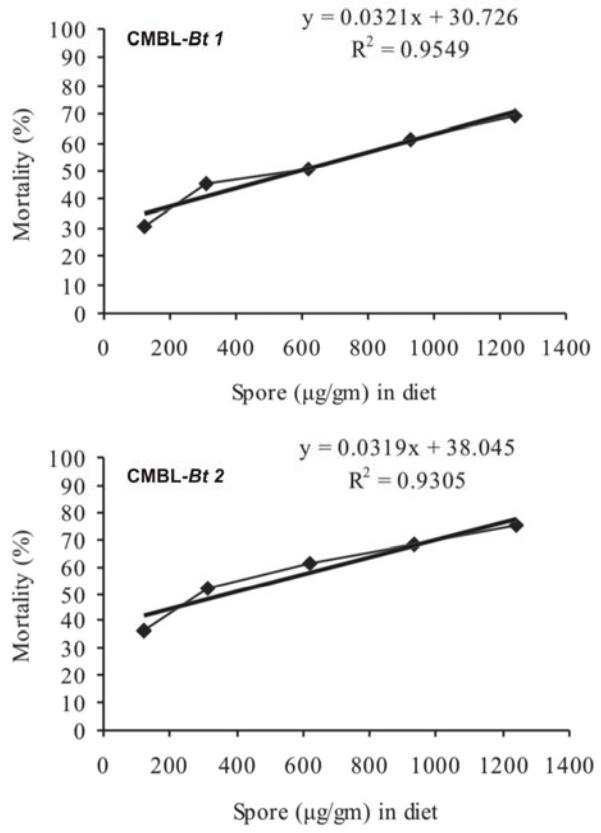

Fig. 4. Toxicities of *B. t.* strains CMBL *Bt*1 and CMBL *Bt*2 against *Musca domestica*. Graphs also show equation for Y-axis and R^2 value.

Bioassays with Helicoverpa armigera

Toxicity assays of sporulated *Bt* strains were performed with *Helicoverpa armigera* (American bollworms). Strain CMBL-*Bt*4 was the most toxic, causing 100% mortality at 62.5 µg/ml, while HD29 caused 91.56% mortality. Whereas amongst rest of the strains, CMBL *Bt*2, CMBL *Bt*3 and CMBL *Bt*5 were highly toxic having LC_{50} value of less than 62.5 µg/ml. SBS *Bt*5 and SBS *Bt*1 were more toxic with LC_{50} value of 82 and 147 µg/ml, respectively (Table V).

Bioassays with Musca domestica

Toxicity assays of sporulated *Bt* strains were performed with *Musca domestica.* Strains SBS *Bt*1, SBS *Bt*2, SBS *Bt*4 and SBS *Bt*5 were the most toxic having LC_{50} value less than 124 µg/g, while LC_{50} value for HD29 was 479 µg/g. CMBL *Bt*1 and CMBL *Bt*2 had LC_{50} value of 692 and 430 µg/g, respectively (Fig. 4). Amongst rest of the strains, CMBL *Bt*4 and CMBL *Bt*5 were the least toxic having LC_{50} values of 621 and 1172 µg/g, respectively (Table V).

DISCUSSION

cry *gene profile of local* Bt *isolate*

Genotyping of these Pakistani isolates reveals abundance of *cry*4-type genes (71.42%), followed by *cry*1-type (39.28%). *cry*2-type were the least abundant (19.64%) genes. *cry*1Ac was found only in 9.82% of isolates (Baig, 2007; Bukhari, 2007). Makhdoom (1998) had also reported that cry4 gene was the most abundant (59%) among 438 *Bt* isolates followed by *cry*1-type genes. These profiles are very different from those described from other parts of the world. Bravo *et al*. (1998) found that the cry1 genes were the most abundant, followed by the *cry*3, *cry*11, *cry*4, and *cyt* genes in the Mexican strain collection. Chak *et al*. (1994) reported that cry1A genes were the most abundant, followed by *cry*1C and *cry*1D genes in Taiwan. Święcicka and Mahillon (2005) have reported *Bt* harboring *cry*1 (30%) as the most frequent strain followed by *cry*2 (14%) and *cry*4 (14%) genes from among 103 isolates in Poland. In our collection *cry*1Ac constituted only 9.82% of the total isolates. Chak *et al*. (1994) have found that *cry*1Aa with *cry*1Ac, *cry*1Ac, and *cry*1C with cry1D are three major *cry* gene profiles.

Generally, insecticidal toxin genes of *Bt* reside on large plasmids, often as part of composite structures that include mobile genetic elements (Święcicka and Mahillon, 2005). Occurrence of identical *cry* gene profile among Pakistani isolates emphasizes the occurrence of plasmid transfer among natural *Bt* strains. The plasmids can be transferred by either conjugation or mobilization, which have been described as a frequent process among *Bt* in insect larvae and in soil under laboratory conditions (Jarrett and Stephenson, 1990; Thomas *et al*., 2000; Hu *et al*., 2004). *cry* gene

exchanges can thus occur in the environment, generating strains with new combinations of protein crystals that may enhance the pathogenicity of *Bt.*

Profile of cry*2A subtypes*

Cry2Aa protein is toxic to both lepidopteran and dipteran larvae, whereas Cry2Ab, Cry2Ac and Cry2Ad are only toxic to lepidopteran insects. It is of great interest to investigate the different *cry2* gene profiles of native *Bt* isolates in order to define their distribution, predict their insecticidal activity and detect novel genes or combinations thereof. CMBL *Bt*1 harbors *cry2Aa, cry2Ab* and *cry2Ac* genes, while CMBL *Bt*2 and CMBL *Bt*5 harbor *cry2Ab, cry2Ac* and *cry2Ad* genes (Table II). Both of them are novel combinations and being reported for the first time. On the other hand, each of CMBL *Bt*3, SBS *Bt*1 and SBS *Bt*6 harbors *cry2Ab* and *cry2Ac* genes. CMBL *Bt*4 harbors *cry2Ac* gene only, while SBS *Bt*2 contains *cry2Aa* gene only. SBS *Bt*3, SBS *Bt*4 and SBS *Bt*5 showed amplification with general primers for *cry2* gene, but did not show positive signals for *cry2* subtypes. These isolates need to be investigated further as they might harbor some novel cry2-type genes.

If combinations are neglected, frequency of individual *cry2Ac* genes is the highest (63.63%) among these Pakistani isolates, followed by *cry2Ab* (54.54%), *cry2Ad* (18.18%) and *cry2Aa* (18.18%) genes. *cry2Ab/Ac* combination is the most frequent (27.27%) followed by *cry2Ab/Ac/Ad* (18.18%), *cry2Aa/Ab/Ac* (9.09%), *cry2Aa* (9.09%) and *cry2Ac* (9.09%) alone. Prevalence of multiple *cry2A* genes in most of isolates reveals a close association of these genes. They might be part of the same operon or occur on the same plasmid. These results are very different from those reported before by Ben-Dov *et al*. (1997) and Sauka *et al*. (2005). Ben-Dov *et al*. (1997) could not find strains containing either *cry2Aa*, *cry2Ac*, or the two combinations between them with and without *cry2Ab*. While analyzing 61 *Bt* isolates from soil samples from Israel, Kazakhstan and Uzbekistan, they found highest frequency of *cry2Ab* alone (42.62%) followed by *cry2Aa/Ab* (34.42%) and *cry2Ab/Ac* (22.95%).

Sauka *et al*. (2005) have described distribution of *cry2* genes in 59 isolates, 94.9% of which had *cry2Aa/cry2Ab* profile irrespective of source. The *cry2Ab* gene alone was found in 2 *Bt* isolates (3.4%), while the *cry2Aa* gene was found in just one isolate (1.7%). They could not find any strain that harbors *cry2Ac*, *cry2Ad* or combinations with them. Sauka *et al*. (2005) have found that more than 90% of the *Bt* isolates harbored *cry2Aa/cry2Ab* genes which is not at all consistent with the *cry2* content of the isolates from our collection.

None of the isolates contains *cry2Ab* alone. Also occurrence of two other combinations *i.e. cry2Ab/Ac/Ad* and *cry2Aa/Ab/Ac* have never been reported before. It is possible that this combination of genes is common in this region, but the biological significance of this association has still to be studied. Also the presence of a *Bt* isolate with only the *cry2Ac* gene has never been found and this isolate should be further characterized.

Toxicity and cry *genes contents*

All these isolates reported here, except SBS-*Bt*1 and SBS-*Bt*3, carry multiple cry genes from *cry*1, *cry*2 and/or *cry*4 families, while rest of the >45 *cry* gene families have not been checked and are expected to be toxic to lepidopteran as well as dipteran insects on the basis of *cry* gene contents. However, PCR cannot distinguish between expressed and silent genes (Swiecicka and Mahillon, 2005). Therefore, isolates possessing different cry genes need to be characterized further by bioassay against insects from various orders. All the isolates have shown varying degree of toxicity against *Helicoverpa armigera* as well as *Musca domestica.* Only *Cry*2Aa2 and *Cry*1Ab3 have been reported toxic to *Helicoverpa armigera,* while none of the Cry protein has been reported toxic to *Musca domestica* in specificity database (http://www.glfc.forestry.ca/bacillus/BtResults.cfm).

There are a number of other factors, apart from Cry toxins, in *Bt* which show insecticidal activity or act as enhancers for Cry or Cyt toxins, which act by a different mechanism, are also found within the crystal. *Bt* produces various virulence factors other than the crystal proteins, including secreted insecticidal protein toxins, α-exotoxins, β-exotoxins, hemolysins, enterotoxins, chitinases and phospholipases (Hansen and Salamitou, 2000). The spore itself contributes to pathogenicity, often synergizing the activity of the crystal proteins

(Johnson *et al*., 1998). All of these factors might have a role in insect pathogenesis under natural conditions, helping the bacterium to develop in the dead or diseased insect larvae, but the exact contribution of each factor is often unknown (Schnepf *et al*., 1998).

Other factors are needed to be checked as well before making formulations for biological control of insect pests. Insects develop resistance against chemical as well as biological insecticides with the passage of time. In order to circumvent development of resistance against one bio-control agent, multiple bio-control agents like multiple *cry* genes could be tried.

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