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Identification of Some *Bacillus thuringiensis* Isolates by PCR and their Potential against the Cotton Leaf Worm

Marwa Azzam[1*] • Khaled Soliman[2] • Fatma Talkhan[1] • Jaime A. Teixeira da Silva[3] • Atef Abdel-Razek[4] • Hosni Aboassy[1]• Aly Abdelsalam[2]

[1] Genetics and Cytology Department, National Research Centre, 12311, Giza, Egypt
[2] Genetics Department, Ain Shams University, 11241, Cairo, Egypt
[3] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[4] Pests and Plant Protection Department, National Research Centre, 12311, Giza, Egypt

Corresponding author: * **marwa_azzam@yahoo.com**

ABSTRACT

Bacillus thuringiensis produces unique crystalline cytoplasmic inclusion bodies during the process of sporulation. Both spores and inclusion bodies are released upon lysis of the parent bacterium at the end of sporulation and if ingested these spores and crystals act as poisons for certain insects. Therefore, *B. thuringiensis* is referred to as a stomach poison. Twenty five isolates of *B. thuringiensis* were tested for the presence of both crystal protein by a microscopic examination and the *cryI (Aa)* gene using PCR. The microscopic examination revealed that nine of the 25 isolates had the bipyramodial shaped crystal protein, a distinguishing feature of *B. thuringiensis*, and which is active against insects of the order Lepidoptera. Detection of the *cryI (Aa)* gene with PCR, using specific primers CJI-1 and CJI-2, revealed that all nine isolates which have crystal protein, also had the *cryI (Aa)* gene. Screening of these isolates with the cotton leaf worm (*Spodoptera littoralis*, order Lepidoptera) showed that M_5, ATCF and M_8 isolates were the most toxic.

Keywords: *cryI (Aa) gene*, crystal protein, insect bioassay, *Spodoptera littoralis*

INTRODUCTION

Bacillus thuringiensis is a common Gram-positive motile aerobic entomopathogenic endospore-forming soil bacteria. The spores and crystals are active against larvae of Lepidopteran, Dipteran, Coleopteran (Beegle and Yamamoto 1992) and Hymenopteran (Feitelson 1993). Numerous laboratory studies have been conducted on the infectivity and toxicity of *B. thuringiensis* isolates and it was demonstrated that these isolates which are used in commercial products are safe (Siegel 2001). These studies have been validated by 40 years of *B. thuringiensis* usage in the field. *B. thuringiensis* has proven to be a valuable alternative to conventional insecticides; it is highly active and harmless to the environment owing to its specificity. Formulations of *B. thuringiensis* spore-crystal mixtures are commercially available for use as biological insecticides in agriculture and forestry. Over the last decade, PCR has been widely exploited to determine the *cry* genes (genes that encode for insecticidal crystal proteins) content of many *B. thuringiensis* strain collections. Ceron *et al*. (1995) described a PCR strategy designed to identify strains that harbor any of the known *cryI* or *cryIII* genes by using general primers for these genes selected from a region that is highly conserved in both genes. Strains with unique PCR product profiles can be easily characterized by performing additional PCR with specific primers that they described previously and the novel specific primers. Using this method, they identified 10 different *cryI* genes and five different *cryIII* genes. One feature of this screening method is that each *cry* gene should produce a PCR product having a unique molecular weight. Strains that produce products of different sizes probably contain novel genes. Ceron *et al*. (1995) also illustrated evidence which suggests that novel *cry* genes can be identified by the PCR method. Furthermore, Porcar and Juárez-Pérez (2003) compared the largest *B. thuringiensis* PCR-based screenings, and reviewed the natural occurrence of *cry* genes among native strains. They also discussed the use of PCR for the identification of novel *cry* genes, and they provided a considerable amount of data that they reviewed and analyzed. Furthermore, the future prospects of the prediction of toxicity of *B. thuringiensis* were discussed.

To identify novel *cry I*-type crystal protein genes, 100 *B. thuringiensis* isolates were selected on the basis of their toxicity against lepidopteran insect larvae. About 2.4 kb PCR fragments were amplified using universal oligonucleotide primers, ATG1-F and N400-R, designed to probe the toxic fragment regions of all known and possible *cryI*-type genes (Kim *et al*. 2005). Also, Gough *et al*. (2005) identified several isolates of *B. thuringiensis*, collected from various Australian soil samples, that produce crystals containing 130 and 28 kDa proteins to control sheep blowfly (*Lucilia cuprina*). They detected a novel cry protein and found that it was present in all the strains that were highly toxic in the larval assay. On the other hand, Martins *et al*. (2007) detected the *cryIB*, *cryIVA*, *cryIVB*, *cryX*, *cryXI*, *cytI* and *cytII* genes in five isolates of *B. thuringiensis* against the cotton boll weevil (*Anthonomus grandis*), the major cotton pest in the Americas. Furthermore, a *B. thuringiensis* strain exhibiting specific activity to locusts was isolated from a soil sample in China and characterized. The sequence analysis of a DNA fragment produced by PCR amplification with degenerate *cry*-selective primers revealed that the fragment encoded a δ-endotoxin segment (Song *et al*. 2008).

A large number of *B. thuringiensis* isolates have been obtained from soil samples in China. The flagellar antigen serotypes, *cry* genes and crystal proteins of 570 *B. thuringiensis* isolates were determined, and the pesticidal activity was assayed against the insects, *Plutella xylostella*, *Heliothis armigera*, *Phaedon brassicae* and *Locusta migratoria manilensis*, and the snail, *Oncomelania hupensis* (Gao *et al*. 2008).

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Table 1 Characteristics of *cryI (Aa)* genes specific primers used in PCR.

Primer pair	Sequence	Gene recognized	Positions	Product size (bp)	Accession no.
CJI-1	5'TGTAGAAGAGGAAGTCTATCCA	*cryIAa*	3263-3285	272	D17518
CJI-2	5'TATCGGTTTCTGGGAAGTA		3515-3534		

In Thailand, Thammasittirong and Attathom (2008) analyzed 134 isolates of *B. thuringiensis* obtained from different geographical and ecological origins to determine the distribution and diversity of *cryI*, *cryII* and *cryIX* genes encoding for Cry proteins toxic to lepidopteran insects. Seifinejad *et al*. (2008) characterized 70 *B. thuringiensis* strains isolated from different agro-ecological regions of Iran. Characterization was based on PCR analysis using 25 general and specific primers for *cryI*, *cryII*, *cryIX* and *vip3Aa* genes encoding proteins active against Lepidoptera, crystal morphology, plasmid profiles, and protein band patterns as well as their insecticidal activity on *Heliotis armigera*. Also, characterization of the strains containing Coleopteran-specific and putative novel *cry* genes in an Iranian native *B. thuringiensis* collection was mentioned. Characterization was based on PCR analysis using 31 general and specific primers for differnt *cry* genes, protein banding patterns as well as their insecticidal activity on *Xanthogaleruca luteola* Mull larvae (Nazarian *et al*. 2009).

Valicente *et al*. (2010) used a total of 165 *B. thuringiensis* strains that showed larval mortality above 75% and were selected from 4459 isolates previously evaluated with respect to *Spodoptera frugiperda* activity. Molecular characterization was performed based on PCR using specific *cryI* primers. Furthermore, Zhu *et al.* (2010) characterized a novel *B. thuringiensis* isolate native to China that showed a spherical crystal harboring two major proteins of about 70 and 130 kDa that contained three novel *cry* genes that exhibited larvicidal activity against *Aedes aegypti* (Diptera), *A. aegypti* and *P. xylostella* (Lepidoptera) as insecticidal activity tests showed.

The aim of this work was to identify some isolates of *B. thuringiensis* through microscopic examination, to examine the presence of crystal protein, and using PCR with specific primers, to detect the presence of *cryI(Aa)* that has a specific range of activity against different lepidopteran insects. Moreover, a test of the pathogenicity and potential of these isolates against the cotton leaf worm, *Spodoptera littoralis*, was conducted.

MATERIALS AND METHODS

Bacillus thuringiensis isolates

Eleven bacterial isolates were kindly supplied by the Agricultural Centre of Genetic Engineering and Biotechnology (ACGEB), Faculty of Agriculture, Ain Shams University (coded as A, B, C, D, E, F, G, I, J, K and L). Another 14 bacterial isolates were kindly obtained from the Pests and Plant Protection Department, National Research Centre (Abdel-Razek 1994) (coded as M_1, M_2, M_3, M_4, M_5, M_6, M_7, M_8, M_9, M_{10}, M_{11}, M_{12}, M_{13} and ATCF).

Morphological characterization

Isolates were cultured in LB media (5 g yeast extract, 10 g tryptone and 10 g NaCl, pH 7.0). The presence and morphology of crystals was recorded by direct examination with a light microscope (Nikon®) after 24, 48 and 72 hrs. Results were confirmed by crystal staining with Coomassie brilliant blue according to Ammons and Rampersad (2002) and observed with bright-field microscopy using 100X immersion oil. According to the results of light microscopy examination, some isolates of *B. thuringiensis* were examined by a transmission electron microscopy (type JOEL JEM-1230® operating at 120 kV attached to a CCD camera). A copper grid pre-covered with a very thin amorphous carbon film was used for each sample examination. This copper grid was immersed gently on the surface of saline suspension of the sample ready for the examination by TEM.

DNA isolation

Total genomic DNA was isolated according to Azcárate-Peril and Raya (2001). Then, 10 µl of isolated DNA was loaded in a 0.5% agarose gel in 1X TBE buffer [20 ml of TBE buffer 5X (0.29 g EDTA Na, 5.4 g Tris base (pH 8), 2.75 g boric acid up to 100 ml distilled water) + 80 ml distilled water] at 100 V for 60-90 min and stained with ethidium bromide and visualized under UV light.

Detection of *crylAa* gene by PCR using specific primers

Specific PCR primers were used (**Table 1**) to detect the *cryI (Aa)* gene in the isolated strains after microscopic examination of insecticidal crystal protein to identify these isolates as *B. thuringiensis*, i.e., active against Lepidopteran insects. These primers were described by Ceron *et al*. (1995). The PCR reaction mixture was carried out using lyophilized PCR beads (Biron®). Lyophilized primers were diluted to 100 µM. 1 µl of each primer, 1 µl of DNA and 22 µl of distilled water were added to the lyophilized PCR beads.

PCR amplification was performed with a DNA thermal cycler (MWG, Biotech Primuse® model, Whatman Biometra, Germany) by using a single denaturation step (2 min at 95°C), followed by a 30-cycle program, with each cycle consisting of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min; the final extension step was at 72°C for 5 min. A total of 15 µl of each PCR product was electrophoresed on a 1.2% agarose gel in 1X TBE buffer at 100 V for 60-90 min, stained with ethidium bromide and visualized under UV light.

Isolates toxicity tests

1. Maintenance of cotton leaf worm, Spodoptera littoralis

Egg-masses of cotton leaf worm were collected from some cotton fields on Egypt that were not sprayed before with any chemical or natural insectisides and kept in clean glass jars until hatching. After egg hatching, the larvae were fed daily fresh clean castor bean (*Ricinus communis*) leaves. Glass jars were also cleaned daily. After pupation, pupae were collected, and kept in other clean jars until the emergence of adults. Adult males and females were kept together in a 1: 2 ratio in glass jars containing castor bean leaves for oviposition. Adults were fed with a piece of cotton wetted with sugar solution (10%).

2. Fermentation and production of highly toxic isolates

A special fermentation medium (like a beer) (10 g soybean, 2 g peptone, 15 g dextrose, 2 g yeast extract, 0.3 g Mg $SO_4 \cdot 7H_2O$, 0.02 g Fe $SO_4 \cdot 7H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 5 g NaCl and 1 ml Tween 60, pH 7) (Dulmage 1970) was used to obtain maximum production of spore-δ-endotoxin complex for screening and bioassay experiments. *B. thuringiensis* isolates and a *Bt* commercial product called Diple 2X (as standard) were prepared as powders according to Dulmage *et al*. (1970) as follows: the fermentation medium was adjusted to pH 7.0 with 1 N HCL before centrifugation. Medium samples were centrifuged at 3500 rpm for 20 min. The supernatant, which contained less than 1% of the spores and crystals, was discarded, and the thick, creamy residues were resuspended in a small amount of 4-6% lactose solution and diluted with additional lactose solution until the final volume was between $1/10^{th}$ and $1/20^{th}$ that of the original medium. Then the mixture was stirred 15-30 min to obtain an even distribution of the suspended cream, 4 volumes of acetone were added gradually to each volume of the suspension while stirring was continued, and the resulting aqueous acetone suspension was stirred for an additional 30 min and filtered with filter paper. The suspension

was first allowed to settle for about 10 min before the filtering process was performed. The acetone precipitate was dried overnight at room temperature. Then the powder was obtained and preserved in clean dry tubes, at room temperature, for screening or bioassay experiments.

3. Screening experiments

Only one high concentration (1500 µg/ml) was used for screening experiments of the nine *B. thuringiensis* isolates against 3rd instar larvae of cotton leaf worm. Powder of this concentration was dissolved in distilled water. Disks of fresh castor bean leaves (5.5 cm diameter) were soaked in a suspension (one disk per replicate) for 5 min then left to dry. Then, *S. littoralis* larvae (20 larvae/ replica) were introduced. Three replicates were used for each isolate.

4. Bioassay of highly toxic isolates

Results of screening experiments showed that M_5, ATCF and M_8 isolates were highly active against *S. littoralis*. So, these isolates were used for bioassay experiments at 5 different concentrations (1500, 1000, 500, 250, 125 µg/ml) to evaluate the LC_{50} value. Each concentration and each isolate had 3 replicates; each replicate had 10 cotton leaf worm larvae (3rd instar). The powder of each concentration was dissolved in distilled water as a suspension. The diet was fresh castor bean leaves as one disk (5.5 cm diameter) for each replicate soaked in the suspension for 5 min, left to dry then presented to the larvae (20 larvae/replicate). Results of larval mortality were recorded daily for 4-5 days. The percentage mortality was estimated and corrected according to Abbott's formula (1925):

Corrected mortality (%) = (T-C) / (100-C) × 100

where T = % mortality in treated replicates and C = % mortality in control replicates. Values of LC_{50}, slope factors, confidence limits, variance and χ^2 of the bioassay results were calculated using a computerized electronic version of statistical program called "State" that have been designed by some researchers and engineers at the electronic institute of the NRC (Abdel-Razek 1994).

RESULTS AND DISCUSSION

Morphological and growth characteristics

Twenty five bacterial isolates were examined morphologically according to Thiery and Frachon (1997). Nine isolates (M_1, M_5, M_6, M_8, M_{12}, M_{13}, C, J and ATCF) were identical to the main characteristics of *B. thuringiensis*: colony morphology (white, rough, spread out and could expand over the plate very quickly); the crystal protein with a bipyramidyal shape was detected. *B. thuringiensis* strains were distinguished from other *Bacillus* spp. by their ability to produce parasporal crystalline inclusions (crystal) during sporulation. *B. thuringiensis* crystal proteins have many morphological shapes: bipyramidial, cuobiodal, rectangular. Thiery and Frachon (1997) mentioned that there is a correlation between crystal shape, the activity spectra and the insect order. For example, crystals with a bipyramidial shape (**Figs. 1, 2**) are active against insects of the order Lepidoptera while crystals with a cuobiodal shape are active against insects of order Lepidoptera/Diptera. Therefore, colony morphology, crystal shape and quantity were considered for the examination of isolates in this study. Single colonies were examined by a phase contrast microscope after 72 hrs of incubation. Spores and crystal proteinsof the *B. thuringiensis* isolates were clearly detected. The presence and morphology of crystals were confirmed by staining with Coomassie blue stain (Ammons and Rampersad 2002). **Table 2** illustrates the results of microscopic examination of different isolates showing that most of them were *Bacillus* spp. while one of them was a coccus (M_2). The presence of crystals varied among the isolates; some isolates – A, E, F, I, K, M_4, M_7, M_9 and M_{10} – did not have crystals at all while others had crystal protein but with different shapes. D and L isolates had crystals with a rectangle shape, G had crystals had a cubic shape while M_3 and B isolates had an irregularly shaped crystal. Other isolates that had crystals with an ideal bipyramidial shape like M_1, M_5, M_6, M_8, M_{12}, M_{13}, C, J and ATCF isolates. Therefore, these isolates were selected to test their toxicity against cotton leaf worm.

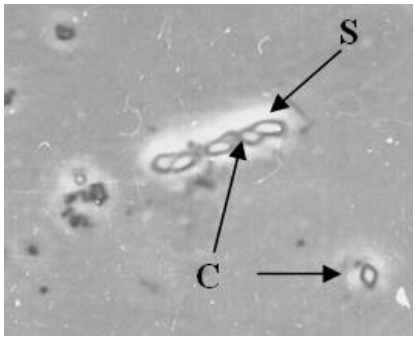

Fig. 1 A phase contrast microscopic photo showing the crystal with bipyramidal shape and spore of *B. thuringiensisI* after 72 hrs at 37°C. S and C refer to spores and crystals respectively.

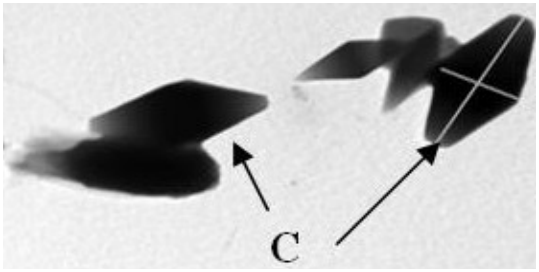

Fig. 2 A photo with transmission electron microscope to M_5 isolate showing the diameter of a crystal protein, with bipyramidial shape, with 20KX amplification bar. C refers to crystals.

Table 2 Results of microscopic examinations of 26 isolates.

Sources of isolates	Code	Microscope examination	
		Cell shape	Crystal existence
ICGEB	A	Bacilli	(-)
	B	Bacilli	(+) irregular shape
	C	Bacilli	(+) bipyramidal
	D	Bacilli	(+) rectangle not bipyramidal
	E	Bacilli	(-)
	F	Bacilli	(-)
	G	Bacilli	(+) cuboidal
	I	Bacilli	(-)
	J	Bacilli	(+) bipyramidal
	K	Short Bacilli	(-)
	L	Bacilli	(+) rectangle not bipyramidal
NRC	M_1	Bacilli	(+) bipyramidal
	M_2	Cocci	(-)
	M_3	Bacilli	(+) irregular shape
	M_4	Bacilli	(-)
	M_5	Bacilli	(+) bipyramidal
	M_6	Bacilli	(+) bipyramidal
	M_7	Bacilli	(-)
	M_8	Bacilli	(+) bipyramidal
	M_9	Bacilli	(-)
	M_{10}	Bacilli	(-)
	M_{11}	Bacilli	(-)
	M_{12}	Bacilli	(+) bipyramidal
	M_{13}	Bacilli	(+) bipyramidal
	ATCF	Bacilli	(+) bipyramidal

Total genomic DNA isolation

Chromosomal DNA and plasmids with low molecular weight, and not high molecular weight (i.e. mega or large)

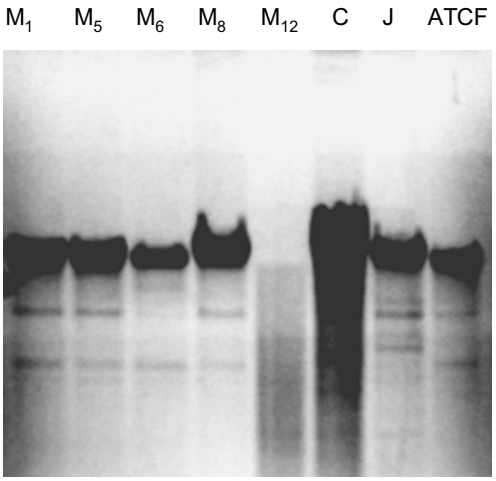

Fig. 3 Agarose (0.5%) gel electrophoresis for isolated total genomic DNA, with low molecular weight plasmids.

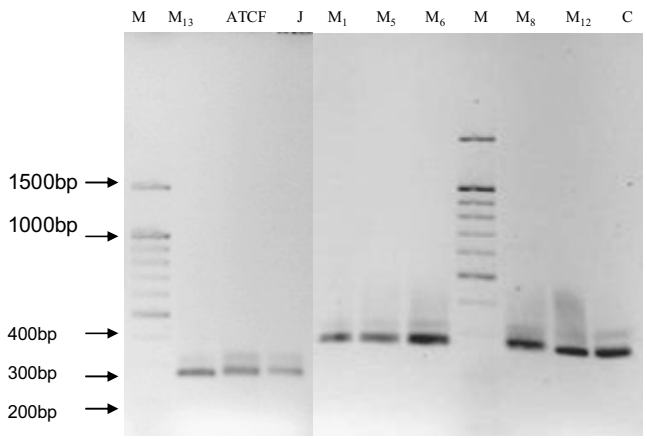

Fig. 4 Agarose (1.2%) gel electrophoresis analysis of PCR products obtained by using the *cryI (Aa)*-specific primers.

plasmids, were extracted according to Azcárate-Peril and Raya (2001). 4-6 low molecular weight *B. thuringiensis* plasmids were detected (**Fig. 3**). This result is in accordance with the findings of Chattopadhyay *et al*. (2004), who noted that in a single strain of *B. thuringiensis* five or six plasmids may be present, and which encode the Cry toxin genes. On the other hand, Lereclus *et al*. (1982) and McDowell and Mann (1991) reported that *B. thuringiensis* strains usually exhibit a complex plasmid profile, with up to 17 plasmids in size ranging from 2 to 250 kb.

Detection of *cryI (Aa)* gene by PCR using specific primers

PCR was used to screen *B. thuringiensis* isolates for the *cryI* gene, which is specific to insects of the order Lepidoptera (Schnepf *et al*. 1998). Specific PCR primers were used to detect isolates that harbor the *cryI (Aa)* gene, whose PCR product was 272 bp. **Fig. 4** shows the results obtained with the isolates under study; all the selected isolates harbored the *cryI (Aa)*, gene which means that all of them are *B. thuringiensis* isolates and are toxic to insects of the order Lepidoptera. Many different PCR screening methods were designed to predict the protoxin genes of previously uncharacterized *B. thuringiensis* strains (Ceron *et al*. 1994; Porcar and Juárez-Pérez 2003; Kim *et al*. 2005; Martins *et al*. 2007; Nazarian *et al*. 2009; Zhu *et al*. 2010). By using this screening procedure, selected *B. thuringiensis* isolates were identified as they contain the *cryI (Aa)* type of crystal protein gene. These isolates produced the expected PCR product when they were assayed with the *cryI (Aa)* general primers (CJI-1 and CJI-2) with 272 bp molecular weight (**Fig. 4**).

Mortality and calculation of LC_{50} values

Assessment of larval mortalities was expressed as percentage mortality of total larvae in each treatment, as well as in the control, after 1 to 4 days. A constant concentration (1500 µg/ml) from each isolate was used for the screening experiments to detect the most toxic isolate. Results of screening experiments are shown in **Fig. 5**. From 9 isolates used to screen activity against the 3rd instar larvae of cotton leaf worm, only 4 had a mortality that ranged between 73 and 100% where the isolates M_5, M_8, ATCF and M_{12} gave 100, 100, 97 and 73% mortality, respectively; these were considered to be the most active isolates. When these isolates were compared with the *B.t.* commercial bio-insecticide (Diple 2X), they showed the same toxicity. In contrast, the other isolates had a mortality that ranged between 17 and 63%; isolates M_6, M_1, J, M_{13}, and C had 63, 60, 37, 27 and 17% mortality, respectively and the control had 7% mortality. Results of the bioassay experiments, shown in **Table 3**, using the highest 3 toxic isolates (M_5, M_8 and ATCF) at 5 different concentrations (1500, 1000, 500, 250 and 125 µg/ml) were tested against 3rd instar larvae of *S. littoralis*. Corrected mortality was calculated according to Abbott's formula (Abbott 1925). M_5 isolate resulted in 94, 83, 75, 50 and 38% mortality at 1500, 1000, 500, 250 and 125 µg/ml, respectively. For M_8 isolate, the mortality values were 63, 56, 50, 38 and 25% while for ATCF they were 81, 50, 50, 31 and 25% at 1500, 1000, 500, 250 and 125 µg/ml, respectively. Statistical analysis of the bioassay results, shown in **Table 4**, indicates that the LC_{50} values were 213, 541 and 603 µg/ml for M_5, M_8 and ATCF, respectively. Values of slope factors, confidence limits, variance and χ^2 are also mentioned in **Table 4** for each isolate separately. The variance and χ^2 values for the bioassay data of three isolates (M_5, M_8 and ATCF) showed that the mortality percentage at the diagnostic LC_{50} value for M_5 in the susceptible population of *S. littoralis* was significantly different to that of the other two isolates (M_8 and ATCF).

Table 3 The bioassay data of mortality of *S. littoralis* larvae treated with different concentrations from the best three *B. thuringiensis* isolates.

Isolates	Conc. (µg/ml)	% Mortality	% Corrected Mortality
M_5	1500	95%	94%
	1000	87%	83%
	500	80%	75%
	250	60%	50%
	125	50%	38%
ATCF	1500	85%	81%
	1000	60%	50%
	500	60%	50%
	250	45%	31%
	125	40%	25%
M_8	1500	70%	63%
	1000	65%	56%
	500	60%	50%
	250	50%	38%
	125	40%	25%

CONCLUDING REMARKS

The results or present work indicate that three isolates (M_5, ATCF, M_8) have the *cryI (Aa)* gene, which is specific for the order of insects Lepidoptera. From these isolates, M_5 was the most promising one to be used as a highly sufficient bio-insecticide against cotton leaf worm.

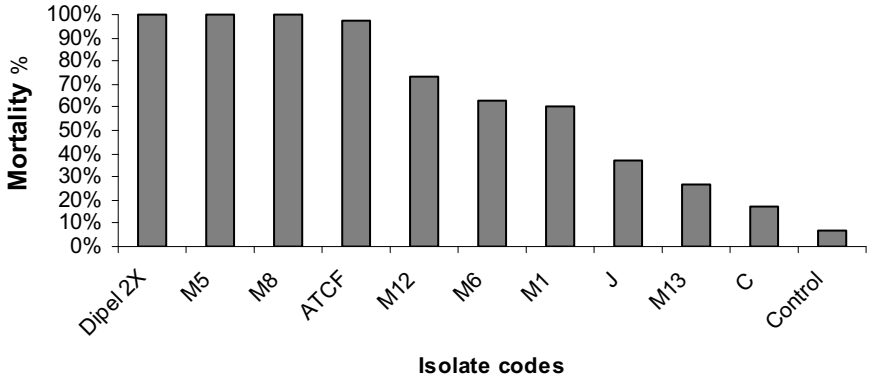

Fig. 5 A comparison between toxicity of *B. thuringiensis* isolates and the commercial product Diple 2X against 3rd instar of *S. littoralis*.

Table 4 Statistical analysis of the bioassay results using State program.

Isolate No.	LC_{50}	Slope	Confidence limits		Variance	Chi2
			Low	High		
M_5	213	1.611	57.65	368.98	2.249343 E-02	5.43944 E-05
ATCF	541	1.22	138.97	943.48	2.322067 E-02	2.574921 E-05
M_8	603	0.892	56.6733	1262.66	0.043965	3.123 E-05

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