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Bacillus thuringiensis Cry1C resistance development and its processing pattern in Egyptian cotton leaf worm: *Spodoptera littoralis* (Boisd.) (Lepidoptera:Noctuidae)

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Abstract

Biopesticides based on the entomopathogenic bacterium, *Bacillus thuringiensis* toxins, have shown high ability to control several pests belonging to order Lepidoptera. Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera:Noctuidae), is a major pest that attacks various crops in Egypt, and Cry1C toxin-based formulations are heavily used to its control. A laboratory investigation was conducted to study the resistance development in *S. littoralis* against Cry1C toxin. Thus, a field strain of *S. littoralis* was selected and maintained under laboratory conditions for 12 generations. The selection pressure resulted in 32.12-fold of resistance ratio after 12 generations. The hydrolysis of Cry1C toxin by the midgut extracts of the susceptible line, and the resistant line was compared. Results revealed that Cry1C toxin was hydrolyzed more rapidly in the resistant line than the susceptible one. The Cry1C toxin was processed till 1 hour after incubation in the susceptible line; but in case of resistant line, the Cry1C toxin was degraded in 1 hour after incubation.

Keywords: *Bacillus thuringiensis* toxins, Cry1C toxin, Resistance development, *Spodoptera littoralis*

Background

The Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera:Noctuidae), is an important polyphagous and destructive pest that attacks various crops in Egypt and many other countries all over the world. Continuous and repetitive use of pesticides caused environmental contamination, and the pests raised insecticidal resistance (Koul, 1982). Therefore, the use of biological control for management of the insect pests (as a safe alternative control method) became an urgent requirement to preserve the environment and natural enemies (Bale et al., 2007).

The biopesticides based on insecticidal crystal protein of *B. thuringiensis (Bt)* toxin had been widely used in insect control. In the midgut of sensitive pests, the *Bt* protoxin is activated by the gut proteases into active toxin, which binds to specific receptors like cadherin and aminopeptidase-N or alkaline phosphates in the peritrophic membrane, forming pores in the midgut epithelial cells (Fortier et al. 2007, Abdullah *et* al. 2009, and Talaei-Hassanloui et al. 2014).

Currently, *Bt* formulations that are promoted for lepidopteran control contain the *Bt* subsp. *aizawai*. This subspecies produces various Cry1 toxins including Cry1C, which has been shown to be highly toxic to *S. littoralis* (Chaufaux et al. 1997).

The first report of resistance to *Bt* toxins was by McGaughey (1985) in *Poldia interpunctata* (Hubner), and later, many other cases were reported either in field or after laboratory selection, e.g., *S. littoralis* (Müller-Cohn et al. 1996), *Helicoverpa zea* (Luttrell et al. 2004 and

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Tabashnik et al. 2008), *Spodoptera frugiperda* (Kruger et al. 2009), and *Busseola fusca* (Strydom et al. 2019).

Recently, dependence on *Bt*-pesticides to control cotton leaf worm in Egypt has been increased, and due to the economic importance of the cotton crop, these compounds are applied repeatedly for several times per season in the cotton fields. However, very little information is available on the status of *Bt* resistance in *S. littoralis* in Egypt.

This research therefore reports studies on the resistance development to *Bt* Cry1C toxin in the Egyptian cotton leaf worm and its processing pattern.

Materials and methods

Insect culture

Larvae of the cotton leaf worm, *S. littoralis*, originally collected from cotton fields located in Kafer Elsheikh Governorate, Egypt, were reared on the artificial diet described by Kranthi (2005) at 27 ± 1 °C, 70% RH, and a photoperiod of 14:10 (L:D) h. After pupation, the pupae were collected and kept in glass jars until adult emergence. The adults were allowed to feed on 10% sugar solution and lay eggs in the same jars. The eggs were collected on tissue paper and kept in small jars along with a wetted cotton piece as a source of moist for hatching.

Preparation of *B. thuringiensis* Cry1C toxin

Cry1C toxin was cultured and purified as described by Abdullah et al. (2009) and modified by Moussa (2009). The bacterial cells were inoculated in a 5-ml culture tube for overnight. The overnight culture was then subcultured in a 1-L flask to grow further for 3–5 days in T3 medium (3.0 g Trypton, 2.0 g Tryptose, 1.5 g yeast extract, 0.0005 g $MnCl_2$, 8.9 g NaH_2PO_4). The growth was harvested at 5200 rpm for 10 min at 4 °C. The pellets were collected and washed in (50 mM EDTA) buffer for 4–6 times/each with 10,000 rpm for 10 min at 4 °C. The obtained pellet was re-suspended in 5 ml of (50 mM Tris, HCl, 5 mM EDTA, pH 7.0) buffer. These inclusion bodies were examined by 10% SDS-PAGE gel electrophoresis. The protein concentration was determined using the Bradford method according to Bradford (1976). The inclusion bodies were then aliquoted in 1.5 ml Eppendroff tubes and stored at − 20 °C.

Bioassay of Cry1C toxin

Bioassay Cry1C toxin was conducted using diet incorporation method. Five different concentrations of Cry1C toxin in water were prepared, and 4 replicates for each concentration were represented. Moreover, the control replicates were treated by dH_2O instead of Cry1C toxin. Twenty newly hatched neonates were placed onto the surface of the diet in each replicate, using a thin brush and then kept under laboratory conditions. The larval mortality was recorded 7 days after treatment, and the median lethal concentration LC_{50} was calculated according to Finney (1971).

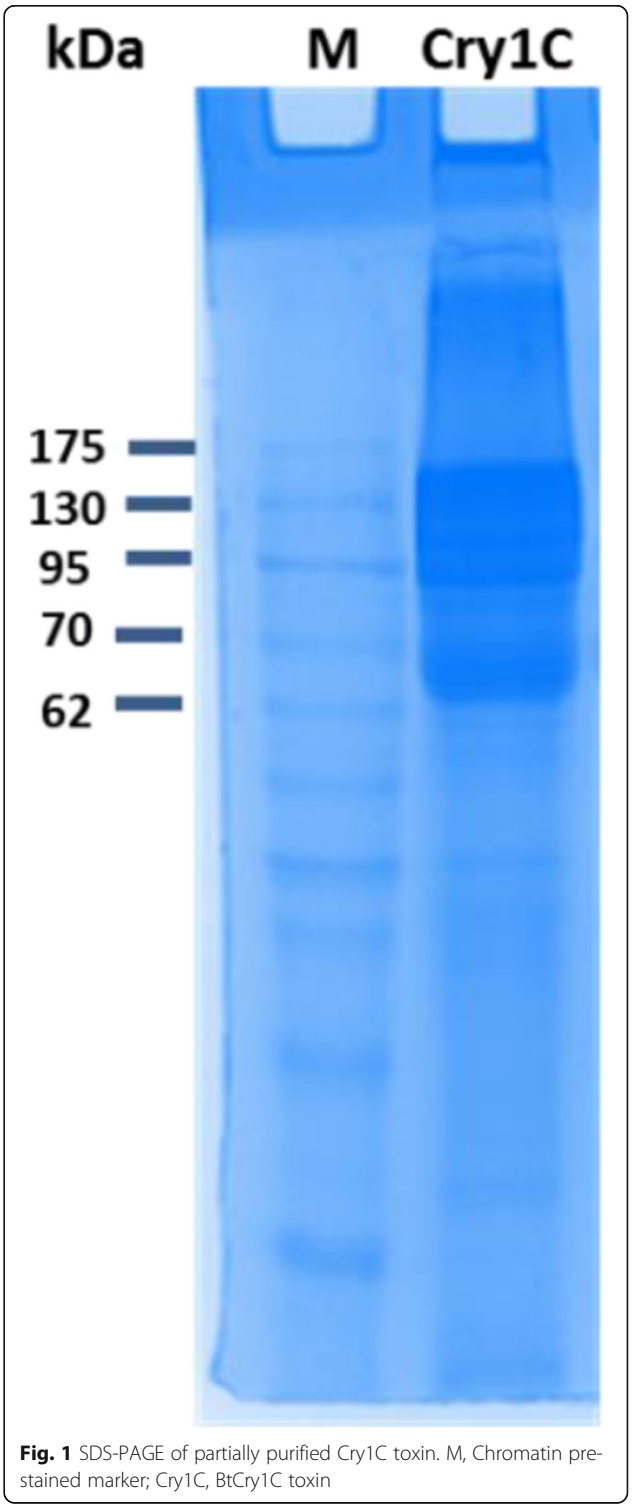

Fig. 1 SDS-PAGE of partially purified Cry1C toxin. M, Chromatin pre-stained marker; Cry1C, BtCry1C toxin

Selection pressure

Selection of *S. littoralis* neonates for resistance was initiated by transferring of 500 neonates onto the surface of artificial diet incorporating Cry1C toxin for 4 subsequent days with care always taken to obtain about 75% mortality or higher. The survived larvae were then transferred to feed on toxin-free diet until pupation. The emerged adults were allowed to feed on 10% sugar solution. The laid eggs were collected and kept in plastic cups along with wetted cotton piece until hatching. The above selection procedure was repeated on the newly hatched neonates of the second generation, and this regular work was performed at every generation until 12 generations. The bioassay was conducted at F1, F3, F6, F9, and F12 in order to calculate the LC_{50}. Resistance ratios were calculated by dividing the LC_{50} of selected generation by the LC_{50} of F1.

Preparation of gut extract

Gut extract was performed referring to the method described by Moussa (2009). Ten 4th instar larvae were dissected on ice, and their guts were pooled in 300 µl dH_2O in microcentrifuge tubes. An amount of 1.5 phenyl methane sulfonyl fluriode (PMSF) was added to inhibit proteinase enzymes. The guts were grinded gently then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was carefully transferred into sterilized Eppendorf tubes and kept at − 20 °C for further use.

Digestion and processing of Cry1C toxin

In order to compare the processing of Cry1C toxin in the midgut of susceptible (F1) and resistant line (F12) of the cotton leaf worm, an amount of 50 µl gut extract sample was mixed with 10 µg of Cry1C toxin in microcentrifuge tube, and the volume was completed to 25 µl, using universal buffer (11.5 mM boric acid, 7.8 nM citric acid, 18.7 mM Na2HPO4, and 68.9 mM NaOH, PH 9.75) (Frugoni 1957). Samples were incubated at room temperature at different time intervals, viz., 5, 15, 30 min, and 1 h with Cry1C toxin. Toxin processing was terminated by heating the sample at 100 °C for 5 min. The samples were cooled down at room temperature, and sample buffer was added. The samples were again boiled for 5 min for protein digestion (Moussa 2009). Finally, the samples were analyzed using SDS-PAGE (4% stalking gel and 10% separating gel).

Results and discussion

Cry1C toxin-based insecticide is being repetitively used to control *S. littoralis* in Egypt. In 1996, a strain of *S. littoralis* was selected with Cry1C toxin that developed 500-fold resistance (Muller-Cohn et al. 1996). Another case of Cry1C toxin resistance was reported by Chaufaux et al. (1997) as after selection that the 12th generation of *S. littoralis* showed more than 500-folds of resistance than the control. In the present experiment, neonates of *S. littoralis* were selected against Cry1C toxin. Cry1C-toxin was purified from *Bt* culture, and the concentration of Cry1C toxin stock solution was 0.5 µg/µl. The stock solution of Cry1C toxin was checked on SDS-PAGE gel and its band appeared at 65 kDa molecular weight (Fig. 1).

Development of resistance

Before selection, the larvae of *S. littoralis* (F1) were sensitive to Cry1C toxin with LC_{50} of 0.17 µg/g diet (Table 1). LC_{50} of Cry1C toxin increased to 2.40 µg/g diet in F3 than F1 with resistance ratio of 14.12 folds. µg/g diet. The resistance ratio increased from generation to another until it reached 32.12 folds in F12. The present data cleared that *S. littoralis* had the ability to develop the resistance to *Bt* toxins, while exposed to diet mixed with the toxin for subsequent generations, as other lepidopteran pests (Tabashnik *et al*., 1997 and Moussa and Gujar, 2005).

The earlier studies proved that laboratory strain of the cotton leaf worm had evolved moderate resistance in 8 subsequent generations of selection pressure against inclusion bodies of Cry1C toxin. The resistance ratio reached 5.8 and 3.4-folds for the Egyptian local *Bt* isolates of DI29 and *entomocidus*, respectively. Additionally, when the cotton leaf worm was selected against commercialized *Bt* formulations of Agerin and Diple 2^x, the LC_{50} increased to 20.5 and 16.3-folds, respectively (Mohammed 1997). The spore/crystal mixture used in the previous study might contain associated particles that may delay resistance

Table 1 Resistance development in *Spodoptera littoralis* laboratory selected line against Cry1C toxin

Generation	LC50 (µg/g diet)	95% confidence limit	Slope ± Se	Resistance ratio (RR)
F1	0.17	0.11–0.24	1.39 ± 0.16	
F3	2.40	2.06–3.01	2.87 ± 0.59	14.12
F6	3.74	2.84–5.35	1.80 ± 0.37	22.00
F9	5.39	4.33–7.61	2.33 ± 0.47	31.71
F12	5.46	4.75–6.60	2.79 ± 0.47	32.12

RR resistance ratio (LC_{50} of resistance/LC_{50} of F1 strain)

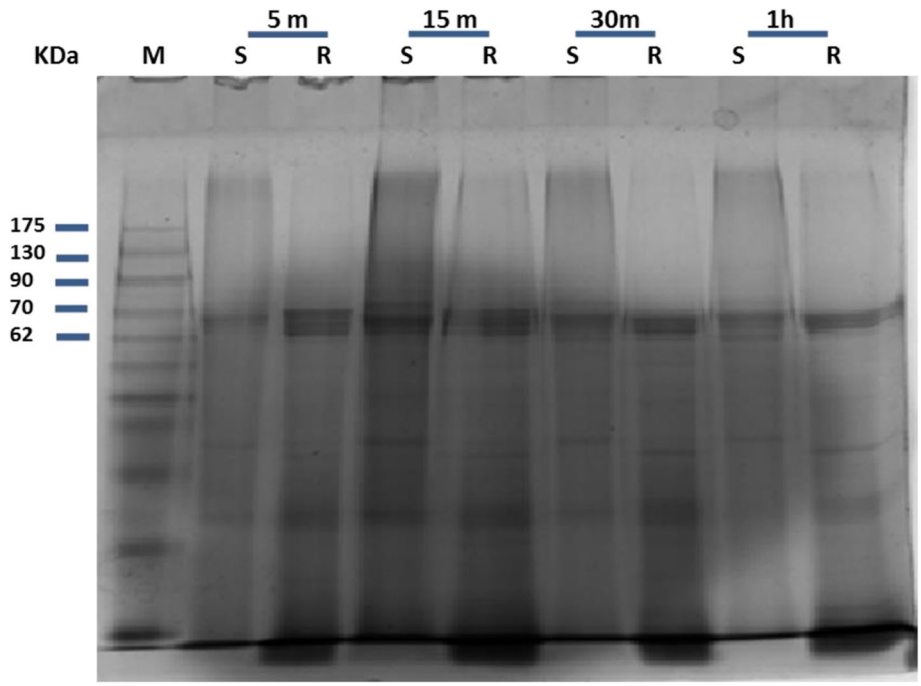

Fig. 2 Hydrolysis of Cry1C toxin by the gut extract of *Spodoptera littoralis* susceptible and resistant lines incubated at different time intervals, viz., 5, 15, 30, and 60 min. M, page ruler marker; S, susceptible lane; R, resistant lane

development in cotton leaf worm strain compared to purified toxins in the present investigation.

Processing of Cry1C by *S. littoralis* midgut extract

In the present study, the hydrolysis of Cry1C toxin by the midgut extracts in the susceptible line of *S. littoralis* (F1) and the resistant line (F12) was studied to determine if there were differences in protoxin activation in both lines. The Cry1C partially purified toxin was incubated with their gut extracts at different time intervals, viz., 5, 15, 30 min, and 1 h. Then, the samples were separated on SDS-PAGE. Results revealed that at 5 min after incubation, one fragment of 65 KDa appeared in susceptible line, while 3 fragments were observed between 62 and 70 KDa in resistant line (Fig. 2).

After 15 min of incubation, 3 fragments between 65 and 70 kDa appeared in the susceptible line. On the other hand, after 30 min of incubation with gut extract of resistant line, one more lowering band at 65 kDa appeared but did not appear in the susceptible line. When Cry1C was incubated by gut extract for 1 h, the upper band at 70 kDa position in resistant line was totally disappeared, and the lowest band became thicker; however, the same band in susceptible line was not totally degraded despite the lowest band was increased. The above observation revealed that the degradation of the Cry1C fragment in resistant line was faster than in the susceptible line. The processing of Cry1C toxin with susceptible and resistant lines varied at different time intervals. In susceptible line, the Cry1C toxin was processed till 1 h after incubation. But in case of resistant line, the Cry1C toxin was degraded in 1 h after incubation. This data is in agreement with that reported by Moussa (2009) who mentioned that Cry1Ac toxin was digested and processed in *Helicoverpa armigera* (H.) within an hour after treatment.

Conclusions

The present study proved that the Egyptian cotton leaf worm, S. *littoralis*, has the ability to build up the resistance gradually, while exposure to *Bt* toxin for subsequent generations. Additionally, the *Bt* toxin was hydrolyzed more rapidly in the resistant line than in the susceptible one.

Abbreviation

Bt: *Bacillus thuringiensis*

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Authors' contributions

SM planned the outline of the research work. AOA prepared the manuscript, while all authors equally did the bioassay experiments. The authors have read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
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Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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