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Efficacy of Cry1Ac toxin from *Bacillus thuringiensis* against the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae)

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Abstract

Transgenic *Bacillus thuringiensis* (*Bt*) cotton is engineered to express delta endotoxin (Cry toxin) proteins in lepidopteran midgut when ingested by lepidopteran larvae such as the beet armyworm, *Spodoptera exigua* (Hübner). The efficacy of *Bt* technology depends on stage, age, and susceptibility of the tested population to Cry proteins. The baseline susceptibility bioassay test for first, second, and third larval instars of *S. exigua*, collected from Faisalabad, Multan, and Bahawalpur, compared with susceptible laboratory population was carried out in 2015 and 2016. The LC_{50} ranged from 0.45 to 2.52 μg ml^{-1}, 1.08 to 5.74 μg ml^{-1}, and 2.01 to 7.85 μg ml^{-1} for first, second, and third larval instars, respectively. The Bahawalpur population was highly resistant and showed 5.63, 5.30, and 3.89 variations than the susceptible population, followed by Multan 3.01, 3.71, 3.10, and Faisalabad 1.93, 2.41, 2.31 population for first, second, and third larval instars, respectively. The molt inhibitory concentration (MIC_{50}) ranged from 0.04 to 0.56 μg ml^{-1}, 0.08 to 0.99 μg ml^{-1}, and 0.10 to 1.42 μg ml^{-1} for the three instars, respectively. The trend in lethal concentration and its respective resistance level was higher in 2016 than in 2015.

Keywords: Transgenic *Bt* cotton, Cry toxin, *Spodoptera exigua*, Bioassay

Background

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous pest of various field crops including vegetables, cotton, and ornamentals. *S. exigua* attacks the cotton plants throughout the whole growing season causing yield losses (Osoria et al. 2008). The widespread adoption of *Bt* cotton has led to several non-target insect pests such as mirids (Lu et al. 2010) aphids (Zhang et al. 2012), and armyworms including the beet armyworm that becomes one of the major pests in *Bt* cotton fields (Arshad and Suhail, 2011). However, *Bt* cotton expressing only CrylAc proteins are unable to provide effective control against *Spodoptera* spp. (Ponsard et al. 2002; Hofs et al. 2004; Selvi et al. 2012). Farmers have to spray insecticide even in *Bt* crops for effective control of *S. exigua* (Smith, 1997). The susceptibility of tested insects to Cry toxins and changes in the baseline, due to the selection pressure of Cry1Ac crops, can be monitored through regular bioassays of the field populations (Heckel, 1993).

Insecticidal protein such as Bt protein is one of the integral parts of good management practices (GMP). It has been regarded as intra-complementary approach for natural enemies establishment when chemical control has been based on selective insecticides. Bt Cry proteins solubilize the midgut receptors and insert toxin to apical membrane and epithelium. This action causes death of treated larvae. These management tactics enable the insect to establish its colony. But the level of the toxin may vary in consistency due to adulteration and impurity. This action of breeders made the lepidopteran insects possible to infest the Bt plants and to establish their population. Under these circumstances, the beet armyworm gained substantial importance by regularly damaging the Bt plants.

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A susceptibility test in view of baseline bioassay has been addressed in few parts of the world including Indian and Chinese *Spodoptera* population to Cry1Ac. The scientists studied monitoring of resistance in Pakistan but still Cry1Ac susceptibility on *S. exigua* is lacking. Therefore, the present study was carried out to evaluate the efficacy of Cry1Ac toxin from *Bt* against the beet armyworm, *S. exigua*, under laboratory conditions.

Material and methods

Larval collection

The larvae of *S. exigua* were collected from Faisalabad, Bahawalpur, and Multan to develop laboratory colonies in 2015 and 2016. The field-collected populations were assigned as Faisalabad (FSD), Bahawalpur (BWP), and Multan (MLT). The collected larvae were placed in glass vials provided with semi-synthetic diet and transported to the Insect Biodiversity and Biosystematics Laboratory, Department of Entomology, University of Agriculture Faisalabad. The susceptible laboratory population was obtained from the Nuclear Institute of Agriculture and Biotechnology (NIAB), Faisalabad.

Artificial diet

The field and susceptible populations were reared on artificial diet as reported by (Shorey and Hale, 1965; Hamed and Nadeem, 2008)

Rearing procedure

Larvae were reared at 25 ± 5 °C and 75 ± 5% RH under 16 h/ 8 h L/D cycle on the artificial diet and allowed to pupate. Pupae were collected and placed in a Petri plate. The pupae were disinfected in a 0.2% sodium hypochlorite solution. Petri plates containing pupae were placed in plastic containers until adult emergence. Adults were kept in open plastic containers covered with white netting. Adult diet (10% (v/v) honey solution) was provided by soaking the cotton wool pad in the honey solution and placed on the top of the netting cage. Every 48 h, egg-laden netting was replaced, and this process was repeated until egg production decreased or no further eggs were required. The netting was cut into small squares, and the netting containing eggs was surface sterilized in 0.05% sodium hypochlorite solution before being placed in a 250-ml round plastic cups sealed with plastic lids. The cups were placed in a controlled environment room until eggs hatching. From the first (F1) generation neonates, three larval instars (L_1), (L_2), and (L_3) were used for bioassays.

Bacillus thuringiensis toxin

Bt Cry1Ac toxin was obtained from Genralpest Biotech Research Co. Ltd, Beijing, China and was stored at − 20 °C. It had been expressed as crystalline inclusions in *Escherichia coli*, with the protoxins purified by sonication and successive washes with 0.5 M NaCl and water as described by Sayyed et al. (2008). The toxin was freshly prepared in distilled water for diet incorporation.

Diet incorporates bioassays

Diet incorporation method was used for conducting bioassay, which is similar to the methods established for the baseline susceptibility of *Helicoverpa armigera* to *B. thuringiensis* toxins (Wei et al. 2017). Seven concentrations (0.25, 0.50, 1, 2, 4, 8, 16 μg ml^{-1}) of Cry1Ac and a control were prepared. The artificial diet preparation was similar to that as described earlier except for the exclusion of ampicillin and a 10% reduction in a distilled water. Approximately 5 ml of the diet containing a toxin concentration was placed into a small aerated cup. Four replications were used for each bioassay. All bioassays were carried out under controlled environment in a growth chamber at 25 ± 2 °C, 65 ± 10% (RH) with a 16:8 (light/dark) cycle.

Data statistical analysis

The molt inhibitory and mortality rates were recorded after 7 days of bioassay. Molt inhibition was recorded when the larvae were unable to molt to the next larval instar. These counts were considered dead larvae. The mortality rate was recorded when larvae failed to respond with a gentle touch of a fine brush and considered as dead. The molt inhibition and mortality data were corrected from control mortality by Abbott formula (Abbott 1925), where needed. Probit analyses were done with PoloPlus (LeOra Software, 2003). The LC_{50} and MIC_{50} values, with their related fiducial limits at 95%, were assessed. The resistance ratios were determined by dividing LC_{50}/MIC_{50} of field with susceptible laboratory population. LC_{50} and MIC_{50} values were considered significantly different when they did not overlap each other with their respective 95% fiducial limits.

Results and discussion

Lethality of Cry1Ac toxin against *S. exigua* in 2015

L_1, L_2, and L_3 larvae of *S. exigua* collected from FSD, MLT, and BWP exhibited variable responses to different levels of Cry1Ac toxin in terms of obtained lethal concentration values (LC_{50}). The BWP population showed a maximum LC_{50} value for all instars, followed by MLT while the lowest value was estimated for FSD population. The susceptible population showed the least LC_{50} (Table 1). The lethal concentrations ranged from 0.45 to 2.52 μg ml^{-1} for L_1, 1.08 to 5.74 μg ml^{-1} for L_2 and 2.01 to 7.85 μg ml^{-1} for L_3 (Table 1). The field populations showed a variation in susceptibility

Table 1 Lethal concentration of susceptible and field population of *Spodoptera exigua* to Cry1Ac toxin during 2015

Larval instars	POP	LC_{50}	Fiducial limit	Equation	χ^2	RR	*P* value (df = 5)
L_1	SS	0.45 ± 0.03	0.02–0.99[a]	0.17 + 1.09	9.82	1	0.08
	FSD	0.86 ± 0.06	0.23–1.95[ab]	0.16X + 0.67	9.50	1.93	0.09
	MLT	1.35 ± 0.08	0.31–3.09[abc]	0.15X − 0.21	14.09	3.01	0.01
	BWP	2.52 ± 0.09	1.01–4.87[bcd]	0.14X + 0.44	11.81	5.63	0.04
L_2	SS	1.08 ± 0.06	0.46–2.75[a]	0.17 + 0.94	6.19	1	0.29
	FSD	2.62 ± 0.51	1.21–4.51[ab]	0.16X + 0.57	7.73	2.41	0.17
	MLT	4.02 ± 0.89	3.04–6.81[bc]	0.13X + 0.30	6.83	3.71	0.23
	BWP	5.74 ± 1.13	3.76–7.14[bcd]	0.14X − 0.09	7.60	5.30	0.02
L_3	SS	2.01 ± 0.95	1.00–4.02[a]	0.13 + 0.609	1.21	1	0.94
	FSD	4.65 ± 1.02	2.02–8.11[ab]	0.14X + 0.39	5.49	2.31	0.36
	MLT	6.24 ± 1.38	2.72–9.48[abc]	0.14X + 0.20	9.57	3.10	0.09
	BWP	7.85 ± 3.28	4.45–10.76[bcd]	0.16X − 0.02	8.78	3.89	0.12

Different letters in the same column indicate significant differences due to non-overlapping basis of 95% CI

levels across all populations up to a 5.63-fold for L_1, 5.30-fold for L_2 and 3.89-fold for L_3. The molt inhibitory concentration ranged from 0.04 to 0.56 μg ml^{-1}, 0.08 to 0.99 μg ml^{-1} and 0.10 to 1.42 μg ml^{-1} for L_1, L_2, and L_3. The data also recorded 12.51-fold (L_1), 12.92-fold (L_2), and 13.66-fold (L_3) variations in susceptibility levels among all the tested populations (Table 2).

Lethality of Cry1Ac toxin against *S. exigua* in 2016

The collected *S. exigua* population in 2016 showed a considerable variability in LC_{50} basis of all bio-assayed instars (Table 3). The LC_{50} values ranged from 0.45 to 2.83 μg ml^{-1}, 1.10 to 5.91 μg ml^{-1}, and 2.01 to 7.85 μg ml^{-1} for L_1, L_2, and L_3, respectively. L_1 showed 6.25-fold variations, followed by L_2 (5.35-fold) and L_3 (3.89-fold) in susceptibility across all populations. The molt inhibitory concentrations ranged from 0.05 to 0.63 μg ml^{-1}, 0.08 to 1.23 μg ml^{-1}, and 0.11 to 1.71 μg ml^{-1}, for L_1, L_2, and L_3, respectively. The data also identified 13.43-fold (L_1), 15.63-fold (L_2), and 15.44-fold (L_3) variations in susceptibility levels among all the tested populations (Table 4). The results also clearly indicated that BWP population was highly resistant to Cry1Ac toxin than MLT and FSD populations. The level of resistance increased with time as depicted through 2015 and 2016 analysis.

The toxicological studies (Kashyap and Amla, 2007), by applying the *Bt* against American and armyworms, resulted in LC_{50} for *S. litura* by 0.11 μg. Bernardi et al. (2014) determined the baseline susceptibility test against *S. frugiperda* and *Diatraea saccharalis*. The LC_{50} mortality was 61.18 and 367.86 ng Vip3Aa20 cm^{-2} for the 6 populations of *D. saccharalis*, and between 92.38 to 611.65 ng Vip3Aa20 cm^{-2} for 16

Table 2 Molt inhibitory concentration of susceptible and field population of *Spodoptera exigua* to Cry1Ac toxin during 2015

Larval instars	POP	MIC_{50}	Fiducial limit	Equation	χ^2	RR	*P* value (df = 5)
L_1	SS	0.04 ± 0.00	0.01–0.03[a]	− 0.20X–2.60	1.99	1	0.85
	FSD	0.09 ± 0.00	0.01–0.38[ab]	− 0.14X–1.47	0.62	2.09	0.99
	MLT	0.24 ± 0.03	0.07–0.62[bc]	− 0.14X–1.27	0.18	5.36	0.99
	BWP	0.56 ± 0.04	0.10–1.10[cd]	− 0.15X–1.06	1.54	12.51	0.91
L_2	SS	0.08 ± 0.01	0.03–0.14[a]	− 0.12X–1.89	0.07	1	1.00
	FSD	0.22 ± 0.04	0.07–0.80[ab]	− 0.12X–1.08	1.49	2.83	0.91
	MLT	0.62 ± 0.51	0.09–1.41[bc]	− 0.13X–0.88	1.10	8.06	0.95
	BWP	0.99 ± 0.12	0.33–2.01[cd]	− 0.11X–0.60	0.89	12.92	0.97
L_3	SS	0.10 ± 0.07	0.05–0.31[a]	− 0.13X–1.72	0.38	1	0.99
	FSD	0.49 ± 0.10	0.09–0.81[ab]	− 0.12X–0.94	1.49	4.76	0.91
	MLT	0.80 ± 0.21	0.21–1.43[bc]	− 0.13X–0.73	0.69	7.72	0.98
	BWP	1.42 ± 0.41	0.80–3.05[cd]	− 0.12X–0.46	0.21	13.66	0.99

Table 3 Lethal concentration of susceptible and field population of *Spodoptera exigua* to Cry1Ac toxin during 2016

Larval instars	POP	LC_{50}	Fiducial limit	Equation	χ^2	RR	*P* value (df = 5)
L_1	SS	0.45 ± 0.14	0.12–1.00[a]	0.18 + 1.06	6.68	1	0.25
	FSD	1.03 ± 0.41	0.61–1.98[ab]	0.16X + 0.56	8.61	2.28	0.12
	MLT	1.59 ± 0.53	0.84–2.87[abc]	0.14X + 0.34	9.70	3.53	0.08
	BWP	2.83 ± 0.75	1.53–4.90[bcd]	0.16X − 0.13	12.74	6.25	0.02
L_2	SS	1.10 ± 0.10	0.60–2.77[a]	0.16X + 0.86	1.29	1	0.94
	FSD	2.96 ± 0.72	1.73–4.73[ab]	0.14X + 0.38	5.11	2.68	0.40
	MLT	4.43 ± 1.08	3.11–6.46[bc]	0.14X + 0.21	4.72	4.01	0.45
	BWP	5.91 ± 1.40	3.87–8.49[bcd]	0.15X − 0.01	4.60	5.35	0.46
L_3	SS	2.01 ± 0.95	1.00–4.02[a]	0.13 + 0.61	1.21	1	0.94
	FSD	4.65 ± 1.02	2.02–8.11[ab]	0.14X + 0.39	5.49	2.31	0.36
	MLT	6.24 ± 1.38	2.72–9.48[abc]	0.14X + 0.20	9.57	3.09	0.09
	BWP	7.85 ± 3.28	4.45–10.76[bcd]	0.16X − 0.02	8.78	3.89	0.12

populations of *S. frugiperda*. Moreover, the effective concentration (EC_{50}) ranged from 48.65 to 163.60 and 21.76 to 70.09 ng Vip3Aa20 cm^{-2} for *D. saccharalis* and *S. frugiperda*, respectively. Obtained results are in agreement with those of Hernandez-Martınez et al. (2008) who recorded toxic effects of different toxins from *Bt* on the *S. exigua*. Likewise, Moar et al. (1995) and Ashfaq et al. (2000) concluded that Cry1Ac was the most effective against lepidopterans and reported approximately 500-fold resistance than the susceptible strain. The variability of *Bt* toxin in different cotton genotypes forced the farming community to shift towards continuous spraying of chemical insecticides in southern Pakistan. The repeated sprays resulted to develop other insect pests. The present study showed that good management of *S. exigua* under field conditions should be used only these varieties, which have higher *Bt* expression or dual toxin genotypes. Resistance in *S. exigua* to these types of Cry toxins can be overcome by planting new and better expression genotypes. The penetration of single Cry toxin varieties has been huge that might induce more serious risk and ultimately reflected in control failure.

Conclusion

The present study established a benchmark for the susceptibility of *S. exigua* to *Bt* Cry1Ac toxin. The results provided a background of developing a high-level resistance of transgenic *Bt* cotton varieties against *Spodoptera* spp. The data is important to policy-makers and technology providers; those develop strategies for the exploitation of transgenic *Bt*-cotton varieties as a component of IPM strategy.

Table 4 Molt inhibitory concentration of susceptible and field population of *Spodoptera exigua* to Cry1Ac toxin during 2016

Larval instars	POP	MIC_{50}	Fiducial limit	Equation	χ^2	RR	*P* value (df = 5)
L_1	SS	0.05 + 0.00	0.01–0.07[a]	− 0.21X − 2.23	2.00	1	0.85
	FSD	0.12 + 0.02	0.08–0.51[b]	− 0.12X − 1.38	0.49	2.64	0.99
	MLT	0.40 + 0.06	0.10–0.81[bc]	− 0.17X − 1.19	0.74	8.53	0.98
	BWP	0.63 + 0.08	0.11–1.73[bcd]	− 0.17X − 0.99	0.49	13.43	0.93
L_2	SS	0.08 + 0.01	0.04–0.21[a]	− 0.15X − 1.84	0.04	1	1.00
	FSD	0.32 + 0.06	0.07–0.80[ab]	− 0.16X − 1.03	0.64	4.10	0.99
	MLT	0.70 + 0.09	0.19–1.41[bc]	− 0.15X − 0.83	0.14	8.91	1.00
	BWP	1.23 + 0.30	0.66–2.75[bcd]	− 0.13X − 0.56	0.26	15.63	0.99
L_3	SS	0.11 + 0.07	0.07–0.42[a]	− 0.14X − 1.49	0.52	1	0.99
	FSD	0.66 + 0.13	0.22–1.17[ab]	− 0.16X − 0.89	0.62	5.99	0.99
	MLT	0.93 + 0.21	0.35–1.69[bc]	− 0.16X − 0.69	0.94	8.39	0.97
	BWP	1.71 + 0.54	0.82–3.34[bcd]	− 0.15X − 0.41	0.24	15.44	0.79

Abbreviations
Bt: *Bacillus thuringiensis*; BWP: Bahawalpur; Cry: Crystalline; FSD: Faisalabad; GMP: Good management practices; IPM: Integrated pest management; L:D: Light and dark; L_1: First instar larva; L_2: Second instar larva; L_3: Third instar larva; LC: Lethal concentration; MIC: Molt inhibitory concentration; MLT: Multan; NaCl: Sodium chloride; NIAB: Nuclear Institute of Agriculture and Biotechnology; POP: Population; RH: Relative humidity; RR: Resistance ratio; SS: Susceptible; UK: United Kingdom

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