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Research Article

Fitness cost associated with resistance to *Bacillus thuringiensis* Cry1Ac toxin in *Helicoverpa armigera* (Hübner)

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ABSTRACT: Transgenic cotton producing a *Bacillus thuringiensis* (*Bt*) Cry1Ac toxin is widely used for controlling the cotton bollworm, *Helicoverpa armigera*. The lessons learnt from the usage of insecticides suggest deployment of effective resistance management strategies to preserve the long-term utility of Bt-cotton. Consequently, it is important to understand the interaction of Cry1Ac toxin with distinct populations of the resistant alleles (homozygote resistant RR, susceptible SS and heterozygote RS or SR) keeping in mind the fitness cost associated with resistance. The present studies were under taken to understand the *in vitro* response of all such allelic populations. A critical analysis on the effects of Bt-toxin on different development stages shows that irrespective of the allelic genotype, the toxin exerts inhibitory influence on all the developmental stages. This effect is visualized as an enormous decrease in larval, pupal and adult weight, wing expanse of adults, adult life span and sex-ratio that was coupled with increase in time taken to pupate, pupal duration and total developmental period. Majority of the emerged adults possessed different types of abnormalities (wingless, deformed wings). They did not mate to lay eggs and if eggs were laid, they normally did not hatch, thus resulting in total loss of population(s). Cry1Ac toxin exerts a high cost of fitness on *H. armigera* and in this context susceptible and heterozygous genotypes were the most affected.

KEY WORDS: American bollworm, *Bacillus thuringiensis*, Bt toxin, fitness cost, resistance, transgenic cotton.

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INTRODUCTION

Transgenic cotton incorporating Cry1Ac gene derived from *Bacillus thuringiensis* Berliner is one of the most exciting advances made in cotton pest management in recent times. The cotton bollworm, *Helicoverpa armigera* (Hubner) is one of the main target pests of *Bt*-cotton technology. In India, it had developed resistance to almost all groups of chemical insecticides because of their intensive use (Kranthi *et al*., 2001; Ramasubramanyam, 2004). The failure of insecticides to control *H. armigera* has been a strong incentive for the development and adoption of transgenic cotton (*Bt*-cotton) expressing a *B. thuringiensis* insecticidal protein. Development of resistance to Cry toxin in bollworm is considered to be an inevitable evolutionary eventuality, considering the intense selection pressure that *Bt*-cotton is likely to impose on the insects due to constitutive expression of toxins throughout the plant for the entire growth season. A progressive increase in the concentration of resistance conferring alleles in pest populations due to sustained selection pressure, results in a concomitant decrease in the pest control efficacy of the transgenic crop. Ultimately a complete control failure is expected when the frequency of resistance alleles in the pest population would reach to 0.5 (Kranthi and Kranthi, 2004). The development of resistance to Bt-toxin by *H. armigera* is now considered as a major threat to the long-term effectiveness of environmentally benign Bt-cotton eventually compromising the benefit of transgenic cotton. Effective resistance management strategies are needed to preserve the long-term utility of *Bt*-cotton. Constitutive expression of genes from *B. thuringiensis* in crop plants can cause continuous production of high doses of toxins, contrast to sprayed insecticides that generally degrade rapidly. Such high doses can make pest resistance functionally recessive (i.e. heterozygotes are killed with high doses but survive with low doses), which is one of the conditions for durable resistance management with a refuge/high dose strategy (Jouanin *et al*., 1998; Gould, 1994; Tabashnik *et al*., 2000). To get prolong benefit from this technology, refuge/high dose strategy is recommended by many scientists. Results from models also indicate delaying the evolution of resistance when resistance alleles are rare and there is

extensive mating between resistant and susceptible adults (Tabashnik, 1994; Caprio, 1998; Gould, 1998). Fitness costs can be another key factor influencing the success of refuges in delaying the evolution of resistance (Lenormand and Raymond, 1998; Carrière and Tabashnik, 2001). Fitness costs occur when the fitness of individuals bearing resistant alleles (RR) is less than that of homozygous susceptible (SS) individuals in the absence of toxin. Because resistance alleles are rare in populations not previously exposed to insecticides, fitness of heterozygous individuals (RS) impacts strongly on the early dynamics of resistance evolution. If fitness costs are not recessive, RS would be less fit than the SS individuals in refuges. With recessive resistance, RS and SS individuals are equally fit in transgenic fields. This suggests that the spread of a recessive RR allele with non-recessive fitness costs could be prevented with an appropriate refuge / high dose strategy (Carrière and Tabashnik, 2001; Andow *et al*., 2000; Bentur *et al*., 2000). Thus, a better understanding of traits influenced by fitness costs and the degree of dominance of such costs could be valuable for devising resistance management strategies. In a previous investigation, we found that resistance to Cry1Ac toxin was inferred to be polygenic, autosomal and inherited as a recessive trait (Kaur and Dilawari, 2011). In this paper we compared resistant and susceptible *H. armigera* strains to identify other traits affected by resistance. We also measured fitness of F_1 hybrid progeny between resistant and susceptible strains.

MATERIALS AND METHODS

Insect rearing

The larvae of *H. armigera* were collected from different locations in Punjab. Larvae were reared at 27±2° C and 75±5% relative humidity on semi-synthetic diet composed of wheatgerm (165 g), methyl 4-hydroxy benzoate (2.48 g), sorbic acid (1.28 g), cystine (0.15 g), ascorbic acid (4.125 g), streptomycin (0.37 g), agar agar (12.75 g), dried active yeast (39.75 g), vitamin mixture (0.1 g), linseed oil (5.25 ml) and vitamin E (200µl). The ingredients were added to 800 ml of boiled water and homogenized in a blender. The field-collected larvae were allowed to develop into mature adults. Pupae were sexed and kept singly in polycarbonate vials (Polylab, India) containing sterilized moist sand. The sexing was done by viewing abdomen ventrally using 10 x magnification. The pupae were distinguished on the basis of reproductive slit, which is present near the abdominal segment line in females and in the middle of abdominal segment in males. Adult food (5g sugar in 90 ml sterile water with 0.2 g each of ascorbic acid and methyl 4-hydroxy benzoate) was provided in cotton swabs and suspended in the center of muslin.

Helicoverpa armigera strains

We used two strains: BM-R (resistant strain) and HP-S (susceptible strain). HP-S strain had been kept in the laboratory without exposure to Cry1Ac toxins. BM-R was derived by selection with Cry1Ac toxin in diet at concentration of 1 ìg Cry1Ac per ml of diet. The frequency of resistance allele conferring resistance to Bt-cotton was 0.009 in BM-R at that time. Reciprocal cross was made by using female from resistant strain and male from susceptible strain and *vice versa*. The fitness cost characteristics of *H. armigera* were tested at 14th generations of Cry1Ac toxin selection.

Toxin used for studies

For selection and bioassays we used MVP-II (19.7 per cent Cry1Ac) (Dow AgroSciences (NZ) Ltd, New Plymouth, New Zealand), a liquid formulation containing a hybrid protoxin similar to CryAc that is expressed in Bt-cotton and encapsulated by *Pseudomonas florescens*. Concentrations of Cry1Ac were calculated based on the amount of protoxin per milliliter of liquid formulation.

Bioassay and fitness cost study

Bioassay was conducted for susceptible, resistant and reciprocal cross progenies to estimate the variability in toxicity of Cry1Ac to different genotypes. The variable developmental parameters were also observed in order to study the fitness cost associated with the toxin. The 14th laboratory generation (F_{14}) of BM-R was used in the bioassay and the progeny obtained from this generation was used for the reciprocal crosses. The fitness cost of Cry1Ac on *H. armigera* was analyzed by exposing 8-day-old larvae of different populations to serial concentrations of Cry1Ac toxin and their effect on various development parameters of all the four genotypes – resistant (RR-BM-R), susceptible (SS-HP-S), RS (BM-R male x HP-S female) and SR (HP-S male x BM-R female). The range of serial concentrations was chosen in view of relative susceptibilities of respective genotypes; i.e., for susceptible baseline HP-S strain (0.025 – 2.0 µg/ml), resistant BM-R strain at 14th generation (0.50 – 15 µg/ml), and the two heterozygotes (0.125 – 6 µg/ml). The LC_{50} values of two types of heterozygotes (RS and SR) were statistically at par indicating neither paternal nor maternal influence associated with Cry1Ac resistance in *H. armigera*. Therefore, only one type of heterozygote (RS) was taken into account for observing effect of toxin on different developmental parameters (larval weight, pupal weight, time taken to pupate, pupal duration, adult weight, adult

life span, wing expanse, and sex ratio). For this, 15 individuals from each concentration under respective bioassay were selected and their weight was recorded at seventh day and then at alternate day interval till pupation.

The mortality data were recorded after 7 days and dose-mortality regression was calculated using Probit analysis (Finney, 1971). The data was inferred by computing arithmetic mean and standard deviation of total development parameters, and larval, pupal and adult weight.

RESULTS AND DISCUSSION

LC_{50} of susceptible, resistant and heterozygote population

The LC_{50} of baseline susceptible strain (HP-S) at 14th was 0.106 µg Cry1Ac/ml diet. The LC_{50} of the population after continuously rearing for 14 generations with 1 µg Cry1Ac/ml diet was 4.28 µg/ml of diet. The LC_{50} values of hybrid progeny from either cross [(susceptible male x resistant female) SR–0.232 µg/ml (fiducial range – 0.138 to 0.388) or (resistant male x susceptible female) RS–0.228 µg/ml (fiducial range – 0.143 to 0.346)] were statistically at par. These observations suggest that the gene(s) for resistance were autosomal and not sex-linked. Therefore, only one type of heterozygote (RS) was taken into account for observing effect of toxin on different developmental parameters (Table 1).

Fitness cost in susceptible, resistant and heterozygote strain

Detailed observations on developmental parameters of different genotypes (from larva to adult) in relation to respective concentration of Cry1Ac for the susceptible, resistant and reciprocal cross progeny (14th generation) are given in Tables 2-5. The development of susceptible genotype (HP-S) was arrested by toxin concentration exceeding 0.20 µg/ml of diet, though reciprocal progenies could tolerate 2.5-fold of this concentration. However, as compared to above, complete development of resistant genotype (BM-R) could proceed even at 25-fold concentration compared to one that allowed development of susceptible genotype. The HP-S strain did not survive at 0.40 µg/ml of diet and at this concentration mean larval weight was 74.33 mg whereas at control weight was 419.98 µg. However, mean larval weight of resistant (BM-R) and heterozygote (RS) strain was 316.74 mg and 207.04 mg at 0.50 µg/ml diet, respectively. The growth of heterozygote strain was inhibited by toxin concentration exceeding 0.50 ug/ml of diet. The BM-R strain survived to larval stage at 8.0 ug/ml of diet but the development was arrested by toxin concentration exceeding 5.0 µg/ml of diet. The total developmental period was 59.51 days at 0.40 µg/ml diet and 52.62 days at 0.50 ug/ml diet in HP-S and RS strain, respectively. However, BM-R strain completed total developmental period in 53.78 days at 5.0 µg/ml diet. This indicates that all strains irrespective of their resistance status responded similarly with variation to different levels of toxins. It is clear from data that irrespective of genotype, the larvae – or at least a significant fraction of population could survive on a much higher level of toxin compared to levels that allowed complete development. The data also showed that presence of toxin in semi-synthetic diet had an inhibitory effect on all the developmental parameters and this level of inhibition related directly to the concentration of toxin.

Quantified estimation on inhibition of developmental parameters

In order to have a quantified estimate on the level of inhibition of different parameters, per cent change in all the developmental parameters (relative to respective controls), in the presence of respective maximum concentrations of Cry1Ac (that could support complete development) for all the three genotypes was derived and is summarized in Table 5.

A critical analysis of the mean values on the per cent change in different developmental parameters (keeping aside the mortality cause and the genotype) shows that the presence of toxin exerted a similar but, inhibitory influence on all the developmental stages, though to different levels in the different populations. This effect was visualized as an enormous decrease in larval weight (62.5%), pupal weight (56.6%), adult weight (57.1%),

Table 1. Comparative LC_{50} of susceptible (HP-S), resistant (BM-R) and heterozygote (SR and RS) strain of *Helicoverpa armigera* at 14th generation

LC_{50} (µg/ml diet)			
0.106	4.280	0.232	0.228
(0 .015 – 0.185)	(3.431 – 5.146)	(0.138 – 0.388)	(0.143 – 0.346)

Figures in parentheses are respective fiducial limits

Table 2. Developmental parameters of HP-S strain (susceptible) of *Helicoverpa armigera* from larvae to pupae bioassayed on different concentrations of Cry1Ac incorporated semi-synthetic diet

Concentration of Cry1Ac (µg/ml diet)	Mean larval weight after 17±1.68 days* (mg)	Pupal weight (mg)	Time taken to pupate (days)	Pupal duration (days)	Adult weight (mg)	Wing expanse (cm)	Adult life span (days)	Sex-ratio (male: female)	Total developmental period (days)
0.025	262.83	225.25	25.00	9.67	125.55	3.00	7.61	2.04	42.28
0.050	245.32	221.47	26.13	10.44	117.35	3.01	7.35	1.85	43.92
0.100	194.85	175.45	33.43	14.15	87.40	2.85	6.50	1.59	54.08
0.200	131.64	102.75	36.01	17.66	83.75	2.80	5.85	1.57	59.51
0.400	74.33	–	–	–	–	–	–	–	–
0.800	13.10	–	–	–	–	–	–	–	–
1.000	5.46	–	–	–	–	–	–	–	–
2.000	4.94	–	–	–	–	–	–	–	–
Control	419.98	302.75	19.13	9.46	181.40	3.20	8.75	1.98	37.34
CD ($P = 0.05$)	37.82	46.98	4.14	2.12	8.91	NS	1.03	NS	4.25

Table 3. Developmental parameters of BM-R strain (resistant) of *Helicoverpa armigera* from larvae to pupae bioassayed on different concentrations of Cry1Ac incorporated semi-synthetic diet

Concentration of Cry1Ac (µg/ml diet)	Mean larval weight after 17±1.68 days* (mg)	Pupal weight (mg)	Time taken to pupate (days)	Pupal duration (days)	Adult weight (mg)	Wing expanse (cm)	Adult life span (days)	Sex-ratio (male: female)	Total developmental period (days)
0.50	316.74	256.43	26.50	9.45	154.97	2.97	7.17	1.82	43.12
1.00	224.86	203.12	27.33	11.83	134.79	2.87	6.67	1.89	45.83
2.00	202.62	186.37	27.83	15.80	119.16	2.80	5.75	1.84	49.38
5.00	119.52	105.98	32.60	16.67	57.01	2.67	4.50	1.83	53.78
8.00	9.10	–	–	–	–	–	–	–	–
10.00	7.41	–	–	–	–	–	–	–	–
15.00	7.54	–	–	–	–	–	–	–	–
Control	391.98	296.33	18.80	8.22	179.04	3.20	8.55	2.16	35.57
CD ($P = 0.05$)	48.57	32.22	3.62	1.51	15.87	0.26	1.48	NS	3.97

Table 4. Developmental parameters of heterozygote strain (RS) of *Helicoverpa armigera* from larvae to pupae bioassayed on different concentrations of Cry1Ac incorporated semi-synthetic diet

Concentration of Cry1Ac (µg/ml diet)	Mean larval weight after 17±1.68 days* (mg)	Pupal weight (mg)	Time taken to pupate (days)	Pupal duration (days)	Adult weight (mg)	Wing expanse (cm)	Adult life span (days)	Sex-ratio (male: female)	Total developmental period (days)
0.125	294.37	243.11	24.36	10.14	94.67	3.10	7.57	1.73	42.07
0.250	246.57	224.10	26.12	12.00	86.31	3.01	7.00	1.97	45.11
0.365	226.15	198.10	30.29	12.50	82.41	3.04	6.83	1.78	49.62
0.500	207.04	181.01	33.90	13.12	71.82	2.85	6.60	1.75	53.62
0.750	127.21	101.14	37.78	–	–	–	–	–	–
1.000	12.81	–	–	–	–	–	–	–	–
1.500	10.86	–	–	–	–	–	–	–	–
2.000	4.72	–	–	–	–	–	–	–	–
3.000	4.33	–	–	–	–	–	–	–	–
4.000	3.79	–	–	–	–	–	–	–	–
4.500	3.73	–	–	–	–	–	–	–	–
5.000	3.78	–	–	–	–	–	–	–	–
6.000	3.95	–	–	–	–	–	–	–	–
Control	407.41	299.65	19.86	8.50	176.20	3.18	9.15	2.17	37.52
CD ($P = 0.05$)	40.68	14.85	3.58	2.79	12.65	NS	0.98	NS	3.37

wing expanse (12.5%), adult life span (36.3%) and sex-ratio (17.2%), coupled with increase in time taken to pupate (77.4%), pupal duration (81.4%) and total developmental period (51.2%) (Table 5) The variation was observed in pupal and adult size with respect to different concentrations. The size of pupae and adult reared at 5 µg/ml diet was smaller as compared to that at 1 and 2 µg/ml of diet, and the control. Majority of pupae and adults possessed different types of abnormalities (larval-pupal intermediate; pupal-adult intermediate; no adult emergence; long abdomen in adults; wingless or deformed wings). Thus, Cry1Ac toxin exerted a high cost of fitness on *H. armigera* and in this context susceptible and heterozygous genotypes were most affected, which highlight the potential importance of refuge crops in delaying resistance development in the field.

Our results show that selection with Cry1Ac in *H. armigera* under laboratory conditions was associated with several undefined metabolic and morphological abnormalities and the individuals of different genetic makeup responded differently to the Cry toxin.

The present studies also inferred that toxin in semi-synthetic diet had inhibitory effect on all the developmental parameters and this level of inhibition related directly to the concentration of toxin. Selection with Cry1Ac toxin results in several undefined metabolic and developmental abnormalities in *H. armigera* which interferred with its normal development and growth. Similarly, Konasale and Moar (2007) suggested high level of fitness cost associated with Cry1Ac resistance in *Helicovepa zea* Boddie. The percentage of mating success in AR (laboratory-selected Bt-resistant strain) significantly reduced in treated population as compared with untreated parental strain. Results also revealed that the reciprocal cross progeny of resistant and susceptible strain had fitness costs in terms of significantly longer larval and pupal periods with deformed adult production as compared to individuals on normal diet. Carrière *et al*. (2001) supported the results of high fitness cost in pink bollworm associated with Cry1Ac toxin and observed reduced survival on non Bt-cotton (51.5%) in resistant individuals relative to susceptible ones. They recorded weight and survival of larvae from RS x SS and SS x RS, which were 16.74 and 12.53 mg and 49 and 60 per cent, respectively. Earlier reports showed poor hatching (less than 1%) of eggs after the eight generations of selection and reported that IC_{50} values (concentration producing 50% inhibition of larval development to 3rd instar) ranged between 0.020 and 0.105 µg/ml, 0.016 and 0.099 µg/ml, and 0.016 and 0.080 µg/ml in 1998, 1999 and 2000, respectively (Moar, 2005; Wu *et al*., 2002). Fitness cost associated with Cry1Ac toxin was observed in the form of reduced fecundity, egg hatchability and adult viability in resistant Hawaiian strains of diamondback moth (DBM), *Plutella xyllostella* to Bt. A Japanese strain of this pest also showed lower egg hatchability, longer larval and pupal durations, lower larval, pupal, and adult survivorship and lower fecundity (Groeters, *et al*., 1994; Shirai *et al*., 1998). Variable fitness has been implicated as a factor that has contributed to delayed resistance in insect species, despite the continued widespread use of transgenic cotton (Wu *et al*., 2002; Burd *et al*., 2003). The continuous selection with Cry1Ac improved the adaptability of *H. armigera* with decline in mortality and other deformities with the progression of each generation.

Table 5. Change in different developmental parameters of susceptible (SS), resistant (RR) and heterozygote (RS) strain of *Helicoverpa armigera* on a concentration of toxin permitting full development into adults

Genotype	Cry1Ac (µg/ml diet)*	Per cent change over control								
		LW	PW	TTP	PD	AW	WE	ALS	SR	TDP
Susceptible	0.20	–68.7	–66.1	88.5	86.3	–43.8	–12.5	–33.0	–20.0	59.5
Reciprocal	0.50	–49.2	–39.6	70.4	54.1	–59.3	–9.4	–28.3	–14.3	42.9
Resistant	5.0	–69.5	–64.2	73.4	103.7	–68.2	–15.6	–47.7	–18.2	51.1
Mean ** Change (%)		–62.5	–56.6	77.4	81.4	–57.1	–12.5	–36.3	–17.2	51.2

LW – larval weight (mg); PW – pupal weight (mg); TTP – time taken to pupate (days); PD – pupal duration (days); AW – adult weight (mg); WE – wing expanse (cm); ALS – adult life span (days); SR – sex-ratio (female: male); TDP – total development period (days)

* Maximum concentration of toxin permitting full development into adults

** Values are relative to respective control populations

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