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Inheritance of Cry1Ac resistance and associated biological traits in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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ABSTRACT

The analysis of reciprocal genetic crosses between resistant *Helicoverpa armigera* strain (BH-R) (227.9-fold) with susceptible Vadodara (VA-S) strain showed dominance (*h*) of 0.65–0.89 and degree of dominance (*D*) of 0.299–0.782 suggesting Cry1Ac resistance as a semi-dominant trait. The *D* and *h* values of F_1 hybrids of female resistant parent were higher than female susceptible parent, showing maternally enhanced dominance of Cry1Ac resistance. The progeny of F_2 crosses, backcrosses of F_1 hybrid with resistant BH-R parent did not differ significantly in respect of mortality response with resistant parent except for backcross with female BH-R and male of F_1 (BH-R × VA-S) cross, suggesting dominant inheritance of Cry1Ac resistance. Evaluation of some biological attributes showed that larval and pupal periods of progenies of reciprocal F_1 crosses, backcrosses and F_2 crosses were either at par with resistant parent or lower than susceptible parent on treated diet (0.01 μg/g). The susceptible strain performed better in terms of pupation and adult formation than the resistant strain on untreated diet. In many backcrosses and F_2 crosses, Cry1Ac resistance favored emergence of more females than males on untreated diet. The normal larval period and the body weight (normal larval growth) were the dominant traits associated with susceptible strain as contrast to longer larval period and the lower body weight (slow growth) associated with resistance trait. Further, inheritance of larval period in F_2 and backcross progeny suggested existence of a major resistant gene or a set of tightly linked loci associated with Cry1Ac sensitivity.

1. Introduction

Insect protective transgenic crops that express Cry toxins derived from *Bacillus thuringiensis* (Berliner) (*Bt*), deployed successfully since 1996, occupied about 46 million hectares out of total area of 125 million hectare of transgenic crops in 2008 (James, 2008). In India, *Bt* cotton (*Gossypium hirsutum* L.) expressing Cry1Ac toxin in Bollgard series (Monsanto event 531) was introduced in 2002 and dual stacked Cry1Ac and Cry2Ab in Bollgard II (Monsanto event 15985) in 2006. The area under *Bt*-cotton cultivation has increased ever since its inception from about 38,000 ha to about 7.6 million ha in India out of total area of cotton cultivation of 9.3 million ha in 2008–2009 with concurrent increase in the number of *Bt* cotton hybrids from 3 in 2002 to more than 280 in 2008. The successful introduction of *Bt* cotton has increased cotton production to about 31.5 million bales in 2007–2008 (James, 2008).

One of the most significant concerns associated with the use of *Bt*-toxin based pest management technologies is the possibility of insects evolving resistance to *Bt* crops. Evolution of field level resistance to sprayable *Bt* formulations has already been documented in the diamondback moth, *Plutella xylostella* (L.) in USA and many other countries (Tabashnik et al., 1990; Ferré and van Rie, 2002) and in the cabbage looper *Trichoplusia ni* (Hübner) under the green house conditions (Janmaat and Myers, 2003). To date, field-evolved resistance to *Bt* crops has been documented in three insect species; *Helicoverpa zea* (Boddie) (bollworm), to Cry1Ac cotton in the southeastern United States in 2003 (Luttrell et al., 2004), *Spodoptera frugiperda* (Smith) (fall armyworm) to *Bt* corn producing Cry1F in Puerto Rico in 2006 (Matten et al., 2008), and *Busseola fusca* (Fuller) (stem borer) to *Bt* corn producing Cry1Ab in South Africa in 2006 (Van Rensburg, 2007), although these did not result in failure of *Bt* crop (Tabashnik et al., 2008; Moar et al., 2008).

Bt-cotton cultivation in India has successfully controlled bollworm complex (Bambawale et al., 2004), especially cotton bollworm, *Helicoverpa armigera* which is a polyphagous, multivoltine and economically important pest of cotton and other crops. Although, *H. armigera* has not evolved field level resistance till date, there is an ample evidence of its ability to evolve resistance under selection pressure (Akhurst and James, 1999; Daly and Olsen, 2000; Kranthi et al., 2000; Liang et al., 2000; Akhurst et al., 2003; Chandrashekar and Gujar, 2004; Wu and Guo, 2004). Mahon et al. (2007) detected resistant alleles to Cry2Ab at frequency of

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0.0033 in Australian population of *H. armigera*. Further, a shift towards increased tolerance to Cry1Ac in the larvae of *H. armigera* has been reported in area of extensive *Bt* cotton planting in China (Wu, 2007) and in recent years in India (Kalia et al., 2006; http://www.cicr.org.in). The high dose of toxin expression and refuge strategy has partly contributed to the success of resistance management in *Bt* crops (Bates et al., 2005). This strategy aims at maximum mortality of genetically heterozygous and homozygous susceptible insects on the assumption of resistance trait being recessive. The random mating between the surviving homozygous resistant individuals with susceptible insects emerging from the non-*Bt* refuge crop would produce the susceptible heterozygous progeny, thereby diluting resistance alleles in the succeeding generations. However, if resistant trait is dominant or semi-dominant in their inheritance, there is likelihood of these resistant insects multiplying to a level causing failure of even *Bt* crop. This would further depend up on the biology of resistant insects on the host crops. Moreover, when resistant insects grow on the non-*Bt* refuge crops, a physiological trade-off called fitness costs occurs between resistance development and the other biological attributes that may help in delaying the evolution of resistance under field conditions (Lenormand et al., 1998). Occurrence of fitness costs in response to development of *Bt* resistance has been reported in several insect species under laboratory conditions on artificial diet (Gassmann et al., 2009). However, there is very little information on biological traits associated with resistance in respect of *H. armigera* strains in India.

The present study reports the mode of inheritance of resistance to Cry1Ac and some associated biological traits in *H. armigera*.

2. Material and methods

2.1. Insect strains

H. armigera larvae used in the present study were collected from Bharuch and Vadodara (about 77.3 km apart) in Gujarat during 2006 from India. Insects collected from Bharuch were surviving larvae on *Bt* cotton while those from Vadodara were from cotton and pigeon pea. The initial bioassays with larvae of F_1 generation showed Bharuch population was resistant (BH-R) as compared to the Vadodara population (VA-S).

The larvae were fed on Bengal-gram based diet of Nagarkatti and Prakash (1974) adapted by Gujar et al. (2004) until pupation. The adults emerging from pupae were kept in jars and fed with 10% honey solution. Five pairs of adults were released in each mating jar (15 × 20 cm), which was covered with a rough cloth for egg laying. In each generation 4–5 replicates of such jars were maintained for egg laying and further crossing experiments. The insects were maintained at 27 ± 2 °C and 60–80% R.H.

2.2. Toxin and bioassays

For bioassays MVPII liquid formulation (Monsanto India Ltd., Bangalore) containing 19.7% Cry1Ac, encapsulated by *Pseudomonas flourescens* was used (Gilroy and Wilcox, 1992). Cry1Ac in MVPII is 99% identical to the active toxin sequence of *cry1Ac* gene in the Monsanto's 531 *Bt* cotton (Tabashnik et al., 2002).

Susceptibility of F_1 neonates of *H. armigera* to Cry1Ac was evaluated using diet incorporation method (Gujar et al., 2000). Six concentrations of Cry1Ac ranging from 0.001 to 3.0 μg of Cry1Ac/g of diet were prepared, mixed thoroughly into an aliquot of diet and transferred to small plastic containers (5 × 2 cm). Each concentration had three replicates and each container containing 3 g diet served as one replicate. Ten insects were released in each of the replicate. The control consisted of untreated artificial diet. A minimum of 210 insects was used for each of the bioassays. Mortality was recorded every 24 h till 4 days and the data were used to estimate the toxicity of Cry1Ac.

2.3. Inheritance studies

2.3.1. Development of isofemale populations and selection experiment

F_1 pupae were sexed and single pairing was done for both the resistant and susceptible strains. Out of the four isofemale families obtained from each of the populations, the progenies of the resistant pairs were assayed with six concentrations (0.01–3.0 μg/g diet) with control and those of the susceptible pairs were assayed at concentrations ranging from 0.0001 to 0.1 μg/g diet. On the basis of median lethal concentrations, the most resistant and susceptible lines were selected and maintained in the laboratory. The inbred families that showed stability in response to Cry1Ac were used for genetic analysis studies.

The VA-S population was reared for seven generations without any selection pressure. It was bioassayed at F_1 and F_7 generations to monitor susceptibility status. The isofemale lines of the resistant BH-R strain were selected on Cry1Ac treated diet and their rate of resistance development as well as the stability of resistance was monitored in different generations. The neonate larvae were placed on toxin-treated diet for 4 days after which they were transferred to the untreated diet and reared until pupation. The BH-R strain was selected with Cry1Ac treated diet ranging from 1.0 to 3.0 μg/g diet for six generations before beginning the crosses.

2.3.2. Crosses and backcrosses

Reciprocal F_1 crosses were performed by mating susceptible ♀ × resistant ♂ (SR) and resistant ♀ × susceptible ♂ (RS) by using F_6 adults of BH-R and F_7 adults of VA-S strains. Sibmating was also done for maintaining both the resistant and susceptible pure lines. All the crosses were conducted in masses using five pairs of adults in jars of 15 × 20 cm size with four replications. The F_1 neonates of hybrid crosses were assayed with Cry1Ac treated diet. The hybrids of each cross were reared to adults, which were further used for performing backcross with resistant parent. The progenies of all the backcrosses were subjected to bioassay and their mortality responses observed. The backcrosses were conducted by mating adults of the hybrid cross with the F_7 BH-R parent *viz.*, ♀ of RS × ♂ of BH-R; ♂ of RS × ♀ of BH-R; ♂ of SR × ♀ of BH-R; ♀ of SR × ♂ of BH-R. Adults of the F_1 reciprocal crosses were further sibmated to obtain the F_2 generation.

2.4. Biological traits

A series of biological traits viz., larval and pupal period (days), larval weight (mg), pupation and adult formation (%) and sex ratio (♂:♀) were analyzed to assess their association with resistant and susceptible strains and their inheritance in the progenies of reciprocal crosses and backcrosses. This was evaluated by comparing the performance of susceptible and resistant parents and progenies of F_1 reciprocal hybrids, backcross and F_2 of reciprocal hybrids on treated and untreated diets. Neonates (ranging from 21 to 30) of each cross were allowed to feed on toxin treated and toxin free diets and allowed to complete growth and development until adult formation.

Further, to determine these associations the distribution of larval period was observed for backcross and F_2 reciprocal hybrids. Assuming that larval growth is a dominant trait and is associated with susceptible VA-S population, we expected that F_1 progeny should grow faster; at least with significantly lower larval period than resistant parents and may be either at par with susceptible parent or slightly more than susceptible parent on the untreated diet. Therefore, with a single locus and two alleles, half of the

backcross progeny are expected to be RS heterozygotes, having a larval period similar to F_1 progeny while other half, homozygotes similar to resistant parents (Gould et al., 1995). The higher end of the larval period distribution of F_1 progeny was considered as a threshold for separating larvae into classes: normal larval period (larval period similar to F_1 progeny) and slow growing larvae (larval period similar to the resistant parents). Furthermore, in the F_2 progeny we expected a phenotypic segregation of 3:1, where three-fourth individuals would be similar to F_1 progeny (dominant trait) and one-fourth similar to the resistant parents. Chi-square test was used to determine significant deviation from Mendelian ratio predicted by the single-locus model.

We also used larval weight distribution as a criterion to determine the association between Cry1Ac resistance and fitness of the individuals. Ten larvae fed on treated and untreated diet from each of the resistant, susceptible parents, and their F_1 reciprocal crosses were weighed on the seventh day. Assuming that larval weight is a dominant trait and is associated with susceptible VA-S population, we expected F_1 progeny to have normal weight as compared to resistant parent and at par with the susceptible parent on the untreated diet, and F_1 progeny to have less weight similar to the susceptible parent on the treated diet.

2.4.1. Biological traits during resistance development in BH-R strain

This was studied for the BH-R population which was selected on 1.0 μg/g treated diet (in F_2, F_5) and 3.0 μg/g (in F_3). Upon hatching, the neonates were transferred and reared on treated diet for 4 days and later transferred to regular diet and reared until pupation. Thereafter, in the subsequent generation the hatching were transferred directly on regular diet in the absence of toxin. The total developmental time, percentage pupation, adult emergence and sex ratio were observed. The biological traits were analyzed by comparing the performance of the BH-R strain before the beginning of selection, during selection and after selection, when they were transferred on a regular diet and reared in the absence of toxin.

2.5. Data analysis

The mortality data were analyzed using maximum likelihood program for probit analysis. The median lethal concentrations (LC_{50}s) in terms of μg/g diet of Cry1Ac for different crosses were considered significantly different if their 95% fiducial limits (FL) did not overlap. Resistance ratio was calculated by dividing LC_{50} of resistant population with the LC_{50} of susceptible population.

The degree of dominance (D) was estimated using the formula given by Stone (1968), represented as $D = (2X_2 - X_1 - X_3)/(X_1 - X_3)$; where X_1, X_2 and X_3 are the log LC_{50} values for resistant homozygotes, heterozygotes and susceptible homozygotes, respectively. D value ranges from −1 for complete recessive to +1 for complete dominance. Dominance (h) was calculated as $D + 1/2$ and ranges from 0 for completely recessive, 0.5 for semi-dominant and 1.0 for completely dominant trait (Liu and Tabashnik, 1997). Larval and pupal duration; % pupation and adult emergence were analyzed using ANOVA using Tukey's least significant difference and larval weight of resistant parent, susceptible parent and their F_1 reciprocal crosses were compared using paired t-test (SPSS Inc. 11.5 for windows).

3. Results

3.1. Isofemale strains and response to selection with Cry1Ac

The VA-S population showed an LC_{50} of 0.016 μg/g (FL 0.008–0.265) in the F_1 generation and 0.0028 μg/g (FL 0.0004–0.007) in the F_7 generation for Cry1Ac. The BH-R population showed LC_{50} of 0.44 μg/g (FL 0.233–0.962) in the F_1 generation, 0.638 μg/g (FL 0.411–1.02) in the F_6 generation and 1.26 μg/g (FL 0.86–0.188) in the F_9 generation for Cry1Ac (Table 1). The resistance level increased 3-fold in 11 generations of selection. However, significant differences were not found in LC_{50} during selection on the basis of the overlap of the FL. Stability in mortality responses of the resistant and susceptible strains over the generations indicates the homozygosity of these strains. Hence, sixth generation adults of BH-R population and seventh generation adults of VA-S population were cross-mated for genetic studies.

Although the resistant strains showed relative stability in resistance, change in the biological attributes was observed during selection. In BH-R population the percentage pupation increased from 4.9% to 16.25% in five generations of selection at a dose of 1 μg/g diet, but decreased from 6.22% in F_6 to 2.4% in F_{12}, when the insects were reared in the absence of selection for four generations (F_7, F_8, F_{10} and F_{11}). The resistant strain showed decreased pupation and adult formation during F_3 generation due to selection pressure of 3 μg/g. Performance of selected individuals on untreated diet was also found to decrease in terms of % pupation from 83.33% in F_1 generation before the selection was initiated, to 63% in F_9 after the selection was ceased and individuals reared in the absence of toxin (Table 2). Similarly, adult emergence also decreased from 80% in F_1 to 59% in F_9 generation, suggesting the association of fitness cost with Cry1Ac resistance.

3.2. Inheritance studies

Dose mortality curves of the BH-R strain, VA-S strain and F_1 progeny from the reciprocal crosses were compared. Based on

Table 1
Evolution of Cry1Ac resistance in the *H. armigera* (BH-R) population and associated biological traits.

[a]Generations	No. selected	Selective dose (μg/g)	% Survival (96 h)	LC_{50} (FL at 95%)	% Survival to pupation
F_1	1980	1	10.66	0.44 (0.233–0.962)	4.9
F_2	825	1	11.76	–	10.06
F_3	330	3	6.36	–	2.12
F_4	No dose	–	–	–	–
F_5	80	1	27.5	–	16.25
F_6	740	2	11.62	0.638 (0.411–1.02)	6.22
F_7 and F_8	No dose	–	–	–	–
F_9	1250	2	29.68	–	3.04
F_{10}	No dose	–	–	–	–
F_{11}	No dose only bioassay conducted	–	–	1.26 (0.86–1.88)	–
F_{12}	500	2	9.4	–	2.4

[a] No. of generations of isofemale line of BH-R population.

Table 2
Fitness of resistant *H. armigera* population (BH-R) before, during selection and after selection was stopped.

S. no.	Life history parameters	Untreated diet (before selection)	Treated diet (during selection)			Untreated diet (after selection)
		F_1	F_2	F_3[a]	F_5	F_9
	No. of insects	30	825	330	80	30
1	Total developmental time (days)	30.42 ± 0.09a	42.0 ± 0.58b	32.0 ± 0.58a	28.0 ± 1.0c	40.0 ± 0.1d
2	% Pupation[b]	83.33 ± 0.99a	10.06 ± 0.26b	2.12 ± 0.09c	16.25 ± 0.22d	63 ± 1.0e
3	% Adult emergence	80 ± 1.42a	7.0 ± 0.64b	2.0 ± 0.17c	15.2 ± 0.47d	59 ± 0.58e

Different letters after means within a row indicate significant difference at $P < 0.05$ level by Tukey's test.
[a] Selection in F_3 was given at 3 μg/g diet of Cry1Ac.
[b] % Pupation on treated diet (F_2, F_3 and F_5) taken from Table 1.

Table 3
Inheritance of Cry1Ac resistance in *H. armigera*.

Cross no.	Female (♀) × male (♂)	LC_{50} (μg/g)	FL at 95%	Slope ± SE	*D*-value	*h*-value	χ^2 value
[a]*Crosses between Bharuch and Vadodara*							
1	VA-S × VA-S	0.0028	0.0004–0.0065	0.381 ± 0.33	–	–	1.6
2	BH-R × BH-R	0.638	0.411–1.02	1.038 ± 0.132	–	–	10.11
Reciprocal F_1 hybrid							
3	VA-S × BH-R	0.146	0.089–0.226	1.322 ± 0.167	0.299	0.65	7.14
4	BH-R × VA-S	0.55	0.304–1.09	0.842 ± 0.134	0.782	0.89	4.21
Backcrosses							
5	BH-R × (VA-S × BH- R)	0.333	0.188–0.603	0.904 ± 0.126	–	–	5.28
6	BH-R × (BH-R × VA-S)	0.077	0.041–0.13	1.534 ± 0.263	–	–	4.28
7	(VA-S × BH-R) × BH-R	0.258	0.159–0.405	1.226 ± 0.165	–	–	5.49
8	(BH-R × VA-S) × BH-R	0.282	0.173–0.446	1.196 ± 0.167	–	–	7.32
Reciprocal hybrid (F_2)							
9	(VA-S × BH-R) × (VA-S × BH-R)	0.39	0.23–0.686	0.994 ± 0.139	–	–	21.46
10	(BH-R × VA-S) × (BH-R × VA- S)	0.286	0.18–0.428	1.424 ± 0.205	–	–	1.42

[a] Bharuch and Vadodara resistance fold 227.9.

96 h mortality data (Table 3), the BH-R population (LC_{50} 0.638 μg/g) showed 227.9-fold higher resistance with respect to the VA-S population (LC_{50} 0.0028 μg/g). The responses of F_1 progeny from the reciprocal crosses in both studies differed statistically as observed from the overlap in the FL of the LC_{50} values, thereby indicating that inheritance of Cry1Ac resistance is sex influenced.

The degree of dominance (*D*) values in the two reciprocal crosses VA-S(♀) × BH-R(♂) and BH-R(♀) × VA-S(♂) were calculated to be 0.299 and 0.782, respectively. These are equivalent to the *h* value of 0.65 and 0.89, respectively suggesting a semi-dominant nature of this resistant trait. The F_1 progeny of female's resistant parents showed higher *D* and *h* values than that of the female susceptible parents.

The progenies of all backcrosses involving F_1 hybrid and the BH-R parent showed high tolerance to Cry1Ac similar to the resistant BH-R parent except for backcross of female BH-R and male of F_1 (BH-R × VA-S) which showed high susceptibility to Cry1Ac. The F_2 progeny of reciprocal F_1 parents did not show significant differences in mortality response amongst themselves and were at par with backcrosses between F_1 and BH-R parent.

3.3. Biological traits of resistant and susceptible parents and their F_1 progeny on untreated and treated diets

The F_1 progeny and the resistant parent showed significantly better performance in terms of the biological parameters tested on the treated diet as compared to the susceptible parent, suggesting detrimental effects of Cry1Ac on the susceptible parent (Table 4). For some of biological traits, the performance of F_1 progeny varied with respect to the parental strains vis-à-vis presence and absence of toxin in the diet. The larval period of F_1 progeny was significantly lower than the susceptible parent on the treated diet whereas larval period of F_1 progeny was significantly lower than resistant parent on the untreated diet. The pupal period of F_1 progeny was significantly higher than the resistant parent on the treated diet, but was lower than the resistant parent on the untreated diet. Similarly, pupation and adult formation of F_1 progeny was higher than the susceptible and resistant parents on both treated and untreated diets. The F_1 progeny from female resistant parent appeared to have better biological traits like % pupation and adult formation than the F_1 progeny from female susceptible parent confirming a sex influenced mode of inheritance (Table 4).

3.4. Biological traits of the backcross progeny on untreated and treated diet

In the experimental set using BH-R and VA-S strains all the backcrosses were conducted with the resistant parent. The backcross progeny showed intermediate larval period between susceptible and resistant parents and relatively better performance in terms of pupation and adult formation than susceptible parent on the treated diet. Similarly, backcross progeny showed intermediate larval period and significantly lower pupal period than either of the parents, but better performance in terms of pupation and adult formation on the untreated diet (Table 4).

3.5. Biological traits of F_2 progeny on untreated and treated diets

The larval and pupal periods of F_2 progeny were lower than resistant and susceptible parents on the treated and untreated diets. Fitness of F_2 of BH-R(♀) VA-S(♂) with respect to percentage pupation and adult emergence was better than resistant parent but at par with susceptible parent on the untreated diet. Performance of the F_2 progeny of cross between F_1 VA-S(♀) BH-R(♂) and BH-R(♀) VA-S(♂) was better than the parents and their F_1 cross in terms of all biological parameters studied on treated diet. In

Table 4
Biology of Parents, their F_1, backcross and F_2 progeny *H. armigera* on Cry1Ac treated diet (0.01 μg/g) and untreated diet.

Life history parameters	Parents		Reciprocal F_1 hybrid		Backcross				Reciprocal hybrid (F_2)	
	R	S	R(♀) × S(♂)	S(♂) × R(♀)	R♀ × (S × R)♂	R♀ × (R × S)♂	(S × R)♀ × R♂	(R × S)♀ × R♂	(R × S)♀ × (R × S)♂	(S × R)♀ × (S × R)♂
No. of insects	30	30	30	30	30	30	30	21	30	30
On treated diet (0.01μg/g)										
Larval period ± SE	20.89 ± 0.13d	25.0 ± 0.02 h	20.83 ± 0.046d	18.56 ± 0.148b	23.06 ± 0.1f	23.66 ± 0.02 g	22.34 ± 0.24e	23.7 ± 0.26 g	16.86 ± 0.09a	19.6 ± 0.21c
Pupal period ± SE	13.56 ± 0.16c	14.86 ± 0.06f	14.22 ± 0.13de	14.86 ± 0.08f	14.03 ± 0.07de	13.2 ± 0.06c	13.99 ± 0.05d	14.31 ± 0.06e	10.7 ± 0.16a	12.29 ± 0.11b
% Pupation	30.0 ± 0.46d	20.0 ± 0.47b	40 ± 0.47f	30 ± 1.5d	20 ± 0.55b	14.29 ± 0.67a	33.0 ± 0.64e	30.0 ± 0.58d	23.33 ± 0.34c	33.33 ± 0.60e
% Adult emergence	30 ± 0.27e	20.0 ± 0.55c	30 ± 0.72e	23.33 ± 0.40d	3.3 ± 0.2a	14.29 ± 0.12b	30 ± 1.2e	29 ± 0.06e	23.33 ± 0.34d	23.33 ± 0.3d
Sex ratio (♂:♀)	1.2:1	0:1	0.8:1	1.3:1	0.7:1	0.65:1	1.1:1	0.82:1	0.6:1	1.33:1
On untreated diet										
Larval period ± SE	25.27 ± 0.2 g	16.89 ± 0.04b	17.0 ± 0.06b	16.29 ± 0.11a	21.92 ± 0.13f	17.78 ± 0.35c	19.23 ± 0.14e	18.32 ± 0.22d	18.0 ± 0.15 cd	16.82 ± 0.06b
Pupal period ± SE	14.78 ± 0.26f	13.33 ± 0.17e	11.69 ± 0.1bc	13.25 ± 0.13e	10.29 ± 0.07a	11.8 ± 0.1 cd	12.2 ± 0.07d	12.92 ± 0.12e	11.3 ± 0.15b	11.48 ± 0.133bc
% Pupation	72.8 ± 1.8a	80.0 ± 0.23b	93.33 ± 0.19d	80.0 ± 0.87b	86.67 ± 1.2c	85.7 ± 1.5c	83.2 ± 1.67bc	80.2 ± 1.11b	80.0 ± 1.5b	73.33 ± 0.68a
% Adult emergence	67.17 ± 0.93a	80.0 ± 0.09c	86.67 ± 0.8d	80.0 ± 1.6c	80.0 ± 1.0c	71.43 ± 0.3b	80.0 ± 0.58c	79.36 ± 0.35c	80.0 ± 1.3c	70.0 ± 0.58b
Sex ratio (♂:♀)	0.7:1	1.2:1	1:1	0.9:1	1.18:1	0.67:1	0.88:1	0.76:1	0.7:1	0.85:1

Different letters after means within a row indicate significant difference at $P < 0.05$ level by Tukey's test. Larval and pupal period expressed in days.

most cases, Cry1Ac treatment was found to favor emergence of more females than males.

3.6. Association and Inheritance of larval period and larval weight

The distribution of larval period was almost disjunctive for the resistant and susceptible parents. The larval period ranged from 15 to 18 days in susceptible parents and from 23 to 27 days in the resistant parents on untreated diet.

As expected the F_1 progeny was either at par or performed better than the resistant parents on the untreated diet and showed a larval period in the range of 14–19 days. Based on the distribution of larval period in the F_1 progeny 19 days was considered to be a threshold for separating larvae into two classes: normal growing individuals with larval period <19 days (similar to F_1 progeny) and slow growing with larval period >19 days (similar to resistant parents) (Fig. 1).

Ratio of the normal growing individuals to slow growing individuals in backcross was 1:0.7 which did not significantly differ from 1:1 ratio ($\chi^2 = 2.92$, $df = 1$, at 5% level of significance) and that in the F_2 progeny also did not differ from 3:1 ($\chi^2 = 3.51$, $df = 1$, at 5% level of significance).

The larval weight of the resistant parents on the untreated diet ranged from 11.3 to 22.1 mg each and 27.1 to 40.7 mg each in susceptible parents. The larval weight of the F_1 progeny ranged from 19.6 to 38.4 mg each on the untreated diet. Significant difference was found in the mean larval weight between resistant (17.13 ± 0.95 mg) and susceptible (32.96 ± 1.86 mg) parents ($t = 6.25$, $P = 0.0$), and between the resistant parent and their F_1 reciprocal cross (27.52 ± 1.93 mg) on the untreated diet ($t = 5.1$, $P = 0.001$). However, larval weights of the susceptible parents and their F_1 hybrids did not differ significantly from each other on the untreated diet ($t = 1.82$, $P = 0.102$).

The larval weight of the resistant parents ranged from 3.9 to 8.2 mg each and from 1.1 to 3.3 each in susceptible parents on treated diet, while those of the F_1 reciprocal hybrid ranged from 0.94 to 2.95 mg each on the treated diet. Significant differences were found in the mean larval weight between resistant (5.28 ± 0.41 mg each) and susceptible (2.37 ± 0.24 mg each) parents ($t = 7.72$, $P = 0.0$) and between resistant parent and F_1 reciprocal hybrid (1.26 ± 0.25 mg each) ($t = 3.65$, $P = 0.011$). However, no significant difference was found between the larval weights of susceptible parent and their F_1 reciprocal hybrid on treated diet ($t = 0.66$, $P = 0.535$). Further due to insufficient sample size, frequency of weight distribution could not be used to determine the Mendelian inheritance.

4. Discussion

The rate of resistance evolution and mode of inheritance of resistance have serious implications on resistance management strategies. There have been relatively few reports on the rapid rate of resistance evolution in heliothines. Evolution of resistance has been reported to be quick in *Spodoptera exigua* (Moar et al., 1995) and *H. zea* (Luttrell et al., 1999; Anilkumar et al., 2008a). However, several authors have observed slow rates of resistance development. Gould et al. (1995) found a drastic increase only between 12th and 19th episodes of selection after exposing a maximum of 1760 neonates of *H. virescens* for 30 generations. Kranthi et al. (2000) found no apparent change in susceptibility of *H. armigera* in initial 4–5 generations of selection. However, an increased resistance of 76-fold was observed at the end of 10th generation. Similarly, Lu et al. (2004) found 11-fold of resistance in *H. armigera* after 22 generations of selection. Further, Wu and Guo (2004) also found slow resistance development in *H. armigera* reaching only 6-

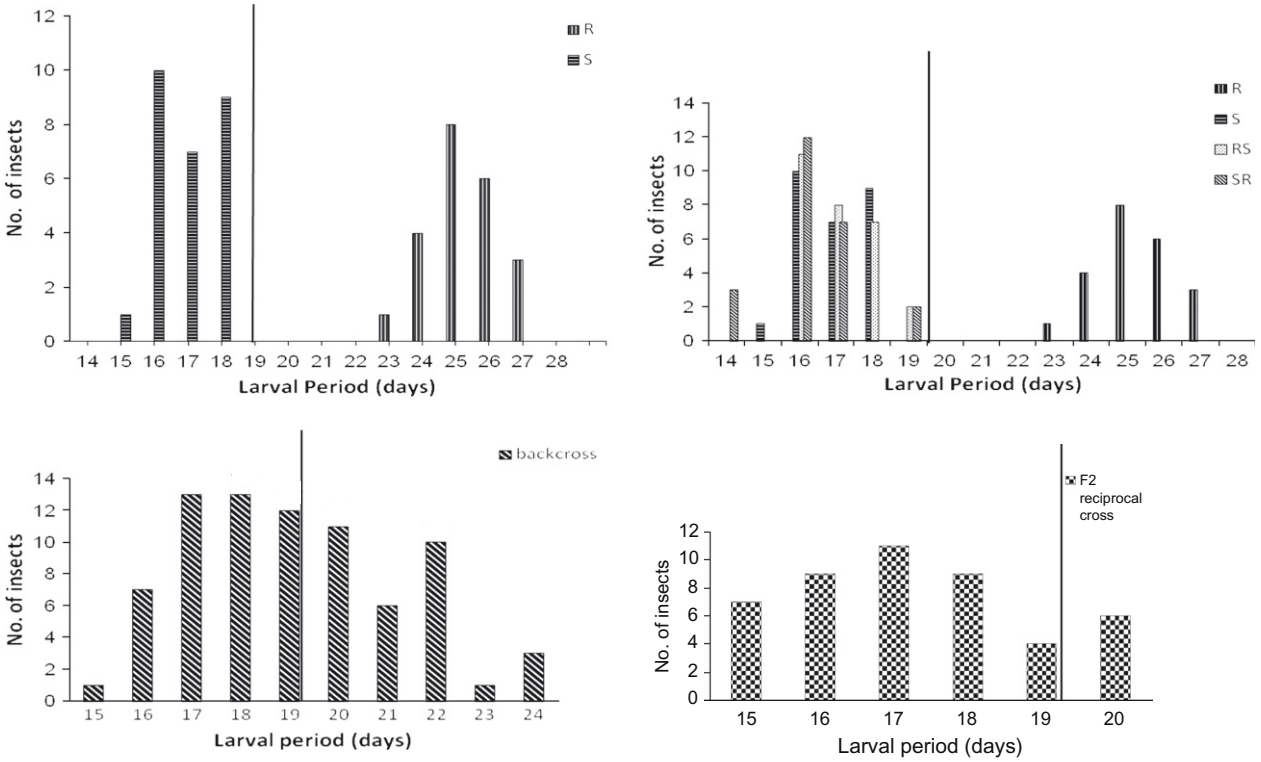

Fig. 1. Histograms showing the distribution of larval period of *H. armigera* on untreated diet for parents, their F_1 reciprocal cross, progeny of backcross and reciprocal F_2 cross. R = resistant parent (BH-R); S = susceptible parent (VA-S); RS = BH-R(♀) × VA-S(♂); SR = VA-S(♀) × BH-R (♂).

fold after 15 generations of selection. However, a rapid increase in resistance level from 16-fold in F_{30} to 106-fold in F_{44} generation was observed. Akhurst et al. (2003) also found no change in susceptibility of *H. armigera* to Cry1Ac despite continuous selection over 12 generations of selection. They found highest resistance levels at 21st and 22nd generations when resistance ratio exceeded 300 after which resistance ratio declined to 57 at 27th generation. They did not find any noticeable effect on survival and growth rate after selection dose was increased 10-fold in the 18th generation and further 3-fold in the 27th generation. Similar instability in resistance was reported by Chandrashekar and Gujar (2004) after continuous selection for six generations. In the present study only 3-fold resistance was observed at the end of F_{11} depicting a relatively slow evolution of resistance. No significant difference in LC_{50} was observed in BH-R strain over 11 generations of selection even after the dose was increased 2-fold from F_6 to F_{11} generation. This is possibly due to selection of single isofemale resistant generation as compared to resistance evolution to the heterozygous population in other studies.

In most of the studies related to heliothines, resistance to *Bt* toxin was inherited as recessive or incompletely recessive trait (Gould et al., 1995; Kain et al., 2004; Akhurst et al., 2003; Bird and Akhurst, 2004; Xu et al., 2005; Liang et al., 2008). In certain other colonies resistance was reported to be more dominant than recessive (Huang et al., 1999; Liu and Tabashnik, 1997; Gould et al., 1995; Daly and Olsen, 2000; Liang et al., 2000; Akhurst et al., 2003). However, resistance was reported to be completely dominant in BKBT strain of *H. armigera* originating from Cote d'Ivoire showing 160-fold resistance to Cry1Ac (Uraichuen, 2002). Our results are consistent with those of Kalia and Gujar (2004) and Kranthi et al. (2006) which further confirms the mode of inheritance of Cry1Ac resistance to be semi-dominant under Indian conditions. Significant difference in the LC_{50}'s of the F_1 progeny of the reciprocal crosses indicates inheritance of resistance to be sex influenced. Our result of a sex-influenced resistance to Cry1Ac in *H. armigera* is inconsistent to those reported in past wherein resistance is reported to be recessive and autosomally inherited (Ferré and van Rie, 2002). However, sex influenced inheritance of Cry1Ac resistance has been reported previously in *Spodoptera littoralis* (Chaufaux et al., 1997), *H. armigera* (Kalia and Gujar, 2004) and *Ephestia kuehniella* (Rahman et al., 2004). Similar observations were also reported in the studies on *H. virescens* for Cry1Ac (Blanco et al., 2008).

The evaluation of the biological traits showed that the resistant strains had a decreased fitness and took significantly longer time to complete development on untreated diet as compared to the susceptible larvae suggesting a probable developmental asynchrony between resistant and susceptible insects. The resistant parent and the F_1 progeny showed a higher fitness as compared to the susceptible strains fed on the treated diets, which could be attributed to the semi-dominant mode of inheritance. The fitness advantage of heterozygotes over susceptible alleles indicates a hastened development of resistance (Curtis, 1981). Assessment of the backcross progeny shows the emergence of more number of females in most of the cases thereby, indicating that Cry1Ac resistance favors the development of females. Although resistance may not be caused by a major sex-linked gene but the results presented in this study suggest the probable influence of females in imparting resistance.

Further, the assessment of the performance on untreated diet of the progenies of F_1 reciprocal crosses, backcross and reciprocal F_2 cross in terms of larval period suggest the probable association between resistance to Cry toxin and the fitness of the individuals. The histograms showing the larval period distribution on untreated diet suggest that detrimental effects of Cry1Ac resistance on the fitness of the individuals in the absence of toxin are based on changes at a single locus or a set of tightly linked loci. Gould et al. (1995) used larval weight of the F_2 and backcross larvae on

diet treated with 0.064 μg/ml Cry1Ac as a criterion to show the existence of a major resistance gene or a set of tightly linked loci in conferring high levels of resistance to Cry1A(b) in *H. virescens* strain. They detected two distinct classes of larval weights (≈1:1) as expected when a major gene for growth on toxic diet is present. Tabashnik et al. (2002) examined larval weight distribution of *P. gossypiella* after 11 days feeding on diet treated with 1 μg Cry1Ac/ml to determine the consistence of growth pattern with expectations from a single-locus model. They found almost completely disjunct weight distribution for resistant and F_1 larvae. Further, they found that the ratio of small to large backcross larvae did not differ significantly from the 1:1 ratio predicted by a single-locus model.

In the present studies, although the consistency of weight distribution with expectations from single-locus model could not be established due to insufficient sample size but, the similarity in the larval weights of the susceptible parent and F_1 progeny and the significant difference between the weights of the resistant parent with both susceptible parent and F_1 progeny on treated as well as untreated diet confirms the association of larval weight with Cry1Ac resistance. Hence, larval period and the weight are the dominant traits associated with susceptibility as contrast to longer larval period and less weight (slow growth) of larvae associated with resistance trait.

The BH-R strain showed increased fitness due to their adaptation on the *Bt* treated diet. Our results are in consistence with Bird and Akhurst (2005) who in one of the experiments also reported a significantly faster development of resistant strain ISOC4 on *Bt* cotton compared with their resistant counterparts on non-*Bt* cotton. Poor performance of the selected individuals on the diet devoid of toxin depicts a significant fitness costs. Longer larval period indicates a decreased fitness of the selected larvae when they were shifted to untreated diet after continuous selection. Similar fitness costs have been reported in *H. zea* (Anilkumar et al., 2008b) and *S. littoralis* (Müller-Cohn et al., 1996). Anilkumar et al. (2008b) observed that the resistant strains produced higher percentage of normal adults when exposed to toxin in selection experiments than when reared on untreated diet. This is in contrast to our results which show that the percentage pupation and adult emergence increased after the selected individuals were reared in the absence of toxin, but when these parameters were compared with the performance of the unselected larvae (before selection) a significant decrease in percentage pupation and adult emergence was observed. Hence, the significant decrease observed in the BH-R strain with respect to all the biological attributes on untreated diets, indicates the prevalence of fitness cost associated with the evolution of resistance. These results strongly show a trade-off in which alleles for *Bt* resistance increase in response to selection of *Bt* toxin but impose a fitness cost when toxin is absent (Gassmann et al., 2009).

Although, in India it is mandatory to have five border rows or 20% non-*Bt* as refuge whichever is maximum as a part of resistance management, farmers do not implement it for reasons of small land holding and ignorance of its necessity. Alternative non-transgenic hosts *viz.* pigeon pea, tomato, okra, sorghum, sunflower and chickpea of *H. armigera,* which serve as important sources of refuges (Ravi et al., 2005), are considered adequate instead of structured refuge in the country. The two key assumptions of the refuge strategy are that inheritance of resistance is recessive and random mating occurs between the susceptible and resistant strains. However, the developmental asynchrony favors assortative mating among resistant moths thereby generating a disproportionately high number of homozygous resistant insects, accelerating the evolution of resistance (Liu et al., 1999). Moreover, the variability in Cry1Ac expression, and its reduction during reproductive phase of crop would enable the heterozygous individuals to overcome the toxin and spread resistant alleles (Kranthi et al., 2005).

In view of the results presented, the contemporary resistance management tactics may be inadequate to delay evolution of resistance. The fitness costs exhibited by homozygous resistant and by heterozygotes over homozygous susceptible alleles appears to be of some merit to delay appearance of *Bt* resistance in the field under Indian conditions. These fitness costs will vary depending upon the availability of non-*Bt* cotton and other alternate host-crops for the resistant insects like *H. armigera* under local conditions. Since fitness costs vary with ecological conditions, refuges designed to increase the dominance or magnitude of fitness costs could be another useful option for delaying pest resistance (Carrière et al., 2001; Gassmann et al., 2009). Furthermore, stacked *Bt* cotton with different sets of genes will compliment these efforts.

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