Frequency of alleles conferring resistance to Bt cotton in North Zone populations of the spotted bollworm, *Earias insulana* (Boisduval)

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Transgenic Bt cotton with insect resistance was introduced for commercial cultivation in India in 2002 (Barwale *et al*. 2004). Bt cotton expressing Cry1Ac is mainly toxic to the bollworm complex (*Helicoverpa armigera*, *Pectinophora gossypiella*, *Earias insulana* and *E. vittella*). The rapid adoption of Bt cotton technology in India imposes a strong selection pressure on the target pest with a risk of resistance development to Cry toxins including Cry2Ab following the introduction of dual gene BG-II cotton in 2006. Spotted bollworm, *Earias insulana* infests the cotton crop during the early season as a shoot borer and damages fruiting bodies during mid-season crop growth. Efficacy of Bt cotton hybrids BG-II (Cry1Ac + Cry2Ab) and Bt (Cry1Ac) through leaf (90–95-day-old crop) and square (80–85-day-old crop) bioassay under laboratory conditions, recorded 97–100 % mortality on leaves of BG-II, 93 % on Bt and 1.3–5.4 % on non Bt against 1-day-old larvae of *Earias insulana*. Observed mortalities on plant squares were 94–100 % in BG-II, 91 % in Bt and 1.3–5.6 % in non-Bt genotypes. Frequency of resistance alleles in field populations of *Earias insulana* to Cry1Ac protein by using F_2 screening procedure was also undertaken. In the present study on F_2 screening on 266 isofemale lines of *Earias insulana* collected from Sirsa (Haryana), Sriganganagar (Rajasthan) and Mansa (Punjab) during 2013–14, 2014–15 and 2015–16, no alleles could be detected conferring major resistance to Cry1Ac in the population of *Earias insulana* from all three locations. However, evidence for partial resistance alleles in F_2 generation of two isofemale lines from Sirsa (Haryana) population was recorded during 2013 but could not be reconfirmed.

Key words: Bt-cotton, F_2 screening, resistance, *Earias insulana*.

INTRODUCTION

Cotton (*Gossypium* spp.) supporting a lengthy fruiting phase is vulnerable to the direct attack of the bollworm complex, *viz*. *Earias insulana* (Boisd.) and *Earias vitella* (Fab.), *Helicoverpa armigera* (Hubner) and *Pectinophora gossypiella* (Saunders) and may lead to severe yield loss. More than 43 % of total cotton production cost was spent on insecticides in the mid-1990s and 80 % of this was for controlling the bollworm complex in India (ICAC 1998a, b). Integrated pest management (IPM) strategies were developed to combat problems of resistance to pesticides, resurgence of secondary pests and environmental contamination. Rapid adoption and dissemination of technologies among farmers have paved the way for the large-scale adoption of Bt cotton.

Bacillus thuringiensis (Berliner) is a soil bacterium that produces a diversity of Cry proteins which are selectively toxic against a wide variety of insect pests (Crickmore *et al.* 1998). Large-scale cultivation of Bt cotton is exerting tremendous selection pressure on target pest species, which has led to the evolution of field resistance (Wolfenbarger & Phifer 2000; Benedict & Ring 2004; Matten *et al.* 2008; Tabashnik *et al*. 2008; Kruger *et al.* 2009; Tabashnik *et al.* 2009), diminishing the benefits of Bt cotton technologies. Several insect species upon prolonged exposure under laboratory conditions have shown high potential of resistance development to Bt (Tabashnik *et al*. 1990; Tabashnik *et al*. 1992; Tabashnik 1994; Gould *et al*. 1995; Chaufaux *et al*. 1997; Gould 1998; McGaughey & Beeman 1988; Liang & Tan Wand Guo 2000; Ferre & Van 2002). Studies conducted by Kranthi *et al*. (2004) on various population of *E. vitella* with Cry1Ac at LC_{90} concentrations showed survival ranging from 0 to 20 %, suggesting the existence of some tolerant individuals which are likely to survive in the Bt-cotton crop when Cry1Ac expression is low. It has therefore become necessary to detect any

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emergent resistant phenotype in population of *Earias* spp. Among the two existing *Earias* spp., *E. insulana* is more abundant in the North Cotton Growing Zone of India and was thus selected for the present study.

The spiny bollworm, *E. insulana*, has an extremely wide range and is found throughout most of Africa and the Mediterranean region and eastwards to India, China and Southeast Asia (Reed *et al.* 1994). This species is an important component of the lepidopteran pest complex of cotton in some regions in Spain and Egypt (Hamed *et al.* 2001), Israel (Horowitz 1997), India and Pakistan (Kranthi *et al.* 1999). Although it is a pest of cotton, it can also grow on other alternate hosts (Abul-Nasr *et al.* 1973). Spiny bollworm causes damage by attacking terminal shoots, flower buds and green bolls. The most serious damage to cotton is caused when larvae bore into the bolls, destroying the fibre, consuming seeds, and producing putrefaction due to the accumulation of faeces and fungus. The roots of cotton plants sprouting in early spring and the fruits of neglected okra left in the field are two important sources of early infestation and multiplication of this pest (Arora & Singh 2004). *Earias insulana* and *E. vitella* (Lepidoptera: Noctuidae) can reduce seed cotton yields by 36–40 % (Dhawan *et al.* 1990; Singh & Lakra 1992). The preferred oviposition site is squares. Virtually no quantitative data are available on the existence of *E. insulana* individuals resistant to B. *thuringiensis* Cry proteins. In the present study, the insecticidal activity of Cry proteins (Cry1Ac and Cry2Ab) in the field was determined in terms of mortality among *E. insulana* neonates larvae fed on various plant parts under laboratory conditions. Since continuous exposure to Cry1Ac may result in the development of resistance to this toxin, we used an F_2 screening method (Andow & Alstad 1998) to determine the frequency of alleles conferring resistance to Cry1Ac in populations of *E. insulana* from Sirsa (Haryana), Sriganganagar (Rajasthan) and Mansa (Punjab) during 2013–14, 2014–15 and 2015–16.

MATERIAL AND METHODS

Details of the location studied in the North Zone of India

The F_2 screening experiments were conducted on *E. insulana* population from Sirsa (Haryana), Sriganganagar (Rajasthan) and Mansa (Punjab) during 2013–14, 2014–15 and 2015–16. These are the major cotton growing districts in North Zone of India cultivating BG-II cotton (*G. hirsutum*) and *G. arboreum* cotton.

Plant part bioassay

Laboratory bioassays were conducted on leaves (90–95-day-old plant) and squares (80–85-day-old plant) of BG-II, Bt and Non Bt genotypes to study the efficacy of Cry toxins against *E. insulana.* Five larvae (one day old) were placed on each leaf/square in one perforated plastic cup and five cups constitute one replication. The leaves were changed every day and squares on alternate days. Mortalities were recorded daily up to 7 days.

F_2 screening method

The methodology described by Andow *et al.* (1998) was followed for screening insect resistance against F_2 populations. It included the following steps (1) Sampling and rearing of natural larval population and establishment of isofemale lines, (2) Rearing and sib-mating F_1 progeny in each isofemale line, (3) screening F_2 neonates to determine susceptibility to Bt toxin, (4) statistical analysis of the data. By sib-mating the F_1 generation, $1/16$ of the F_2 larvae *i.e.* 6.25 % are expected to be homozygous for any resistance alleles that a field-collected female (or her mate) carried. Since each female carries at least four haplotypes (two of her own and two from her mate), therefore each isofemale line enables the characterisation of at least four alleles. In the present study rearing was carried out on artificial diet under laboratory conditions at 27 ± 1 °C at 75 % RH and photoperiod of 12L:12D. Screening of F_2 neonates was done against Cry1Ac.

Collection of natural larval populations of *E. insulana* was done from okra and non-Bt cotton crops. Field sampling was done during the months of August, September, October and November 2013, 2014 and 2015 from different locations. The collected larval populations were reared on an artificial diet (Kranthi 2005) until pupation. Sexual differentiation of pupae was done as described by Gupta (1978). Male and female pupae were kept separately until adult emergence. This was done to restrict/avoid multiple mating of adult females. Males and females of F_0 population were paired in a plastic container provided with 10 % sugar and honey solution as

an adult diet to establish isofemale lines. Fertile females produced eggs in each isofemale line. For each line, the number of F_1 males and females which were sib-mated was recorded.

Bioassay dosages

Since the susceptibility of *E. vitella* and *E. insulana* is more or less the same, F_2 bioassays were conducted after confirmation of the results of the LC_{50} obtained in previous bioassays conducted by Kranthi *et al.* (2004). Cry1Ac protein was provided by CICR, Nagpur. For the Sirsa (Haryana) population, the highest Cry1Ac dose *i.e.* T1 (0.28 μg/ml of Cry 1Ac) selected was twice the LC_{90} and the second highest Cry1Ac dose T2 (0.028 μg/ml) was equal to the LC_{50} for population of *E. vitella* of district Sirsa and T3 (0.0028 μg/ml) was 10 times less than T2. For Ganganagar (Rajasthan) population of *E. insulana*, the highest dose of 0.13 μg/ml of Cry 1Ac was twice the dose of LC_{90} as evaluated for *E. vitella* population of Sriganganagar during 2004 and the second highest dose (0.013 μg/ml) was equivalent to LC_{50}. For the Mansa population, the highest dose 0.11 μg/ml of Cry 1Ac was twice the dose of LC_{90} as evaluated for *E. vitella* population from Mansa during 2004 and the second highest dose (0.011 μg/ml) was equal to the LC_{50} for population of *E. vitella* of district Mansa (Punjab).

Screening of F_2 larvae

For F_2 screening, larval populations collected from the fields were reared to F_1 generation and newly emerged neonates by the sib-mated F_1 females of each line were transferred to an artificial diet for 2 days. Each bioassay was performed in a 25-well plate; a single larva being released in each well. In each bioassay, larvae were transferred on 20 ml fresh artificial diet incorporated with toxin on alternate days. Control treatments consisted of wells containing diet without toxins. Two-day-old F_2 larvae, 17 208 (Sirsa), 17 973 (Ganganagar) and 10 010 (Mansa) were used in F_2 screening to determine resistant allele (Cry1Ac) frequency. Mortality of larvae in individual lines in each test was recorded on alternate days. Evidence of feeding, moulting of larvae and pupation was also monitored throughout the test. Observations were taken on alternate day until 13 days from the start of the experiment.

Analysis

Expected allele frequencies were calculated using the equation from Andow & Alstad (1998).

$$E(q) = \frac{(S+1)}{4(n+1)}$$

where n is the number of female isoline screened and S is the number of female isolines found to have offspring that survive.

RESULTS

The 1-day-old larvae of *Earias insulana* were released on 95–100-day-old plant leaves and 80–85-day-old plant squares of the genotypes carrying single Bt gene (Cry1Ac) and dual gene BG-II (CryIAc + Cry2Ab) and a non Bt cotton under laboratory conditions. The leaves were replaced every day and mortality was recorded.

The larval mortality in plant parts bioassays conducted during 2014–15, was 100 % in BG-II genotypes, 93 % in Bt and 2.4–5.4 in Non Bt leaves. In square at 80–85-day-old plant the larval mortality obtained in BG-II was 100 %, 91 % on Bt cotton and 2.4–3.6 % in non Bt cotton. The surviving larvae in the Bt-cotton expressing Cry1Ac protein could not complete the life cycle as their growth was severely affected and failed to pupate.

The larval mortality recorded during 2014–15 and 2015–16 ranged from 97–100 % in BG-II genotypes, and 1.3–5.4 in non-Bt leaves whereas in squares 94–100 % mortality in BG-II and 1.3–5.6 % in non-Bt squares was recorded. The plant part bioassay studies on Bt-cotton expressing Cry1Ac protein could not be pursued during 2015–16 and 2016–17 due to the non-availability of Bt cotton genotypes (Table 1).

For F_2 screening at Sirsa (Haryana) during 2013–14, a total of 1 457 larvae were collected from okra and non-Bt cotton. A total 549 F_0 male and female pairs were made during September (294), October (156) and November (99) of which only 264 (48 %) isofemale lines produced F_1 fertile eggs within 3–4 days (Table 2). The other pairs (285) died without producing eggs.

For confirmation, the progeny of F_0 (*i.e.* F_1 larvae) from 83 lines out of the 264 isofemale lines established from Sirsa (Haryana) collected population were screened on three diagnostic doses, and showed maximum mortality (%) in T1. Mortality at the highest dose (0.28 μg/ml) ranged from 60.0–100.0 at 13 days after treatment (DAT). All larvae had died after 19 DAT. The range of mortality in the F1 generation on treatment T2

Table 1. Leaf and square bioassay on efficacy of Bt, BG-II and non-Bt cotton against *Earias insulana* under laboratory conditions.

SN	Year	Crop stage	Larval mortality (%) in leaves			Crop stage	Larval mortality (%) in squares		
			BG-II	Bt	Non-Bt		BG-II	Bt	Non-Bt
1	2014–15	95–100	100	93*	2.4–5.4	80–85	100	91*	2.4–3.6
2	2015–16	95–100	100	**	3.6–5.4	80–85	100	**	2.3–5.6
3	2016–17	95–100	97.3–100	**	1.3–2.7	80–85	94–98.7	**	1.3–4.0

*Surviving larvae could not complete the life cycle and failed to pupate properly.

**Single gene products are not in cultivation now.

Table 2. Detail and progress of founder lines established subjected to F_1screening at Sirsa (Haryana) location.

SN	Treatment	Isofemale screened at F_1 (No.)	Pupation by 13th day (%)	Percentage mortality (range)	Avg. wt. (range) of surviving larvae (g)
1	T1 (0.28 µg/ml)*	86	0	89.69 (60–100)	Negligible
2	T2 (0.028 µg/ml)**	86	0	28.56 (0–61.2)	10.34 (5.1–14)
3	T3 (0.0028 µg/ml)	86	0–29.5 (11.99)	3.97 (0–20)	31.02 (20.6–39.3)
4	Control	86	30–62.4 (48.52)	0.18 (0–3.3)	54.32 (42.2–71.6)

*No pupation and weight gain was observed in any of the line screened.

**No pupation observed.

(0.028 µg/ml) and T_3 (0.0028 µg/ml) was 0.0–61.2 % and 0.0–20.0 %, respectively, at 13 DAT. The surviving larvae observed in T2 at 13 DAT were dead by 19 DAT but in case of T3, some of the larvae developed into pupae and adult emergence was also observed (Table 2). In control the minimum mortality (0–3.3, average 0.18 %) and maximum pupation percentage (48.52 average, and range 30–62.4 %) was recorded with an average weight gained by the survivors was 54.32 g (42.2–71.6 g)

Of the remaining 181 lines that were established and used in the F_2 assays, 31 single paired lines produced F_1 eggs but did not reach the pupal stage due to development of either insect pathogens (viruses or bacteria) or moulds in the diet; offspring of F_1 from 39 lines were sib-mated but did not produce F_2 eggs or produce very few eggs and 25 single paired lines produced enough eggs which did not hatch to produce F_2 neonates. Finally we were able to complete F_2 screening on 86 isofemale lines as the availability of neonates to conduct screening was sufficient (Table 3). Over these 86 isofemale lines, an average of 31.8 pupae was formed. Among the 86 lines screened, an average of 13.87 F_1 males and 13.55 F_1 females were obtained for each isoline and sib-mated to get F_2 neonates. An average of 297.2 neonates per isofemale line was subjected to screening over three doses in three replicates to test for their susceptibility to Cry1Ac. Mortality in control or untreated diet was below 5 % throughout the bioassay. Among the 86 lines screened, F_2 larvae that survived up to 13 days ranged from 63.6 to 96.7 % at concentration of 0.0028 µg/ml (T3) of toxin. An averages of 4.1 % pupation per isofemale line occurred in T3 and larvae showed very poor growth with an average mass to 28.5 mg per isofemale line. This was twice less than the average mass observed in the control treatment per isofemale line.

Survival of F_2 neonates from the 86 isofemale lines screened at 0.028 µg/ml (T2) of toxin ranged from 4.3–14.1 %. Larvae exposed to T2 at 0.028 µg/ml did not pupate in any of the 86 lines until the end of the 13th day. Average mass was seven times less than the average body mass of surviving larvae in the control treatment per isofemale line.

Survival of F_2 neonates from the 86 isolines exposed to T1, the highest dose (0.28 µg/ml), ranged from 2.3–25 %. All larvae from 79 of the isolines died by the end 13 DAT. Only five individuals, from only two isofemale lines (# 368 & # 485) from F_2 generation survived on T1 (0.28 µg/ml of Cry1Ac) until 19–20 DAT (Table 4). These surviving

Table 3. Detail and progress of founder lines established and number of lines progressing through the F_2 screening at different locations.

Year	Location	Number of F_0 lines started [a]	Number of F_0 lines mated and produced F_1 eggs [b]	Number (%) of F_0 pairs died without egg laying (%)	F_1 larvae screened	Number of lines produced F_1 adults	Number of lines screened at F_2 larvae level	Percentage of F_0 pairs screened at F_2 level
2013–14	Sirsa	549	264	285 (51.91)	83	*150	86	32.50
2014–15	Sriganganagar	320	245	75 (23.43)	–	*206	110	44.89
2015–16	Mansa	210	138	72 (31.28)	–	*107	70	50.0

[a] Isofemale lines paired during September, October and November.

[b] Line was considered mated if a female laid fertile eggs.

*At Sirsa (Haryana) out of 150 lines, 39 lines did not produce F_2 eggs and 25 lines produced F_2 eggs but did not hatch at Sirsa, At Sriganganagar (Rajasthan) out of 206 lines, 36 lines did not produce F_2 eggs and 60 lines produced F_2 eggs but did not hatch and at Mansa (Punjab) out of 107 lines, 37 lines did not produce F_2 eggs.

Table 4. Survival (%) of F_2 generation at different doses on 13th day and details of surviving lines at highest dose of Cry toxin on 19th day.

District	Year	Dose (µg/ml of diet)	Lines surviving on 13th day of screening	Survival (%) on 13th day of screening	Lines surviving on 19th day of screening at highest dose	No. of live larvae at highest dose	Expected resistance allele frequency
Sirsa	2013–14	0.0028 (T3)	86	63.6–96.7			
		0.028 (T2)	86	4.3–14.1			
		0.28 (T1)	73	2.3–25	2	5	2.8×10^{-3}
Sriganganagar	2014–15	0.0013 (T3)	110	32.4–94.1			
		0.013 (T2)	89	28.2–94.1			
		0.13 (T1)	55	0.3–39.5	0	0	2.2×10^{-3}
Mansa	2015–16	0.0011 (T3)	70	20.0–61.7			
		0.011 (T2)	66	4.3–41.4			
		0.11 (T1)	49	2.2–35.7	0	0	3.4×10^{-3}

individuals did not reach instar 2 (no evidence of moulting), consequently they could not be subjected to further confirmatory bioassays. We observed S = 0 (the number of isofemale line with a major resistance) over the 86 families screened, so the expected resistance allele frequency in Sirsa population is $E(q) = 2.8 \times 10^{-3}$ (Table 4).

During 2014–15 at Sriganganagar (Rajasthan), a total of 1380 larvae were collected from fields of okra and non-Bt cotton. Of the total 320 F_0 male and female pairs made during September (145), October (110) and November (65), 245 (76.50 %) isofemale lines produced F_1 fertile eggs within 3–4 days (Table 2). A total of 110 F_2 two-parent line (220 feral individuals) of *E. insulana* were established where 75 lines died without producing eggs, 39 isofemale lines produced viable eggs which hatched but did not reach the pupal stage due to mould development in the diet. Thirty-six lines produced enough eggs and F_1 adults were sib-mated but did not produce F_2 eggs. Only offspring from 110 isofemale lines were screened for susceptibility to Cry1Ac toxin (Table 2). Survival at the highest dose (0.13 μg/ml of Cry1Ac) was 0.3 to 39.5 %. Surviving larvae had died by 15 DAT, without gaining any mass or exhibiting any feeding behaviour, suggesting the absence of any allele that confers resistance to Cry1Ac. We observed S = 0 over the 110 families screened. Hence, the expected resistance allele frequency in Sirsa population is $E(q) = 2.2 \times 10^{-3}$ (Table 4).

During 2015–16, at Mansa (Punjab), out of 210 isofemale lines established, only females from 138 isofemale lines laid fertile eggs. Individuals from 107 lines developed to the F_1 adult stage (Table 2). From these, only 70 isofemale lines produced neonates and were screened against Cry1Ac. At the highest dose T1 (0.11 μg/ml of Cry1Ac), larval survival (%) ranged from 2.2 to 35.7. None of the larvae had survived past 19 DAT (Table 3), and none of the lines produced larvae that developed to second instar. The expected resistance allele frequency in the Mansa population is $E(q) = 3.4 \times 10^{-3}$ (Table 4).

These results suggest that it is most unlikely that alleles conferring resistance to Cry1Ac toxin exist within the populations of *Earias insulana* (Boisduval) from the three locations of the North Cotton Growing Zone of India, *i.e.* Sirsa (Haryana), Sriganganagar (Rajasthan) and Mansa (Punjab).

DISCUSSION

No reports regarding field survival of the *E. insulana* is available till date in Bt-cotton genotypes under cultivation in this part of country and was also confirmed through the plant part bioassays conducted under laboratory conditions. The population of *E. insulana* collected from North Cotton Growing Zone of India was subjected to F_2 screening to detect the rare resistance allele. The studies were conducted on field-collected population of the pest as described by Andow & Alstad (1998). F_2 screen relies on inbreeding of field-collected individuals so that all recessive alleles are expressed in the F_2 generation where they can be screened for the phenotype of interest. Bt-cotton is effective against *Earias* spp. and bioassays conducted in the present studies on leaves and squares of the plant at 90–95 and 80–85 days after sowing resulted into good mortality of the 1-day-old larvae. Bt-cotton hybrids suffered low shoot damage by spotted bollworm, *Earias vittella* (Sholanki 2011), baseline toxicity of Cry1Ac toxin against spotted bollworm, *E. vittella* using a diet-based bioassay studied by Kranthi *et al*. (2004) indicated good susceptibility of the bollworm towards these Cry toxins. Initially the resistance development is rare so F_2 screen may be a useful monitoring technique for Bt cotton, especially for the detection of rare recessive resistant alleles easily done through collection of small number of target insects to detect potential susceptibility shifts (Andow & Alstad 1998). F_2 screen is probably the only current method available to detect rare recessive alleles. This method has been used to estimate the frequency of resistant allele to Cry toxins from *B. thuringiensis* in European corn borer (*Ostrinia nubilalis*) (Andow & Alstad 1998; Bourguet *et al*. 2003), rice stem borer (*Scirpophaga incertulas*) (Walker) (Bentur *et al*. 2000) and diamond back moth *Plutella xylostella* (L.) (Zhao *et al*. 2002). Two hundred and sixty-six lines of *E. insulana* screened in the present studies are theoretically sufficient to detect the resistant individual in the population. Andow & Alstad (1998) reported a sample of 100 female lines has a precision of ± 0.0025 for dominant alleles and ± 0.0025 for recessive resistance alleles.

The F_2 screening on field-collected population in the present studies was conducted using artificial diet as earlier reported in diamond back moth, validation of the F_2 screen method for each

insect-crop system should be conducted before the routine procedures used in the F_2 to detect rare Bt resistance alleles in field populations (Andow *et al*. 2000).

Based on effective mortality obtained through plant parts bioassays (on the leaves and squares of BG-II, Bt and non-Bt plants) under laboratory conditions followed by the F_2 screening expressing rare presence of resistant allele, Cry1Ac is still found to be effective against *E. insulana* populations. Furthermore, the changed cultivation pattern of BG-II cotton that has outdone the cultivation of Bt-cotton hybrids, with high toxin expression of Cry1Ac as well as Cry2Ab, also seemed to be incremental in maintenance of susceptibility of the Cry toxins to this larva in this region.

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