Frequency of alleles conferring resistance to Cry1Ac toxin of *Bacillus thuringiensis* in population of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) from India

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Detection of major resistance allele may have greater implication in resistance management. A field collected population of the cotton bollworm *Helicoverpa armigera* (Hübner) was used to test F$_2$ screening developed for monitoring the development of resistant alleles to Cry1Ac toxin of *Bacillus thuringiensis*. From four different locations viz., Bangalore, Dharwad, Raichur and New Delhi (IARI), *H. armigera* was collected and 146 isofemale lines were prepared from the field collected population. Out of which, 42 isofemale lines were used for F$_2$ screening of Cry1Ac resistant alleles using diet incorporation method. Other 65 isofemale lines were also prepared separately, for screening susceptibility in order to estimate fold resistance. In the F$_2$ screening, four positive lines were obtained. The fold resistance was observed to be 231.33. The F$_2$ screening results showed that the expected *Bt* resistance allele frequency in the collected populations was 0.085 with 95% confidence interval of 0.009 to 0.256 indicating that the F$_2$ screening method can be used to detect major alleles conferring resistance to *Bt* cotton in insect.

**Keywords**: *Bt* resistance, Cotton bollworm, Cry1Ac, F$_2$ screen, IPM

The cotton bollworm, *Helicoverpa armigera* (Hübner) is one of the most damaging cotton pests’ worldwide$^1$. It is a polyphagous pest with a wide host range. The pest causes losses up to 30 per cent of the total production of cotton in India. Traditionally, the pest is managed by spraying pesticides, 12-13 times. It has the ability to develop resistance to almost all the insecticides used for its control.

Transgenic cotton expressing *Bacillus thuringiensis* (Bt) insecticidal proteins is the primary strategy for controlling lepidopteran insects on cotton in India. It remains the only transgenic crop released successfully in India for the commercial cultivation in the year 2002. Though the insecticidal crystal (Cry) proteins produced by *Bacillus thuringiensis* (Bt) are effective against insect pests, evolution of resistance remains an issue of concern$^2$. Therefore, development of effective management plans has remained a major concern for sustainable use of *Bt* plants. The main strategy for delaying evolution of pest resistance to *Bt* crop relies on adoption of refugia which promotes survival of pests susceptible to *Bt* toxins$^3$. In addition to adoption of refugia other control tactics such as increasing the number and diversity of toxins$^4$; target pest spectrum and dose should also be integrated. Also, different *B. thuringiensis*-like subspecies and strains which produce potent Cry toxins can be identified and used for developing bioinsecticides$^5$. Large-scale cultivation of *Bt* cotton will place a high selection pressure on target insects, and consequently may hasten resistance evolution in pest population$^6$. Studies on mechanisms of resistance to Cry1Ac showed that proteases of resistant insects degraded Cry1Ac faster than those of susceptible insects, which led to the relative unavailability of toxin for binding and perforation of midgut epithelial membrane of the target insect$^7$. Therefore, the alternate way for managing development of *Bt* resistance is to detect continuously the frequency of resistant alleles presence in the field.

Monitoring of resistance allele is essential to ensure the durability of *Bt* cotton technology as an effective pest management tool. To produce accurate information about the presence/absence of early stages of resistance evolution, a reliable method that can detect resistant alleles in field populations is essential. Liu et al.$^8$ suggested using an F$_2$ screening method to detect rare resistance alleles in natural insect populations. When insecticide resistant alleles

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are rare in field populations and are recessive, a second generation (F₂) screen method is necessary to identify them because insects with resistant alleles that are collected from the field are expected to be heterozygous, and therefore, susceptible to the insecticide. Isolines derived from females and/or males can concentrate resistant alleles into homozygous F₂ offspring that can be distinguished by discriminating concentrations of insecticide. Keeping this in view, here, we attempted to monitor and detect the frequency of resistance alleles present in the field population of *H. armigera* so as to delay the development of resistance to *Bt* cotton.

**Materials and Methods**

*Helicoverpa armigera* were collected as eggs or larvae or pupae of host crops i.e. cotton and pigeon pea from different geographical locations viz., Bangalore, Dharwad, Raichur and New Delhi (IARI). The collected eggs were kept in incubator for hatching and the pupae were kept for adult emergence. The larvae were reared in the laboratory on a chickpea-based semi-synthetic meridic diet of Nagarkatti and Prakash adapted by Gujar et al. until pupation. The adults emerging from pupae were fed with 10% honey solution fortified with multivitamins throughout their egg-laying period. The pairing of adults was carried out by keeping the required number of adult pairs in each jar covered with rough cotton cloth for egg laying. The F₁ survived neonates were used for screening of tolerance. A constant temperature of 28±1°C and 80% RH were maintained throughout the rearing period.

F₂ tests generate isofemale lines that produce a proportion of individuals that are homozygous for haplotypes present in their field derived parents. The methods for the F₂ screen used herein are described by Andow and Alstad.

**Parental generation**

Field collected eggs or larvae were reared individually to adult in the laboratory. On pupation, pupae were collected, washed, sexed and set up in mating jars that contained pupae of the same sex from the same place. Emerged adult moths (six males and six females) from the same place were then placed into mating jars. The eggs collected from this mating were reared until the neonates emerged. Bioassays (at different doses ranging 0.001 to 3.0 µg of Cry1Ac/g of diet and at a diagnostic dose of 1 µg of Cry1Ac/g of diet) were carried out of these neonates at 28 ± 1°C and 80% RH in the laboratory using pure toxin of Cry1Ac in order to obtain tolerant strain of the field collected population against *Bt* Cry1Ac toxins.

Susceptibility of F₁ neonates of *H. armigera* to Cry1Ac was evaluated using diet incorporation method. Different 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 (5.5 cm in diameter; 1.5 cm in height). 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. Observations were taken at 7th day and LC₅₀ was calculated using MLP 3.01.

Screening at 1 µg of Cry1Ac/g: In this, the neonate larvae of *H. armigera* were treated with 1µg of Cry1Ac/g of diet and per cent survival at 7th day was recorded. The larvae which survived were considered as the tolerant strains. These tolerant strains are the parental generation. These tolerant larvae were reared individually to adult in the laboratory. On pupation, pupae were collected, washed, sexed and set up in mating jars that contained pupae of the same sex from the same location. Emerged adult moths (one male and one female) from the same place were then placed into mating jars as single pairs. One hundred and forty six isofemale lines (Batch I) were made. For this, pairings were done among tolerant strains, among normal strains and also between tolerant strain and normal population. The use of single pairs ensured that four haplotypes were tested for the presence of resistance alleles. This technique was preferred over testing field mated females because multiple mating occurs in this species, which complicates the interpretation of data.

**F₂ generation**

Jars housing the single pairs were checked at least every two days and fertile eggs were collected and stored at 10°C until the female had ceased ovipositing or at least 130 eggs were collected. The eggs were incubated at 25°C to promote hatching. It was aimed to rear 80-100 neonates from each pair individually. On pupation, pupae were collected, washed and sexed and equivalent numbers of males and females were kept in a single jar and allowed to sib-mate in bulk.

**F₂ generation**

Eggs were collected daily and stored at 10°C. When at least 200 eggs had accumulated over an interval of 4 days the eggs were placed in incubator at
25°C to promote hatching. The neonates obtained from these eggs were the F₂.

**F₂ Screen assay**

Assays were conducted in circular plastic containers (5.5 cm in diameter and 1.5 cm in height) which contain 3 g of rearing diet mixed with Cry1Ac toxin of concentrations of 1 µg and 10 µg of Cry1Ac/g of diet. After 7 days, the larvae were referred as alive which were able to undertake coordinated movement, and those which were moribund or unable to take coordinated movement or killed were discarded. From this screening, four promising lines which showed higher percentage of survivals were selected in order to obtain F₃ generation. The F₃ generation obtained from these selected lines were subjected to 10 µg of Cry1Ac/g of diet and the line with the highest percentage of survival was selected. This selected line was the resistant line (DN×DT).

**Isofemale line (Batch II)**

A total of 65 isofemale lines were made of which 45 isolines were from mixed normal population and 20 isolines were from the remaining survival lines of F₂ screening other than four selected promising lines. F₂ generation from isofemale lines (batch II) was obtained by following the same procedure as was done in isofemale line (batch I).

**F₂ screening**

F₂ neonates were screened using Cry1Ac toxin of concentrations of 1 µg and 10 µg of Cry1Ac/g of diet. From this screening, the line which showed least survival percentage in 1 µg of Cry1Ac/g of diet and 0% survival in 10 µg of Cry1Ac/g of diet were selected. This selected line was the susceptible line (S Line).

LC₅₀ values for different bioassays were calculated using Maximum Likelihood Programme, MLP 3.01. Resistance ratio was calculated by dividing the LC₅₀ of the resistant population with that of the most susceptible population. Bayesian inference was used to calculate the estimated allele frequency and the 95% Credible Intervals (C.I.)

$$E(p) = (S+1)/n+2$$
$$E(q) = (S+1)/4(n+2)$$

where, E(p)=Expected frequency of resistance line and E(q)= Expected frequency of resistance alleles where ‘n’ is number of isofemale lines tested, and ‘S’ is the number of survival successes, in the assumption of uniform prior distribution.

**Results**

Different concentrations of Cry1Ac toxin ranging from 0.001 to 3.0 µg/g were incorporated in diet and LC₅₀ was calculated (Table 1). Dharwad population, Bangalore population and IARI population showed LC₅₀ of 0.54 (FL 0.26-1.35), 0.51 (FL 0.26-0.85) and 0.008 (FL 0.001-0.02), respectively. The neonate larvae of *H. armigera* were treated with 1 µg of Cry1Ac/g of diet and the per cent survival at 7th day was recorded (Table 2). Raichur population showed highest percentage survival of 50.37% and the least percent survival were recorded in IARI population (0% survival).

**F₂ screening of isofemale line Batch I**

About 146 isofemale lines were made among tolerant strains, among normal strain and between tolerant and normal population and were designated as isofemale line batch I. F₂ neonates were screened by using 10 µg of Cry1Ac/g toxin. A total of 1746 F₂ neonates belonging to 57 isofemale lines were used for detecting the presence of alleles conferring resistance in *H. armigera* to Cry1Ac (Table 3). Though a total of 161 F₂ neonates belonging to 30 isofemale line survived in F₂ generation were recorded after subjecting.
to discriminating dose of 10 µg of Cry1Ac toxin, the estimated allele frequency was 0.078. Confidence interval at 95% for the expected allele frequency was 0.044 (lower limit) to 0.281 (upper limit) for the estimated allele frequency (Table 4). From this screening, four promising lines which showed higher percentage of survivals were selected in order to obtain F₃ generation. The four promising lines were BN × BN (f), DT × DT (d), DN × DT (e) and DN × DT (f). The F₃ generation obtained from these selected lines were subjected to 10 µg of Cry1Ac toxin and the line with the highest percentage of survival viz., DN×DT (e) was selected. This selected line DNxDT (e) was the resistant line (Table 5).

**F₂ screening of isofemale line Batch II**

A total of 2300 F₂ neonates from 44 isofemales line during were screened with a discriminating dose of 1 and 10 µg/g of Cry1Ac/g toxin for detecting the presence of alleles conferring resistance in *H. armigera* to Cry1Ac (Table 3). A total of 161 F₂ larvae belonging to 21 isofemale lines survived after 7 days of treatment. The survivors were reared till pupation. Each lines were reared and maintained till F₃ generation. These were utilized for confirming the presence of resistance alleles. The estimated allele frequency was 0.195. Confidence interval at 95% for the expected allele frequency was 0.0201 (lower limit) to 0.586 (upper limit) for the estimated alleles frequency (Table 4).

The most susceptible line (S line) was selected based on the per cent survival rate. The S line showed

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**Table 4 — Expected resistance allele frequency of F₂ screen for identifying Bt resistance alleles in field population of Helicoverpa armigera collected from different location of India**

<table>
<thead>
<tr>
<th>Isofemale line</th>
<th>No. of isofemale lines</th>
<th>Total no. of isoline screen in F₂</th>
<th>No. of isoline selected in F₂ screening</th>
<th>Frequency of resistant line [E(p)]</th>
<th>Frequency of resistance allele [E(q)]</th>
<th>Confidence level at 95% (Lower limit – upper limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch I</td>
<td>146</td>
<td>30</td>
<td>4</td>
<td>0.156</td>
<td>0.078</td>
<td>0.044 – 0.281</td>
</tr>
<tr>
<td>Batch II</td>
<td>65</td>
<td>44</td>
<td>8</td>
<td>0.391</td>
<td>0.195</td>
<td>0.020 - 0.586</td>
</tr>
<tr>
<td>Overall</td>
<td>211</td>
<td>74</td>
<td>12</td>
<td>0.171</td>
<td>0.085</td>
<td>0.009 – 0.256</td>
</tr>
</tbody>
</table>

**Table 5 — Evolution of Cry1Ac resistance in the *H. armigera* (DNxDT) population**

<table>
<thead>
<tr>
<th>Generation</th>
<th>No. selected</th>
<th>Selection dose(µg/g)</th>
<th>% survivals at 7th day</th>
<th>LC₅₀ (FL at 95%)</th>
<th>Chi square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field collected population</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IARI</td>
<td>380</td>
<td>1</td>
<td>0</td>
<td>0.008 (0.001 – 0.02)</td>
<td>14.66</td>
</tr>
<tr>
<td>Dharwad</td>
<td>400</td>
<td>1</td>
<td>10.25</td>
<td>0.54 (0.26 – 1.35)</td>
<td>9.13</td>
</tr>
<tr>
<td>Bangalore</td>
<td>2450</td>
<td>1</td>
<td>4.97</td>
<td>0.51 (0.26 – 0.85)</td>
<td>2.38</td>
</tr>
<tr>
<td>Raichur</td>
<td>135</td>
<td>1</td>
<td>50.37</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F₀ (Parent)</td>
<td>No dose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F₁</td>
<td>No dose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F₂</td>
<td>1 ppm</td>
<td>10 ppm</td>
<td>1, 10</td>
<td>1 ppm 10 ppm</td>
<td>-</td>
</tr>
<tr>
<td>BNxBN</td>
<td>380</td>
<td>340</td>
<td>26.32</td>
<td>13.24</td>
<td>14.28</td>
</tr>
<tr>
<td>DTxDT</td>
<td>220</td>
<td>242</td>
<td>26.82</td>
<td>6.20</td>
<td>26.22</td>
</tr>
<tr>
<td>DTxDN</td>
<td>478</td>
<td>416</td>
<td>25.94</td>
<td>6.97</td>
<td>23.33</td>
</tr>
<tr>
<td>DNxBDN</td>
<td>98</td>
<td>90</td>
<td>44.90</td>
<td>23.33</td>
<td>44.90</td>
</tr>
<tr>
<td>DNxDT</td>
<td>350</td>
<td>348</td>
<td>29.43</td>
<td>5.17</td>
<td>26.22</td>
</tr>
<tr>
<td>DNxDN</td>
<td>100</td>
<td>100</td>
<td>26</td>
<td>13</td>
<td>26.22</td>
</tr>
<tr>
<td>BNxDN</td>
<td>100</td>
<td>90</td>
<td>30</td>
<td>3.33</td>
<td>26.22</td>
</tr>
<tr>
<td>BTxBT</td>
<td>32</td>
<td>20</td>
<td>37.5</td>
<td>15</td>
<td>26.22</td>
</tr>
<tr>
<td>F₃</td>
<td>BNxBN(f)</td>
<td>120</td>
<td>27.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DTxDT(d)</td>
<td>120</td>
<td>14.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DNxDT(e)</td>
<td>160</td>
<td>45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DNxDT(f)</td>
<td>80</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

[BN, Bangalore Normal; BT, Bangalore Tolerant; DN, Dharwad Normal; DT, Dharwad Tolerant]
Table 6 — Fold resistance of resistant strain (DN×DT) against susceptible strain (S Line)

<table>
<thead>
<tr>
<th>Population</th>
<th>LC₅₀</th>
<th>Fiducial Limit (95%)</th>
<th>Slope ± SE</th>
<th>Chi square</th>
<th>Resistant Ratio</th>
<th>7th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN×DT (Resistant line)</td>
<td>6.94</td>
<td>1.15-9.46</td>
<td>4.0</td>
<td>2.44</td>
<td>231.33</td>
<td></td>
</tr>
<tr>
<td>M4 line (Susceptible)</td>
<td>0.03</td>
<td>0.005-0.09</td>
<td>0.46</td>
<td>7.02</td>
<td>1</td>
<td>±0.12</td>
</tr>
</tbody>
</table>

26.6% survival at 1 µg of Cry1Ac/g of diet and 0% survival at 10 µg of Cry1Ac/g of diet. The fold resistance was calculated by comparing the LC₅₀ of resistant strain {DN×DT(e)} against susceptible strain (S line). The fold resistance was observed to be 231.33 (Table 6).

Discussion

To monitor the presence of resistant alleles in a field population, a reliable method that can detect resistant alleles is essential. When insecticides resistant alleles are rare in field population and are recessive, a second generation (F₂) screen method is necessary to identify them because insects with resistant alleles that are collected from the field are expected to be heterozygous, and therefore, susceptible to the insecticide. Isolines derive from females and/or males can concentrate resistant alleles into homozygous F₂ offspring that can be distinguished by discriminating concentrations of insecticides.

The F₂ screen was designed to estimate the frequency of rare resistance alleles in natural population. The concept of F₂ screen is a valid method for obtaining rare Bacillus thuringiensis alleles. This is a labour intensive method but it offers reliability for detection of insect resistance. The methods for the F₂ screen as described by Andow and Alstad were used herein to detect the Cry1Ac resistance in Helicoverpa armigera collected from the field. The F₂ screening was used in the European corn borer (Ostrinia nubilalis), Diatraea saccharalis, Helicoverpa armigera (Hubner), and Spodoptera frugiperda for detecting resistance to Bt transgenic crops.

In this experiment, Helicoverpa armigera was collected at various stages (eggs, pupae, larvae) from Bangalore, Dharwad, Raichur and New Delhi (IARI). The neonate larvae obtained from these populations were treated with 1 µg of Cry1Ac/g of diet. Raichur population showed the highest percentage survival of 50.37% and the least percent survival were recorded in IARI population (0% survival). The 0% survival in New Delhi (IARI) population may be due to various factors such as disease (NPV), very low resistance of the heterozygous collected field population. The size of the population collected also influence the 0% survival.

In the F₂ screening, 146 isofemale lines were made out of which 4 promising resistant lines viz., BN × BN (f), DT × DT (d), DN × DT (e) and DN × DT (f) were observed. Xu et al.20 reported 15 out of 278 isofemale lines carried resistance alleles to Bt cotton in F₂ screen of field populations of Helicoverpa armigera in Qixuan county of China.

In order to obtain accurate result, the F₃ generation obtained from these 4 selected lines were treated with 10 µg of Cry1Ac/g of diet and the line with the highest percentage survival viz., DN×DT (e) was selected to calculate fold resistance. This selected line DN×DT (e) was the resistant line. The fold resistance was observed to be 231.33.

The F₂ screening is not only useful for estimating frequency of rare recessive alleles but it also recovers those alleles for additional testing. F₂ screening has the capability of detecting alleles at discrimination dose assay. F₂ screening was conducted by Pan et al.21 to detect the resistance alleles frequency in the field population of H. armigera in Qixuan county during 2010 to 2012. The annual values of resistance alleles frequency were 0.0389 (95% CI: 0.0064–0.0714), 0.0372 (95% CI: 0.0036–0.0708) and 0.0781 (95% CI: 0.0318–0.1244), respectively. Dourado et al.22 conducted F₂ screen to assess risk of resistance to Cry1Ac protein in H. armigera to Bt Soyabean in Brazil. The estimated allele frequency was reported to be 0.0011. Mahon et al.18 reported that the frequency of resistance alleles for conferring to Cry1Ac was <0.0003 with 95% credibility interval between 0 and 0.0009 in field collected population of H. armigera.

Reference


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