Insect Resistance and Field Evaluation Studies of Transgenic Cotton Lines Harboring Insecticidal Gene (cry1AB)

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ABSTRACT

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Background: The transgenic lines CEMB-3, CEMB-11, CEMB-16 and CEMB-17 developed as an independent transformation event from cotton cultivar MNH-93 harboring insecticidal gene (cry1Ab) were investigated for the resistance against targeted insect pests under field conditions. Objective: The efficacy of the introduced gene (cry1Ab) in transgenic cotton lines was evaluated using various approaches like western dot blot, leaf biotoxicity and artificial field infestation assay. Furthermore, various agronomic (seed cotton yield, plant height, number of bolls per plant, number of sympodial and monopodial branches and average boll weight) and fibre characteristics of transgenic lines along with non transgenic control variety were recorded. Results: The expression of introduced gene was found to be variable as quantified by Image Quant software, it ranged from 0.2 to 0.4 percent of the total soluble protein; however it conferred full protection against targeted insect pests. Laboratory leaf biotoxicity and artificial field assays were also performed to evaluate the efficacy of insecticidal genes against targeted insect pests by calculating the mortality %age of Helicoverpa larvae. Most of the transgenic lines showed up to 60-100% resistance against targeted insect pests. Morphological, agronomic and fibre data of these transgenic lines was recorded and analyzed statistically. Conclusion: Based on molecular, agronomic and fibre characteristics data, it was concluded that these transgenic lines are an excellent source of germplasm to be used in conventional breeding programme.

INTRODUCTION

The application of biotechnology tools to agriculture has allowed scientists to transform plants without the need for sexual compatibility between species, thus establishing the possibility of rapidly producing new crop varieties with traits beneficial to human health and the environment. Plants have been transformed successfully to improve their pest and disease resistance, herbicide tolerance, nutritional qualities, and stress tolerance. The rapid transformation of plants with enhanced traits holds great promise for increased efficiency of land use, a development that can help feed the expanding world population using sustainable growing practices [1, 2].

Cotton is an important economic and fibre crop worldwide and likewise it is of immense importance for Pakistan’s economy because of its major share in GDP [3]. Therefore the cotton plant has always been subjected to extensive research aimed at improving its genetic architecture to obtain greater benefits. The modern techniques of biotechnology offered potential to overcome this problem by the introduction of genes encoding insecticidal proteins from Bacillus thuriengenesis into plants to develop insect resistance. Transgenic cotton expressing insecticidal proteins from B. thuriengenesis has been one of the most rapidly adopted GM crops in the world [4, 5, 6] containing cry gene(s) such as cry1Ac, cry1Ac + cry2Ab or cry1Ac + cry1F. In 2010, genetically modified crops were cultivated on 148 million hectares globally. In Pakistan, insect resistant cotton was grown on 2.4 million hectares out of 2.8 million hectares allocated land [7].

Transgenic Bt cotton has been documented as an effective strategy in to combat lepidopteran pests, and is highly beneficial to the growers and the environment by reducing chemical insecticide sprays and preserving population of beneficial arthropods [8, 9]. Transgenic Bt cotton has become an increasingly important tool for...
farmers around the world. Large and small acreage farmers benefit from increased productivity, convenience, and time savings. The majority of farmers using Bt cotton around the world are smallholder farmers who may reap economic, environmental, and social benefits from adoption of this important tool for agriculture [10].

The present study was conducted to evaluate advance transgenic lines (CEMB-3, CEMB-11, CEMB-16 and CEMB-17) harboring insecticidal gene (cry1Ab) developed from local cotton cultivar MNH-93 by Agrobacterium mediated transformation. The various molecular and agronomic approaches were adopted to evaluate these transgenic lines along with control (CEMB-C: MNH-93) under field conditions. Experimental fields were surrounded with 5 rows of untransformed MNH-93 and CIM-482 (another locally approved cotton cultivar) to serve as refugia to prolong insect resistance. Sorghum bicolor was grown around the field to isolate field from surroundings following recommendation and biosafety guidelines [11].

MATERIALS AND METHODS

MNH-93 (a good yielding local cotton variety but susceptible to lepidopterans insect pests) was transformed with Bacillus thuriengenis gene (cry1Ab) using Agrobacterium tumefaciens strain C58C1 [12]. The positive transgenic cotton plants out of these transformation events were further used to develop pure lines in field and green house for successive generations. After molecular screening and recording of agronomic data, seed of the transgenic plants having same agronomic characteristics was bulked; hence lines were further named as CEMB-3, CEMB-11, CEMB-16 and CEMB-17.

The transgenic lines along with control (CEMB-C) were planted using randomized complete block design with three replications. The plant to plant and row to row distance was kept 30 and 75cm, respectively. No insecticidal spray against lepidopteran insects was applied. The crop was however, protected from the attack of sucking pests by applying suitable insecticides. All agronomic practices were adopted as per recommendation of the Punjab Agriculture Department, Punjab, Pakistan.

Estimation of Insecticidal Protein (cry1Ab):

The insecticidal protein (cry1Ab) contents in transgenic lines were quantified. About 0.5 gram fresh terminal leaves was ground in liquid nitrogen. One millilitre protein extraction buffer (0.04 M EDTA, 10% glycerol, 0.15 M NaCl, 0.01 M Tris-CIH, 10 mM NH4Cl, 20 mM PMSF phenylmethylsulfonyl fluoride), 10 mM DTT (dithiotheritol) was added and centrifuged at 14,000 rpm at 4°C. The supernatant was harvested. The protein concentration was estimated by comparing OD value at 595 nm with an already plotted curve of known concentrations of BSA (bovine serum albumin). An equal amount of protein of each sample (10ng) was loaded directly onto a Hybond-C membrane and the procedure of western blotting was followed. After processing, the blot was scanned and the Bt contents quantified using ImageQuant TL software {Amersham BioSciences (Pvt) Limited}.

Leaf Biotoxicity Assay:

The laboratory biotoxicity assays of transgenic and non transgenic cotton leaves with Helicoverpa larvae (2nd instar) were conducted to evaluate the efficacy of endotoxins against targeted insect pests. The five fresh leaves from the upper, middle and the lower portion of each transgenic and non transgenic lines were detached and placed on moist filter paper in a petri plate. One 2nd instar larva, pre-fasted for 4-6 hours, was released in the each plate and allowed to feed on the leaf. The data on insect mortality were taken on daily basis up to fourth or fifth day. The mortality rates of larvae were calculated as follows:

\[
\%\text{Mortality} = \frac{\text{No.of dead larvae}}{\text{Total no. of larvae}} \times 100
\]

Artificial Field Infestation:

Artificial field infestation assays were conducted with 1st/2nd instar american bollworm (Helicoverpa armigera) e.g. placing the larvae on leaves of the plants with the help of camel hair brush. For this purpose, a large number of Helicoverpa larvae were reared in the CEMB insectary. Ten larvae of 1st/2nd instar were placed in a small glass vial and tied to the middle of the stem of each plant in the experiment. The glass vial was opened afterwards to let the insects travel to all parts of the plant. The plant health condition and number of bolls per plant were recorded before and after each infestation. After 5-6 days of infestation, boll damage %age was calculated.

Agronomic Characteristics of Transgenic Lines:

Along with molecular aspects, agronomic characteristics of the transgenic lines along with control were recorded. Different morphological and agronomic chracteristics including seed cotton yield (g), plant height (cm), number of bolls per plant, number of sympodial and monopodial branches and average boll weight was recorded. The data collected on the above-mentioned characters were subjected to analysis of variance and mean comparisons.
**Ginning Outturn (%age) and Fibre Characteristics:**

- The produce of each plant was cleaned and dried. A sample of 100 grams from each plant was taken and ginned separately with a Single Roller Electric Gin. The lint obtained was weighed and the following formula was used to calculate Ginning Outturn (GOT):-
  
  \[ \text{GOT %age} = \frac{\text{Weight of lint}}{\text{Weight of seed cotton in the sample}} \times 100 \]

- Fibrograph Model 530 (electronic) was used for measurement of the staple length. The Fibre Fineness was measured with the help of “Sheffield Micronaire-complete with Air Compressor” and expressed in microgram per inch. The data collected on the above-mentioned fibre characters were subjected to analysis of variance and mean comparisons.

**RESULTS AND DISCUSSION**

Although it is well documented now that transgenic cotton lines harboring insecticidal proteins from *Bacillus thuringiensis* has led to the reduction of conventional broad-spectrum pesticides to a great extend against targeted pests. However, there is always a risk that insects could develop resistant to this toxin after prolonged and repeated field exposure. One of the most practical approach to prolong the effectiveness of *Bt* crops against targeted pests has been reported as refugia strategy and pyramiding of two or more genes in the same cultivar [13].

- The insecticidal protein (*cry1Ab*) contents in transgenic lines were quantified using Image Quant TL software (Amersham BioSciences). The data were subjected to Analysis of Variance and it was revealed that the transgenic lines had highly significant differences among themselves (Table-2). The mean comparisons given in the (Table-4) showed that these transgenic lines were statistically different from the control. Among the transgenic lines, CEMB-17 had the highest level of *Bt* contents (0.298% of the total protein). The line CEMB-11 and CEMB-16 had 0.287% and 0.261% of the total protein respectively, and were statistically not different from CEMB-17. Similarly, CEMB-3 had the lowest level of *Bt* content (0.216%) among all transgenic lines but it was statistically not different from CEMB-11 and CEMB-16 (Table-4). The variation in insecticidal gene expression among transgenic lines was found variable; the results are in confirmation of the findings of Sachs et al., [14] who found that *cry1A* gene expression was variable and strongly influenced by environmental factors. These results are also in agreement with previous studies conducted by Wu et al., [15], Mahon et al., [16], Xia et al., [17], Adamczyk et al., [18], Bakhsh et al., [19, 20, 21] who have reported inconsistency in insecticidal gene expression over the crop growth period and in various generations.

**Table 1:** Analysis of Variance: Mean Squares for Different Characters of the *Bt* Trials 2004-2005.

<table>
<thead>
<tr>
<th>Source Of Variation</th>
<th>Yield Per Plant</th>
<th>Plant Height</th>
<th>No. Of Monopodial Brancahes</th>
<th>No. Of Sympodial Branches</th>
<th>Natural Infestation Of Spotted Bollworm</th>
<th>Field Bioassay (Heliothis)</th>
<th>Got %Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>224.20 *</td>
<td>413.22 *</td>
<td>12.89 *</td>
<td>9.33 *</td>
<td>0.19 **</td>
<td>0.01 ns</td>
<td>0.10 ns</td>
</tr>
<tr>
<td>Genotypes</td>
<td>24.59 ns</td>
<td>653.30 **</td>
<td>6.09 ns</td>
<td>13.11 **</td>
<td>0.09 **</td>
<td>0.01 ns</td>
<td>0.04 **</td>
</tr>
<tr>
<td>Error</td>
<td>58.96</td>
<td>614.24</td>
<td>2.23</td>
<td>2.59</td>
<td>0.02</td>
<td>0.01</td>
<td>2.11</td>
</tr>
</tbody>
</table>

** indicates significant differences at P< 0.05 probability level.

* indicates significant differences at P< 0.01 probability level.

ns = Non-significant

The analysis of variance was done with the help of the techniques mentioned by Steel and Torrie [28].

**Table 2:** Analysis of Variance: Mean Squares for Different Characters of The *Bt* Trials 2004-2005.

<table>
<thead>
<tr>
<th>Source Of Variation</th>
<th>No. Of Bolls Per Plant</th>
<th>Boll Weight</th>
<th>Staple Length</th>
<th>Fibre Fineness</th>
<th>Bt Protein Content</th>
<th>Lab Bioassay (Heliothis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>34.77 ns</td>
<td>0.69 ns</td>
<td>0.65 ns</td>
<td>0.05 ns</td>
<td>0.00470 ns</td>
<td>24.00 *</td>
</tr>
<tr>
<td>Genotypes</td>
<td>12.3 ns</td>
<td>1.15 ns</td>
<td>0.84 ns</td>
<td>0.03 ns</td>
<td>0.045343 **</td>
<td>181.73 *</td>
</tr>
<tr>
<td>Error</td>
<td>13.07</td>
<td>0.42</td>
<td>0.54</td>
<td>0.04</td>
<td>0.001435</td>
<td>43.33</td>
</tr>
</tbody>
</table>

** indicates significant differences at P< 0.05 probability level.

* indicates significant differences at P< 0.01 probability level.

ns = Non-significant

The analysis of variance was done with the help of the techniques mentioned by Steel and Torrie [28].

To determine the efficacy of the transgenic lines against targeted insect pests, leaf bioassocity and artificial field infestation were conducted. The laboratory bioassocity assays with 2nd Instar *Helicoverpa* larvae showed that expression of the introduced genes (*cry1Ab*) transgenic lines is sufficient to kill the targeted insects. In laboratory bioassocity assay, most of the transgenic lines were showing 60-100% larval mortality while no any larval mortality was observed in non-transformed control plants (Table-4). For artificial field infestation, American bollworm (*Helicoverpa armigera*) was reared in the laboratory and released in the transgenic field to study the insect resistance level of the *Bt* cotton lines in comparison with the control lines. For this purpose, the...
The number of surviving Helicoverpa larvae after one week of 2nd and 3rd field infestations respectively was recorded. The number of surviving larvae in control line was much higher than Bt lines. The Bt line CEMB-11 showed 67% less surviving Helicoverpa population than control. The lines CEMB-3, CEMB-16 and CEMB-17 had 33%, 53% and 20% less insect population of surviving Heliothis larvae than the control line. The mean squares data have been presented in (Table-3) which however, indicated that there were statistically non-significant differences among the transgenic lines.

Difference in resistance level in laboratory biotoxicity and artificial infestation assay can be attributed to the variation in expression of insecticidal gene in transgenic progenies. These results were in agreement with the previous studies conducted by various researchers (Fitt et al.,[22], Greenplate et al.,[23], Chen et al.,[24]; Mahon et al.,[16], Xia et al.,[17], Manjunatha et al.,[25], Bakhsh et al.,[19, 21] have reported inconsistency in insecticidal gene expression in cotton.

The transgenic line CEMB-3 gave 22.93 % more yield than the control line. Similarly, CEMB-11 and CEMB-17 gave 9.15% and 10.86% more yield than control, respectively. The line CEMB-11, however, showed 3% less yield than the control. Statistically, the transgenic lines had non-significant differences among themselves in yield per plant (Table-1). It means that the Bt gene had exerted no significant positive or negative effect on seed cotton yield. It was as per expectations because the Bt gene is not a yield contributing gene.

### Table 3: Mean Comparisons for Different Characters of the Bt Trials.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Yield Per Plant (G)</th>
<th>Plant Height (Cm)</th>
<th>No. Of Monopodial Branches Per Plant</th>
<th>No. Of Sympodial Branches Per Plant</th>
<th>Natural Infestation Of Spotted Bollworm (No. Of Insects Per Plant)</th>
<th>Field Bioassay (Heliothis) (No. Of Insects Per Plant)</th>
<th>Got %Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEMB-3</td>
<td>24.44 a</td>
<td>110.00 b</td>
<td>4.08 a</td>
<td>12.32 b</td>
<td>0.69 b</td>
<td>0.10 a</td>
<td>34.23 a</td>
</tr>
<tr>
<td>CEMB-11</td>
<td>21.70 a</td>
<td>95.17 b</td>
<td>3.98 a</td>
<td>12.47 b</td>
<td>0.76 b</td>
<td>0.05 a</td>
<td>34.28 a</td>
</tr>
<tr>
<td>CEMB-16</td>
<td>19.31 a</td>
<td>102.50 b</td>
<td>5.82 a</td>
<td>11.82 b</td>
<td>0.76 b</td>
<td>0.07 a</td>
<td>34.15 a</td>
</tr>
<tr>
<td>CEMB-17</td>
<td>22.04 a</td>
<td>96.83 b</td>
<td>4.42 a</td>
<td>13.50 b</td>
<td>0.72 b</td>
<td>0.12 a</td>
<td>34.04 a</td>
</tr>
<tr>
<td>CONTROL</td>
<td>19.88 a</td>
<td>131.47 a</td>
<td>6.12 a</td>
<td>9.52 a</td>
<td>0.99 a</td>
<td>0.15 a</td>
<td>31.29 b</td>
</tr>
</tbody>
</table>

* Means followed by the same letter are statistically non-significant

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>No. Of Bolls Per Plant</th>
<th>Boll Weight (G)</th>
<th>Staple Length (Mm)</th>
<th>Fibre Fineness (µg/In)</th>
<th>Bt Protein (%Age Of Total Protein)</th>
<th>Lab Bioassay (Heliothis Mortality %Age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEMB-3</td>
<td>15.00 a</td>
<td>2.93 a</td>
<td>24.96 a</td>
<td>4.63 a</td>
<td>0.215987 b</td>
<td>41.333 a</td>
</tr>
<tr>
<td>CEMB-11</td>
<td>12.50 a</td>
<td>2.91 a</td>
<td>25.91 a</td>
<td>4.59 a</td>
<td>0.287177 ab</td>
<td>33.333 ab</td>
</tr>
<tr>
<td>CEMB-16</td>
<td>11.03 a</td>
<td>4.02 a</td>
<td>26.13 a</td>
<td>4.80 a</td>
<td>0.260833 ab</td>
<td>40.667 a</td>
</tr>
<tr>
<td>CEMB-17</td>
<td>13.37 a</td>
<td>2.70 a</td>
<td>25.87 a</td>
<td>4.64 a</td>
<td>0.298333 a</td>
<td>34.667 ab</td>
</tr>
<tr>
<td>CONTROL</td>
<td>16.17 a</td>
<td>2.38 a</td>
<td>26.35 a</td>
<td>4.82 a</td>
<td>0.000000 c</td>
<td>22.000 b</td>
</tr>
</tbody>
</table>

* Means followed by the same letter are statistically non-significant

To establish the level of significance among various genotypes, New Duncan’s Multiple Range Test (5% level) was applied to compare the means for all parameters.

The number of surviving Helicoverpa larvae after one week of 2nd and 3rd field infestations respectively was recorded. The number of surviving larvae in control line was much higher than Bt lines. The Bt line CEMB-11 showed 67% less surviving Helicoverpa population than control. The lines CEMB-3, CEMB-16 and CEMB-17 had 33%, 53% and 20% less insect population of surviving Heliothis larvae than the control line. The mean squares data have been presented in (Table-3) which however, indicated that there were statistically non-significant differences among the transgenic lines.

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A perusal of the (Table-1) indicates that the lines did not differ in respect of number of monopodial branches per plant. The number of monopodial branches per plant, however, ranged from 3.98 in CEMB-11 to 6.12 in control. The results clearly indicated that the transformation had not affected this character of the plants. The un-alteration of the characters other than for which transformation was done is highly desirable. The
number of sympodial branches per plant ranged from 9.52 in control to 13.50 in CEMB-17. The analysis of variance showed that the transgenic lines differed highly significantly in number of sympodial branches per plant (Table-1). The Duncan’s New Multiple Range Test was applied to separate the means. The mean comparison presented in (Table-3) showed that the means of all transgenic lines differ from the mean of the control. The average numbers of bolls per plant ranged from 11.03 in CEMB-16 to 16.17 in CEMB-control. The analysis of variance data have been presented in (Table-4). The number of bolls per plant differed non-significantly among the transgenic lines. Similar results were reported by Bakhsh et al. [26] who found significant differences in transgenic lines transformed with two insecticidal genes (cry1Ac & cry2a) as compared to control variety. The boll weight ranged from 2.38g to 4.02g. The lowest boll weight (2.38g) was recorded in control line whereas the highest (4.02g) was recorded in the CEMB-16 (Table-4). However, the transgenic lines had non-significant differences among themselves regarding boll weight (Table-2).

The Ginning Outturn Percentage ranged from 31.29 (control) to 34.28 (CEMB-3). The mean squares given in the (Table-3) shows that the transgenic lines differed highly significantly among themselves. When the means were compared following DMR test, it was revealed that CEMB-3 had highest GOT %age (34.28), followed by CEMB-11 (34.23), CEMB-16 (34.15) and CEMB-17 (34.04). The transgenic lines were statistically non-significantly different from each other (Table-1). The lowest GOT %age (31.29) was recorded in the control line which was significantly different from all transgenic lines. The staple length ranged from 24.96mm (CEMB-3) to 26.35mm (control). The mean squares presented in the (Table-4) showed that the transgenic lines differed non-significantly from one another in respect of staple length. The fibre fineness ranged from 4.59 µg/in (microgram per inch) for CEMB-11 to 4.82 µg/in for CEMB-Control. The mean squares for fibre fineness presented in (Table-2) showed that the transgenic lines did not differ significantly from each other.

The transgenic lines were also studied for a number of other characters besides the primary insect resistance traits. The lines were found to be statistically different at a significant level from the control in Number of Sympodial Branches per Plant, Plant Height and Ginning Outturn Percentage (GOT). A careful study of the data showed that after transformation, the Bt plants had got other positive effects as well in addition to insect resistance, i.e. the GOT percentage and Number of Sympodial Branches per Plant had increased significantly from the non-Bt parent. The increase in these two characters is much desirable from the breeders’ point of view. Similarly, the Bt plants had shown a significant reduction in height which is also a desirable change. Based on the molecular data obtained from the laboratory and agronomic data recorded from the field it is believed that these transgenic progenies are an excellent source of germplasm to be used in conventional breeding programme.

REFERENCES


