

## Insect Resistance and Risk Assessment Studies in Advance Lines of Bt Cotton Harboring *Cry1Ac* and *Cry2A* Genes

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**Abstract:** Advance generations of different transgenic lines of locally approved cotton cultivar CIM-482 expressing two insecticidal genes (*Cry1Ac* and *Cry2A*) were confirmed for the integration and expression of insecticidal genes, evaluated for the resistance against cotton boll worms especially american boll worm (*Heliothis armigergera*) under field condition for three consecutive years. Homozygous lines showing high insect resistance, morphological and agronomic characteristics were selected, risk assessment studies of these transgenic lines were also undertaken. Laboratory biotoxicity assays were performed to calculate the mortality %age of *Heliothis* larvae as a result of expression of insecticidal genes. Most of the transgenic lines showed up to 70-100% resistance against targeted insect pests. After artificial infestation of targeted insects, transgenic lines showed significant resistance levels. Data were recorded in terms of boll damage percentage. Morphological and yield data analysis were recorded. All the characters were stably inherited in advance generations. Experiments conducted to evaluate potential risk of transgenic lines on non target insects and soil organisms showed that these lines have no significant effect on non target insects (insects belonging to orders other than Lepidoptera). No any evidence of transgene flow was found from crop to soil organisms.

**Key words:** Insect resistance • Risk assessment • Bt Cotton • *Cry1Ac* and *Cry2A*

### INTRODUCTION

Cotton is the most important cash crop and backbone of textile industry of the world. Pakistan is the fifth largest producer of cotton in the world, the third largest exporter of raw cotton, the fourth largest consumer of cotton and the largest exporter of cotton yarn.

Cotton being “Golden fibre” of the world is susceptible to attack by more than 15 economically important insects, the major Lepidopteron being, American Boll worm (*Heliothis armigera*), Pink Boll worm (*Pectinophora gossypiella*), Spotted Boll worm (*Earias insulana/vitella*), Army Bollworm (*Spodoptera lithura*). Cotton breeders have continuously been looking to improve cotton through conventional plant breeding which has introduced numerous improvements in crop yield during past centuries. However, resistance to insect pests and diseases does not exist in available germplasm; this has led to a limit in availability of new genetic information into plants and to create plant varieties with novel characters through plant breeding techniques [1].

Current approaches to cotton improvements include use of genetic engineering that is gaining momentum in developed as well as developing countries.

The most significant breakthrough in plant biotechnology is the development of the techniques to transform genes from unrelated sources into commercially important crop plants to develop resistance against insect pests [2, 3]. *Bacillus thuringiensis* (*Bt*) is perhaps, the most important source of insect resistant genes. Genes from *B. thuringiensis* encode for crystal proteins, which are toxic against larvae of different insects, e.g. Lepidopterans [4-6], Coleopterans [7, 8] and Dipteran insects [9]. These genes are generally safe for human consumption [10].

Bt cotton is considerably effective in controlling lepidopteran pests and is highly beneficial to the grower and the environment by reducing chemical insecticide sprays and preserving population of beneficial arthropods [11, 12]. A variety of issues regarding risk assessment i.e. the effect of transgenic crops on non target insects, horizontal gene flow, vertical gene flow and development

of resistance against toxins in targeted insect pests must be considered when developing insect resistant transgenic plants.

To prolong the resistance development time, it is recommended that lines expressing two Bt genes should be released in environment [4]. Studies have shown that *Cry1Ac* and *Cry2A* is good combination for lepidopterans insects [13]. In addition to gene pyramiding described above, the possibility of target insect evolving resistance could also be mitigated through the use of planting refugia crop all around the transgenic crop to dilute the insect resistance.

Gene pyramiding has been hailed as a lasting Bt resistance management strategy [14, 15]. However, it reveals that pyramiding was developed as a practical strategy to broaden the range of insect species that were not being adequately controlled by a single toxin cotton variety. The strategy of Bt gene pyramiding rests on some core assumptions [16]. The first assumption is that insects resistant to only one toxin can be effectively controlled by a second toxin produced in the same plant.

The second assumption is that strains resistant to two toxins with independent actions cannot emerge through selection pressure with one toxin alone. Karim *et al.* [17] contend that the use of multiple toxins to impede evolution of resistance is premised on the idea that if insects homozygous for one resistance gene are rare, insects homozygous for multiple resistant genes are extremely rare. When using multiple crystal proteins, even insects homozygous for one or two resistant genes but heterozygous for another resistance gene would still be controlled by crops expressing multiple Bt toxins [18, 19].

CIM-482, a locally approved cultivar was transformed with two insecticidal genes *Cry1Ac* and *Cry2A* by Agro bacterium mediated transformation [20]. Insect resistance and Risk assessment studies of transgenic lines developed out of this transformation event were evaluated for three years 2006, 2007 and 2008 at the campus of National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore,

Pakistan with the purpose to determine stable integration of insecticidal genes in advance cotton lines and to evaluate the resistance level of these transgenic lines in field along with its biosafety aspects.

## MATERIALS AND METHODS

**Transformation of Cotton Variety:** Agrobacterium-mediated genetic transformation of a local cotton cultivar CIM-482 (provided by Central Cotton Research Institute, Multan, Pakistan) was achieved using shoot apical meristems isolated from seedlings as explants and a synthetic genes encoding *Cry1Ac* and *Cry2A* endotoxins of *Bacillus thuringiensis* cloned in plasmid pk2Ac (Fig. 1). Regeneration of shoots was carried out in selection medium containing kanamycin (100 mg/l) after co-cultivation of the explants with Agrobacterium tumefaciens (strain LB4404). Progeny obtained by selfing To plants was grown in the greenhouse and screened for the presence of neomycin phosphotransferase (*nptII*) and *Cry1Ac* and *Cry2A* genes by polymerase chain reaction (PCR) and Southern blot hybridization [20]. The transgenic plants were further multiplied in field with the purpose to evaluate their insect resistance in open field trials.

**Plant Material and Experimental Design:** Seed of lines in T4 progeny was taken from CEMB cotton Store. It was delineted with commercial H<sub>2</sub>SO<sub>4</sub> at a rate of 100 ml/kg of seed. After removing the fuzz, seed was washed with tape water four to five times to remove the remaining acid from seeds. Ten advance homozygous lines 3001, 3009, 3010, 3012, 3013, 3016, 3033, 3038, 3036, 3043 were selected on the basis of agronomic and morphological performance under field conditions and insect resistance being good in expression of endotoxins, lesser boll damage %age in field after being artificially infested. These lines were sown along with their parent variety (untransformed CIM-482) for three consecutive years (2006-2008) according to randomized complete block design (RCBD) with four replications.

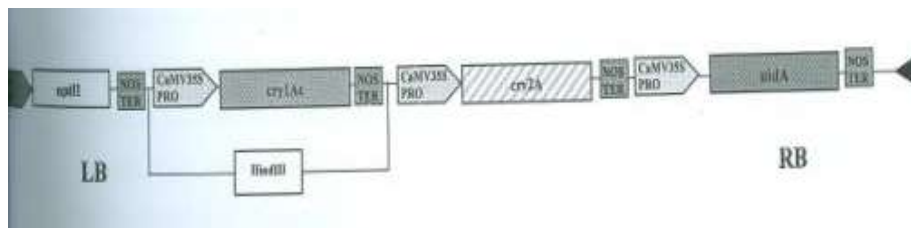


Fig. 1: Vector construct map of Pk2Ac containing *Cry1Ac* and *Cry2A* genes

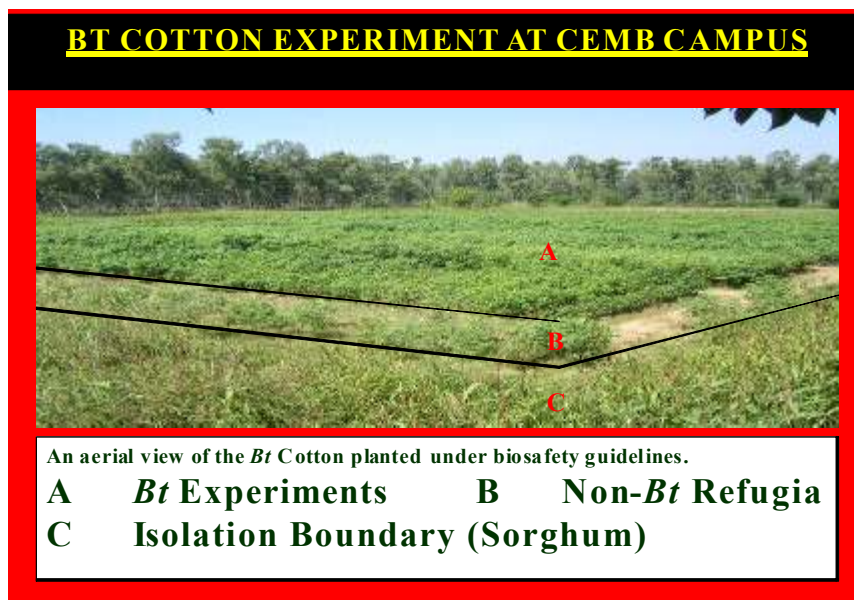


Fig. 2: Layout of Bt Cotton field under Biosafety guidelines at CEMB Campus

Table 1: Primer list for PCR amplification of Cry1Ac and Cry2A genes

Primer Name	Primer Sequence	Annealing Temp.	Product Size
Cry1Ac Forward	5'-ACAGAAGACCCTTCAATATC-3'	52°C	565bp
Cry1Ac Reverse	5'-GTTACCGAGTGAAGATGTAA-3'	52°C	---
Cry2A Forward	5'-AGATTACCCAGTTCAGAT-3'	52°C	600bp
Cry2A Reverse	5'-GTTCCCGAAGGACTTTCTAT-3'	52°C	---

Experimental fields were surrounded with 5 rows of untransformed CIM-482 and MNH-93 (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 [21] (Fig. 2).

**Confirmation of Stable Gene Integration:** For the purpose to confirm the stable integration of insecticidal genes *Cry1Ac* and *Cry2A* in advance cotton lines, molecular analysis like PCR and Southern Blot were performed:

**Polymerase Chain Reaction (PCR):** Genomic DNA was isolated from fresh cotton leaves using the method described by Dellaporta *et al.* [22]. PCR was run for the detection of integrated *Cry1Ac* and *Cry2A* gene by amplifying internal fragments of 565bp and 600bp respectively by a modification of the method by Saiki *et al.* [23]. DNA extracted from untransformed plants was used as negative control

and that of plasmid pk2Ac as positive control. The PCR was performed at 94°C for 4 minutes 94°C for 1 minutes 52°C for 1and 72°C for 1 minutes followed by 35 times (Table 1). The amplified PCR fragments were resolved on 1% agarose gel and observed under UV light. For amplification of genes, following primer sequences were used.

**Southern Blot Analysis:** PCR positive plants were further analyzed for southern blot. Genomic DNA was digested with HindIII enzyme and rest of the procedure was followed as described by Southern [24]. Gene specific Probe of *Cry1Ac* was labeled using Fermentas Biotin DecaLabel™ DNA Labeling Kit (Cat #K0651). Detection procedure was followed as provided in Fermentas Biotin Chromogenic Detection Kit (Cat# K0661).

**Protein Extraction:** About 500mg leaves from plants of different transgenic cotton lines were taken, wrapped in aluminum foil, labeled properly and taken to lab in liquid nitrogen. Leaves were grinded to fine powder in pre-chilled pestle mortar in the presence of liquid nitrogen.

Transferred the powder in 1.5ml tubes and added about 300 µl ice cold extraction buffer (10mM Tris HCl pH 7.5, 40mM EDTA, 150mM NaCl, 10% Glycerol, 1mg/ml DTT and 1mM PMSF). Kept the tube on ice for 20 minutes and centrifuged at 4°C for 5 minutes at 14,000 rpm. Transferred the supernatant in new 1.5 ml tube, it contains extracted protein.

**SDS- PAGE:** SDS-PAGE was carried out by the method of Laemmli *et al.* [25]. About 20 µg of extracted protein was mixed with loading dye denatured by heat shock in boiling water bath for 5 minutes and quick chilled on ice. Prepared 12% resolving and 4% stacking gel in gel assembly. Loaded the protein on gel and run in mini Biorad gel apparatus at 50 V. Stained the gel with Commassie stain (0.25% Coomassie brilliant blue R 250, 45.5% methanol and 9% glacial acetic acid) for 30 minutes and destained it with destaining solution (25% ethanol, 7% glacial acetic acid). Captured the photograph of gel in gel documentation apparatus.

**Biotoxicity Assay and Artificial Field Infestation:**

To check the efficacy of endotoxins against targeted insect pests, laboratory bioassay and artificial infestation of cotton field with *Heliothis* larvae was conducted each year. Ten leaves from upper, middle and lower portion of each lines were taken in petri plate, taken to laboratory and 2<sup>nd</sup> instar larvae of *Heliothis* was fed to them. After 2-3 days mortality rate was noted which was appreciable having a mortality range of 70-100% while it was 0% in control CIM-482. Mortality rates was calculated as follows

$$\%Mortality = \frac{\text{No.of dead larvae}}{\text{Total no. of larvae}} *100$$

Entomology section of CEMB provided *Heliothis* larvae. Their moths were collected from far off places in Punjab (A Province of Pakistan) and reared in laboratory. The egg masses were collected and hatched in glass vials under the laboratory conditions. Field artificial infestations were performed twice in August and September (Boll worms activity is optimal at this stage in Pakistan) by taking ten 2<sup>nd</sup> instar larvae of *Heliothis* in a glass vial and then fastenteing this vial with the plant in the field. Almost 10,000 larvae were released per infestation. Transgenic crops were exposed to artificial infestation deliberately to check the level of resistance in these cotton lines against boll worms. Number of bolls

was calculated before and after each infestation and plant and bolls health was recorded. After 5-6 days of infestation, the number of boll was again counted and boll as well plant health condition was again recorded. Boll damage %age was calculated as follows

$$\%Boll\ damage = \frac{\text{No. of damaged bolls on plant}}{\text{Total Number of bolls on plant}} *100$$

**Agronomic and Morphological Characteristics:**

Agronomic characters are very important with respect to crop performance in field conditions. Different morphological and agronomic characteristics were recorded including plant height, number of bolls, number of sympodial and monopodial branches, boll damage %age after infestation, days to maturity and average yield. Yield of transgenic plants was calculated as a percent increase or decrease relative to control plants.

**Risk Assessment Studies:**

Ten plants per replication in 5 lines (4 transgenic lines and one parent non Bt line CIM-482) were selected to study the effect of transgenic cotton of non targeted insects. Firstly, non targeted insects of cotton were identified; insects were collected and counted on both transgenic and control lines. Analysis of variance was performed to see the effect on non target insects. Effect on Ladybird beetle, Red cotton bug, wasps, jassid, whitefly and common flies was studied.

Horizontal gene flow was studied to determine the flow of gene to other unrelated organism; DNA was isolated from the soil after 15 and 30 days of crop harvesting. Polymerase Chain Reaction (PCR) was run to amplify Cry gene using soil DNA as template. Plasmid DNA pk2Ac was used as positive control in this reaction.

Soil from field after 15 and 30 days of harvesting was collected. 1 g of soil was grinded to fine powder in pre-chilled pestle mortar in the presence of liquid nitrogen. Transferred the powder in Eppendorf tubes and added about 1.5 ml ice cold extraction buffer (10mM Tris HCl pH 7.5, 40mM EDTA, 150mM NaCl, 10% Glycerol, 1mg/ml DTT and 1mM PMSF). Isolated protein was quantified as described by Bradford [26] and subjected to Dot blot assay to detect the Cry protein in soil samples. Protein isolated from soil bacterium *Bacillus thuringiensis* strain (HD-73) was used as positive control and protein isolated from untransformed CIM-482 was used as negative control.

## RESULTS

### Amplification of Insecticidal genes by PCR:

Polymerase chain reaction (PCR) of both genes *CryIAc* and *Cry2A* confirmed the stable inheritance of these genes to subsequent generations. 565 bp and 600 bp internal fragments for *CryIAc* and *Cry2A* respectively were amplified (Fig. 3). No amplification was detected in negative control.

**Integration of *CryIAc* gene in Cotton Plants:** Few of the PCR positive plants were further proceeded and the presence and stable integration of *CryIAc* gene in plant genome was confirmed by Southern blot analysis. Gene integration was detected by gene specific probe after the plasmid pk2Ac DNA was digested with HindIII restriction enzyme. Plant genomic DNA digested with the same restriction enzyme and hybridized with *CryIAc* specific probe showed the integration of *CryIAc* gene in Plant genome. Non transformed CIM-482

plant DNA was used as negative control while that of plasmid DNA pk2Ac was used as Positive control (Fig. 4).

**SDS-PAGE:** Total protein was extracted from transgenic plants samples along with control and about 20 µg of extracted protein was loaded on 12% polyacrylamide gel. A 68 kDa band of *CryIAc* protein was observed in transgenic plant samples (Fig. 5) and this specific protein band was absent in control sample.

**Biotoxicity Assay and Field Infestation:** Laboratory Biotoxicity assays with 2<sup>nd</sup> Instar *Heliothis* larvae showed that expression of both genes is sufficient to kill the targeted insects. In Laboratory Biotoxicity assay 5 lines 3001-1, 3016-1, 3012-3 and 3038-3 were showing 100% mortality of larvae. While other lines showed a mortality rate of larvae that ranged between 70-90 % as shown in Fig. 6. The larvae which survived in few cases were too inactive or sluggish to be harmful for the plant. While in case of non transformed control CIM-482, no any

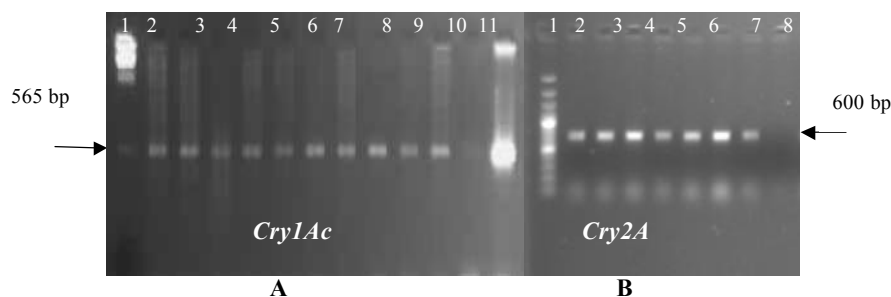


Fig. 3: PCR amplification of *CryIAc* and *Cry2A* in transgenic plants

(A)	(B)
Lane 1 : Lamda HindIII Marker	Lane 1 : 50bp Ladder (Fermentas)
Lane 2-11 : Representative transgenic plant of line 3001, 3009, 3010, 3012, 3013, 3016, 3033, 3038, 3036, 3043	Lane 2-7 : Representative transgenic plants of lines 3001, 3009, 3010, 3012, 3013, 3016
Lane 12 : -ve control (CIM-482)	Lane-8 : +ve control (CIM-482)
Lane 13 : +ve control (pk2Ac plasmid)	Lane 9 : -ve control (pk2Ac plasmid)



Fig. 4: Southern blot of *CryIAc* gene in transgenic plants

- Lane 1 : Negative Control (Non transformed CIM-482 plant)
- Lane 2 : Positive Control (Plasmid DNA Pk2Ac)
- Lane 3-5 : Representative plants of three transgenic Lines 3001-1, 3010-14 and 3016-1

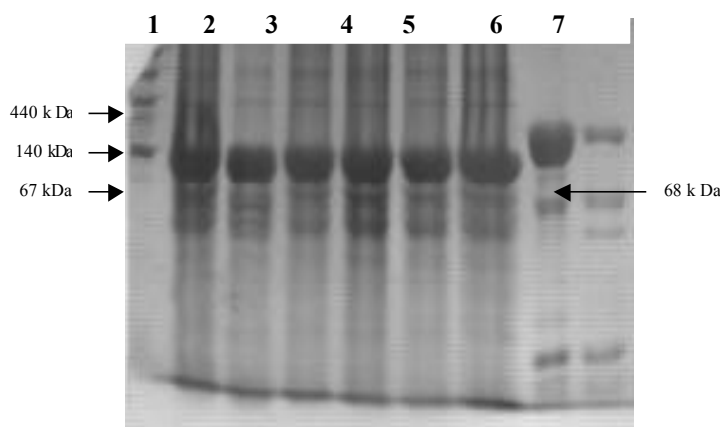


Fig. 5: SDS-PAGE of extracted protein from different transgenic lines of cotton

- Lane 1 : High Molecular wt. Protein Marker
- Lane 2-7 : Transgenic plant of line 3001, 3009, 3010, 3012, 3013, 3016
- Lane 8 : Positive Control (Isolated protein of *Bacillus thuringensis* HD-73.
- Lane 9 : Non Transformed Control (CIM-482)

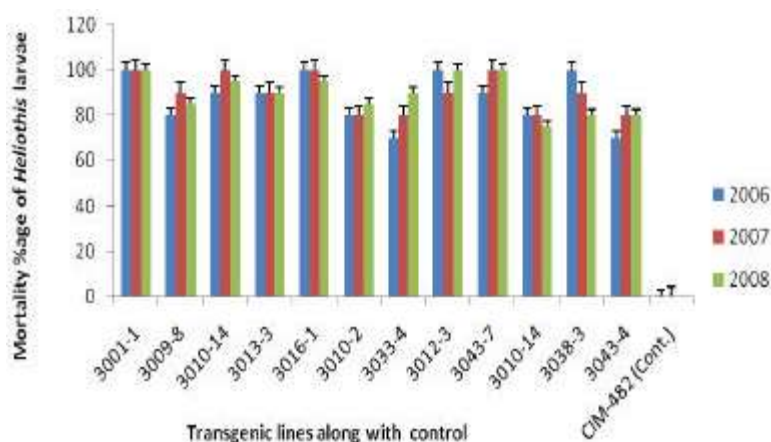


Fig. 6: Graph Showing Mortality rates of *Heliothis* Larvae in different transgenic lines (laboratory Biototoxicity assay)

mortality of larvae was noted. Field infestation carried out twice in season in August and September respectively. These months were selected for the infestation as boll worm activity is maximum during these months in Pakistan. Lines showed appreciable level of resistance against the targeted insects and boll damage varied between 0.09- 4.5% in case of transgenic lines while in control CIM-482, boll damage was calculated to 25%. (Fig.7). Transgenic lines also varied in boll damage %age when analyzed statistically (Table 2), transgenic 3016-1 showed excellent result regarding boll damage %age. The resistance against lepidopterans was stably inherited during both years and all plants showed high level of resistance against lepidopterans.

**Agronomic and Morphological Characteristics:** Agronomic characters are very crucial in evaluation of crops in field conditions. Highly remarkable statistical differences were observed for plant height, number of bolls, days to mature, boll damage %age and average yield between different transgenic lines and untransformed control (Table 2). Boll damage %age varied among transgenic lines when analyzed statistically but was highly significant as compared to untransformed CIM-482 which was heavily attacked and had serious boll attack. Another positive aspect of transgenic lines found during these years was the early maturity as compared to control variety. A difference of 30-35 days was recorded which is a highly desirable for farming community in Pakistan especially in cotton growing area because

Table 2: Comparison of Yield contributing factors between transgenic lines and untransformed CIM-482 (2006-08)

Line No.	Average Number of bolls/plant			Boll Damage %age			Days to mature			Average Yield/Plant(Grams)
	2006	2007	2008	2006	2007	2008	2006	2007	2008	
3001-1	42.8 <sup>b</sup>	45.2 <sup>b</sup>	44.8 <sup>b</sup>	0.95 <sup>a</sup>	1.22 <sup>b</sup>	1.10 <sup>a</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	141.5 <sup>b</sup>
3009-8	43.2 <sup>b</sup>	46.2 <sup>b</sup>	42.4 <sup>b</sup>	1.20 <sup>b</sup>	1.47 <sup>b</sup>	1.28 <sup>b</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	139.3 <sup>b</sup>
3010-14	37.6 <sup>b</sup>	39.5 <sup>c</sup>	41.1 <sup>b</sup>	2.21 <sup>b</sup>	0.09 <sup>a</sup>	1.05 <sup>a</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	136.9 <sup>b</sup>
3013-3	43.5 <sup>b</sup>	45.5 <sup>b</sup>	40.9 <sup>b</sup>	2.29 <sup>b</sup>	2.05 <sup>b</sup>	1.87 <sup>b</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	139.8 <sup>b</sup>
3016-1	53.2 <sup>a</sup>	56.8 <sup>a</sup>	55.5 <sup>a</sup>	0.09 <sup>a</sup>	0.95 <sup>a</sup>	1.05 <sup>a</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	152.2 <sup>a</sup>
3010-2	42.0 <sup>b</sup>	40.8 <sup>c</sup>	43.5 <sup>b</sup>	3.50 <sup>c</sup>	1.55 <sup>b</sup>	2.50 <sup>c</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	124.5 <sup>c</sup>
3033-4	41.9 <sup>b</sup>	41.0 <sup>c</sup>	38.4 <sup>c</sup>	2.83 <sup>b</sup>	1.66 <sup>b</sup>	2.44 <sup>c</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	124.3 <sup>c</sup>
3012-3	36.0 <sup>c</sup>	38.2 <sup>c</sup>	37.5 <sup>c</sup>	1.38 <sup>b</sup>	2.24 <sup>b</sup>	1.33 <sup>b</sup>	110 <sup>a</sup>	115 <sup>a</sup>	115 <sup>a</sup>	111.2 <sup>d</sup>
3043-7	35.0 <sup>c</sup>	40.2 <sup>c</sup>	37.6 <sup>c</sup>	4.05 <sup>c</sup>	1.28 <sup>b</sup>	2.15 <sup>c</sup>	125 <sup>b</sup>	125 <sup>b</sup>	125 <sup>b</sup>	112.5 <sup>d</sup>
3010-14	39.0 <sup>c</sup>	38.0 <sup>c</sup>	36.0 <sup>c</sup>	3.30 <sup>c</sup>	3.12 <sup>c</sup>	2.40 <sup>c</sup>	110 <sup>a</sup>	115 <sup>b</sup>	115 <sup>b</sup>	115.5 <sup>d</sup>
3038-3	36.9 <sup>c</sup>	42.6 <sup>b</sup>	38.9 <sup>c</sup>	4.12 <sup>c</sup>	2.25 <sup>b</sup>	3.00 <sup>c</sup>	125 <sup>b</sup>	125 <sup>b</sup>	125 <sup>b</sup>	118.5 <sup>d</sup>
3043-4	34.5 <sup>c</sup>	37.5 <sup>c</sup>	34.5 <sup>c</sup>	3.17 <sup>c</sup>	3.52 <sup>c</sup>	2.42 <sup>c</sup>	125 <sup>b</sup>	125 <sup>b</sup>	125 <sup>b</sup>	120.2 <sup>d</sup>
CIM-482 (Control)	26.9 <sup>d</sup>	27.2 <sup>d</sup>	25.4 <sup>d</sup>	28.5 <sup>d</sup>	25.0 <sup>d</sup>	26.0 <sup>d</sup>	150 <sup>c</sup>	150 <sup>c</sup>	150 <sup>c</sup>	92.4 <sup>e</sup>

Numbers with same letters within column are not significantly different from each other according to LSD test at 5% level of significance

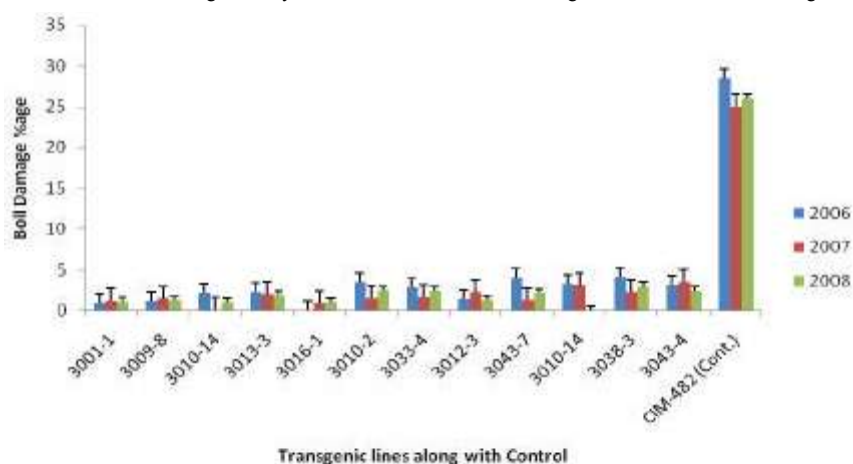


Fig. 7: Graph showing boll damage %age in transgenic cotton lines cotton lines after infesting crop artificially with 2<sup>nd</sup> Instar *Heliothis* larvae

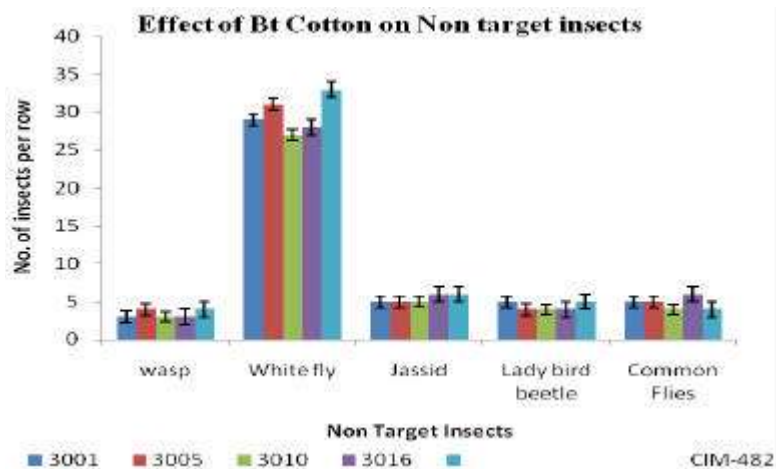


Fig. 8: Effect of Bt Cotton on Non target insects



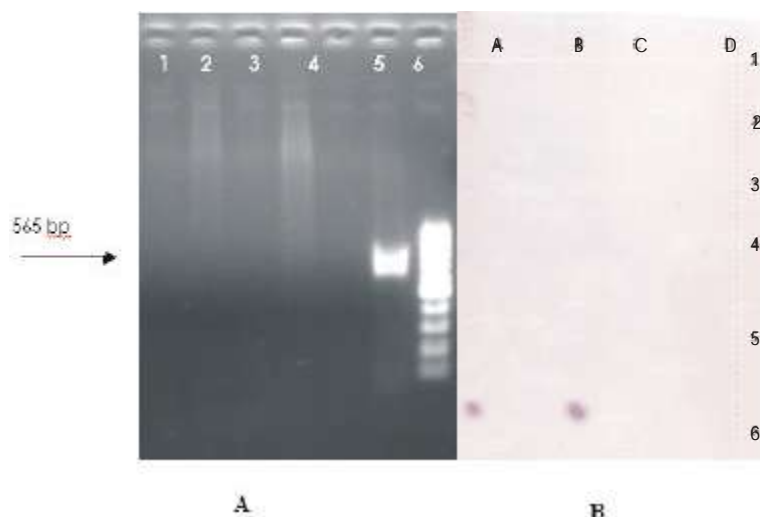


Fig. 9: PCR and Dot blot assay to confirm the confirm horizontal gene flow

farmers can cultivate wheat crop one month earlier in September - October as crop rotation. Otherwise they normally get late and sow wheat crop in the month of late October or early November. Similarly, significant results were obtained in case of number of bolls, plant height and average yield.

**Risk Assessment Studies:** Almost same number of non target insects were found both on transgenic and non transgenic lines. The data was found non significant when analyzed statistically rejecting the hypothesis that transgenic crops can affect the survival of the non target insects or natural enemies of insects (Fig.8). Most of the predators non target insects like ladybird beetle, red cotton bug, wasp and spiders were found throughout the season of crop growth.

Polymerase chain reaction (PCR) was performed using gene specific primers to detect the persistace of *CryIAc* gene in soil particles. There was no detection of *Cry* gene in samples while positive sample plasmid *pk2Ac* amplified a required 565 bp Fragment (Fig. 9). No integration of *Cry* genes suggested that Bt cotton is environment friendly.

To confirm the expression of *Cry* gene, a dot blot assay was performed that did not detect *Cry* protein in soil samples suggesting the degradation of *Cry* protein after crop harvesting. Only Positive protein samples isolated from soil bacterium *Bacillus thuringiensis* strain (HD-73) were detected (Fig. 8). No other detection was found in test samples as well as in negative samples.

## DISCUSSIONS

Transgenic cotton lines encoding two insecticidal crystalline proteins from *Bacillus thuringiensis* can reduce the use of conventional broad- spectrum pesticides dramatically against targeted pests. There is always a risk that insects could become resistant to Bt toxin after prolonged and repeated field exposure. The most practical approach to prolong the effectiveness of Bt crops was a refugia strategy and pyramiding of two or more genes in the same cultivar [4].

The performance of advance cotton lines against targeted pests has been studied for three consecutives years and we found that gene pyramiding could be the best way to delay the resistance level in insect pests against endotoxins. Performance of these lines with two genes has been excellent regarding the insect resistance and yield performance.

PCR, southern blot and SDS-PAGE of extracted protein were undertaken to confirm the integration and expression of inserted insecticidal genes in transgenic lines. Here, the results are given of few representatives transgenic plants of cotton lines. The 565 bp and 600 bp fragments in case of PCR showed the integration of *CryIAc* and *Cry2A* in advance lines and a 68 Kda band of protein, which is a truncated *CryIAc* toxin from integrated Bt gene showed the expression of *CryIAc* gene. This appearance of 68 Kda band indicates the expression of gene in transgenic cotton lines, as this band of protein is absent in non-transgenic control samples.



Some of the transgenic lines provided upto 100% resistance against American boll worm and few of them were showing 70-90% resistance against targeted pest. Similarly boll damage in few lines was almost zero showing against 100% resistance to boll worms especially infested *Heliothis larvæ*. Difference in resistance level in laboratory biotoxicity assay and boll damage %age in field is perhaps due to the expression level of insecticidal protein which varies with the age of plant as well as in different plant parts [27].

Transgenic lines having two Bt genes were superior as compared to untransformed control variety with respect to average number of bolls, boll damage %age, early in maturing and yield of plant. Results suggested the stable integration of these insecticidal genes in subsequent generation as revealed by different molecular analysis.

It is already reported that transgenic lines may be early or late in maturity [28]. Possible reason for this morphological variation may be somaclonal variation [29], breakdown of plant genes caused by transgene insertion or insertion mutagenesis [30], pleiotropy or transgene induced endogenous silencing [31]. Somaclonal variations seems most likely cause of these changes as it took more time to produce transgenic plants as compared to normal tissue culture procedure and the longer the tissue culture time the higher the frequency of somaclonal variation [32].

In short, transgenic lines expressing two unrelated Bt gene *Cry1Ac* and *Cry2A* provided protection against lepidopteran insects throughout the growth period. These lines provided highly resistance against targeted pests till the harvesting and were desirable in agronomic and morphological characteristics. It is very important to note that these are advance lines in T5 to T6 generations, insecticidal genes have been inherited stably and no gene silencing has been observed for Cry genes.

Specificity of Cry genes for particular orders of insects is their most significant attribute. Bt sprays donot disturb the overall predators or parasitoid communities or population trajectories of non targeted herbivores [33]. A common method to check the response of transgenic lines to such insects to compare the number of non target insects on transgenic lines and control [10, 34-37]. Results from present study suggest that Bt cotton is safe for non target insects as well as for the environment.

The risk of horizontal gene flow is associated with deployment of transgenic crops in field. There are two chances of Horizontal gene flow. One is to free-living soil microorganism and other related to microorganism

associated with plants i.e. microorganisms associated with root nodules of nodule forming plants. Results indicate that there is no risk of horizontal gene flow from crop to soil residues or soil organisms. PCR for all the samples taken from transgenic and control lines was consistently negative. De Vries at al [38] also reported similar results that no transgenes can transfer to bacteria even in lab optimized conditions. No sample showed amplification of *cry* gene indicating that either DNA from crop residues are completely degraded or indicate that sequence homology between *Cry1Ac* gene and the related microorganisms is not sufficient to take up DNA from soil.

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