Evaluation of resistance and susceptibility level of single and double gene Bt cotton against Armyworm and American bollworm

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Abstract

Bt cotton was developed against lepidopteran insects and got a significant attraction by the Pakistani farmers to control target pests. The experiment was conducted to determine the resistance and susceptibility level of lepidopteran insects against double gene (Cry1Ac + Cry2A) and single genes Cry1Ac and Cry2A. Six Bt cotton genotypes G1 and G2 with Cry1Ac, G5 and G6 with Cry2A, and G3 and G4 with double gene (Cry1Ac + Cry2A) were studied. G7 was non-Bt which was used as a control. A qualitative test of Bt genes was done with immune-blots strips, and quantification was done by the ELISA technique. In the Armyworm bioassay, G1 and G2 showed 1.187 μg g⁻¹ and 0.927 μg g⁻¹ concentrations of Cry1Ac and 41.66% and 33.33% larval mortality, respectively. The Cry2A genotypes, G5 and G6, showed 7.60 μg g⁻¹ and 7.79 μg g⁻¹ concentrations of Cry2A, respectively, and larval mortality as 83.33% on both genotypes. The double gene genotypes, G3 and G4, showed Cry1Ac concentrations as 0.803 μg g⁻¹ and 0.730 μg g⁻¹, those of Cry2A 7.459 μg g⁻¹ and 7.407 μg g⁻¹, whereas the larval mortality of the two genotypes had been 91.67% and 100%, respectively. In the American bollworm bioassay, G1 and G2 showed concentration of Cry1Ac as 1.049 μg g⁻¹ and 1.052 μg g⁻¹ and larval mortality 33.33% and 41.66%, respectively. G5 and G6 showed Cry2A concentrations as 6.520 μg g⁻¹ and 6.464 μg g⁻¹ and the larval mortality levels as 66.67% and 58.33%, respectively. G3 and G4 showed concentrations of Cry1Ac and Cry2A as 1.460 μg g⁻¹ and 0.968 μg g⁻¹, and 6.025 μg g⁻¹ and 6.651 μg g⁻¹ and the larval mortality as 100% and 91.66%, respectively. Low weight gain was observed on those genotypes which contained double gene rather than any of the single genes. Strong positive correlations existed between insect’s mortality and double gene concentration. A lethal concentration (LC) of the double gene of Armyworm and American bollworm was 7.513 μg g⁻¹ and 7.684 μg g⁻¹, respectively, required to kill 99% of the insect’s population. Double gene Bt cotton genotypes had resistance against target pests, while single-gene Bt cotton genotypes lost their efficacy against the target pests.

Abbreviations: Bt: Bacillus thuringiensis; Cry1Ac, Cry2A: Crystallized proteins synthesized by Bacillus thuringiensis; ELISA: Enzyme-Linked Immunosorbent Assay; LC: Lethal concentration; DAS: Days after sowing

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**Introduction**

Cotton (*Gossypium hirsutum* L.) belongs to Malvaceae family. It is a tetraploid species with a chromosome number of $2n = 52$. More than 50 species of *Gossypium* have been recognized in the tropical and sub-tropical regions (Wendel and Grover, 2015). Cotton, corn, and soybean are the crops that have been genetically engineered with the Bt gene from *Bacillus thuringiensis*. These crops turn out Bt toxin which is fatal for lepidopteran insects (Bravo et al., 2011; Abbas, 2018).

In Pakistan, Bt cotton was commercialized in 2010 for general cultivation by the National Biosafety Committee (NBC) working under the Environmental Protection Agency (EPA) (Cheema et al., 2015). Bt crops trim down the cost of production and increase the farmer’s income (Downes et al., 2016). Many tentative cotton genotypes contain a single Bt gene (Cry1Ac) which produces δ-endotoxin (Macintosh et al., 1990; Jamil et al., 2021). Bt cotton is developed for resistance against lepidopteran insects, i.e., Armyworms, American bollworms, and pink bollworms, but advancement has happened in them against the single Bt gene. The Cry1Ac toxin interacts with cadherin protein in the midgut of lepidopteran pests causing pores in the midgut epithelial layer, which causes insect’s death (Wang et al., 2012). But at present, lepidopteran insects have shown low resistance against the single Bt gene (Cry1Ac) (Tabashnik and Carrière, 2017). The lepidopteran insect’s larvae start to nourish on cotton leaves after hatching and become a source of considerable damage to cotton production (Santos et al., 2003; Guan et al., 2022).

The researchers have stacked the second Bt gene (Cry2A: MON-15985) to delay the resistance in lepidopteran insects against Bt cotton (Pan et al., 2014). Single Bt gene (Cry1Ac) is now being replaced by the double Bt gene (Cry1Ac and Cry2A) for Integrated Resistance Management (IRM) (Selvi and Sakthi, 2015). In Pakistan, double gene (Cry1Ac and Cry2A) in Bt cotton was approved for general cultivation in 2017, but the susceptibility level of the target pest was not determined. Therefore, the present study was designed to assess the effectiveness and expression level of single genes (Cry1Ac and Cry2A), double gene (Cry1Ac + Cry2A), and Cry protein in Bt cotton on target pests in locally bred Bt cotton genotypes, and know about the resistance status of armyworms and American bollworms in Punjab, Pakistan.

**Materials and Methods**

**Collection of seeds of Bt cotton genotypes**

The seeds of six Bt cotton genotypes were collected from CEMB (Centre of Excellence in Molecular Biology, Lahore) in which G1 and G2 contained single Bt gene Cry1Ac and G5 and 6 contained Cry2A. Two genotypes G3 and G4 contained double Bt gene (Cry1Ac + Cry2A). One non-Bt cotton genotype was used as a control.

**Experimental site**

All genotypes were grown in a greenhouse with a controlled environment (Temp. = 30 ± 2 °C, RH = 60 ± 5%) under a completely randomized design (CRD) in the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan during the crop season.

**Leaf sampling**

Three plants were selected randomly for leaf sampling. The immuno-blot strip assay was done for confirmation of Bt genes in the Bt cotton genotypes at the age of 30 days after sowing (DAS). Bt toxin quantification was done at 70 days after sowing (DAS).

**Qualitative test of Bt genes**

Qualitative test was done using the immune chromatographic (lateral flow) strip tests (Stave, 2002) by using immune-blot strips of a commercial kit (QuantiPlate™ Kit, EnviroLogix, Inc., Portland, ME, Cat AP 005). It was done according to the protocol described in the kit manual (Figure 1).

**Bt genes quantification**

Bt quantification was done through the ELISA (Enzyme-Linked Immunosorbent Assay) technique (Stave, 2002) by a commercial quantification kit (QuantiPlate™ Kit, EnviroLogix, Inc., Portland, ME) in PGRL (Plant Genetic Resource Laboratory) in the Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan (as explained in Figure 2). The supernatants were diluted before starting the assay. Extraction buffer at the rate of 500 μl of 1X and 50 μl sample extract were added to dilution tubes labeled for each sample, and then mixed the solutions thoroughly. After that, the ELISA was performed for the quantification of Cry1Ac and Cry2A using the kit’s manual protocol. The amount of Bt toxin (Cry1Ac and Cry2A) was taken as ppm which was converted to microgram per gram (μg/g).
Insect bioassay

Rearing of Insects

Armyworm and American bollworm were reared in an insectary machine on an artificial diet containing 150 g chickpea seed-flour, 2.35 g ascorbic acid, 9.0 g agar-agar, 24.0 g yeast, 6 mL linseed oil, 0.02 g vitamin mixture, 1.5 g methyl-4-hydroxy benzoate, 0.75 g streptomycin, 0.75 g sorbic acid and 700 mL DD water. Honey (10%) and water solution on a cotton swab were supplied to adults for feeding and provided a layer of liner strip hung inside the box for oviposition. Adult insects laid eggs on liner strips. After eggs hatching, 1st instar larvae were transferred on the synthetic diet in aerated jars. The second instar larvae were used for bioassay.

Bioassay methodology

Bioassay was done by randomly selecting three plants of each genotype and detaching two leaves, one from the upper portion and the other from a lower portion of a plant, and then placed these cotton
leaves on wet tissue papers in Petri dishes after taking punches for ELISA to determine toxin level (Figure 3). The second instar larvae were released on leaves and the Petri dishes were covered with a netted cloth and fixed with a rubber band in such a way that none of the insects would escape out from these Petri dishes. After every 24 h, larval mortality data was recorded along with replacing old leaves with fresh ones. This data was taken for six days.

![Figure 3: Rearing and maintenance of insect cultures on artificial diet](image)

**Statistical analysis**

Correlation (Pearson) analysis was done to know about the relations between genes and insect bioassay traits. Lethal concentrations, i.e., LC$_{99}$, LC$_{95}$, LC$_{90}$, and LC$_{50}$ were estimated by the Probit analysis at a 95% confidence interval (CI). The Duncan’s Multiple Range test at 5% probability level was performed to compare the mean values of insect bioassay attributes. All statistical analysis was performed by SPSS version 16.00 and Statistics 8.1.

**Results and Discussion**

**Conformation of Bt genes in cotton**

Out of six Bt cotton genotypes, G1 and G2 contained single gene Cry1Ac, and G3 and G4 double gene (Cry1Ac and Cry2A). G5 and G6 contained a single gene Cry2A, and G7 was a non-Bt cotton genotype used as a control. The confirmation was done with the help of the strip testing (Figure 4).

**Quantification of Bt genes in cotton**

Analysis of Bt gene quantification in different cotton genotypes showed a significant difference in the expression of single genes, Cry1Ac and Cry2A (Figure 4; Figure 5). The Bt cotton genotypes on which armyworm was fed, the range of concentration of single Bt genes Cry1Ac and Cry2A was 0.730 µg g$^{-1}$ to 1.187 µg g$^{-1}$ and 7.406 µg g$^{-1}$ to 7.791 µg g$^{-1}$, respectively (Figure 5). Genotypes of Bt cotton from which American bollworm received food had an appropriate range of concentration of single Bt genes Cry1Ac and Cry2A as 1.031 µg g$^{-1}$ to 1.493 µg g$^{-1}$ and 5.931 µg g$^{-1}$ to 6.521 µg g$^{-1}$, respectively. It was inferred that the concentration of Bt toxin in different genotypes was not constant. The Bt toxin concentration was variable among different and within Bt genotypes. The concentration of Cry1Ac was lower than that of Cry2A (Figure 5).
Figure 4: Strips showed presence or absence of Bt genes

The experiment was conducted by taking 10 genotypes for Bt quantification by the ELISA technique at 30, 60, 90, and 120 DAS. The results revealed that the expression of Bt toxin reduced over time, because highest Bt toxin concentration was noticed at 30 DAS and lowest at 120 DAS, as earlier reported elsewhere (Khan et al., 2018). The level of Bt toxin in the genotype with Cry1Ac was degraded due to high temperature (Chen et al., 2005a) and use of pesticides for killing lepidopteran insects (Spielman et al., 2015). The expression of Cry1Ac was extremely affected by the atmosphere of Pakistan as compared to that of Cry2A being slightly affected (Oliveira et al., 2016). Bt toxin level was erratic even among different plants of the same genotype (Khan et al., 2018). The variation in Bt expression became an issue in transgenic cotton performance to control target pests (Kranthi et al., 2005).

Figure 5: Concentration of Bt toxin (Cry1Ac and Cry2A) on different Bt cotton genotypes

**Insect bioassay**

According to the results presented in **Table 1**, an inversely proportional relationship existed between Bt toxin level and mortality percentage. In the insect bioassay of 2nd instar larvae, the mortality percentage was more in armyworm than that in the American bollworm in all Bt cotton genotypes except G3 and G2 (Table 1). The mortality percentage was higher and the weight gain phenomenon was lower in both G3 and G4 genotypes than those in all other genotypes due to the presence of a double gene in both insects’ bioassay. The weight gain trait was higher in American bollworm than that in armyworm. G3 is the only genotype in which the armyworms lost their weight instead of gaining weight (Table 1).
Bt proteins (Cry1Ac and Cry2A) kill lepidopteran pests. The toxin activates in the midgut of lepidopteran pests by protease enzymes, which in turn binds to the receptor protein in the midgut of target pests (Zhang et al., 2006). The egg-laying capacity and oviposition period could be affected by higher concentration of Bt toxin (Wang et al., 2016). The insects developed resistance against a single-gene either Cry1Ac or Cry2A, but not against both genes at a time; this is the reason why the double gene in Bt cotton showed the highest resistance against lepidopteran insects as earlier observed elsewhere (Tabashnik and Carriere, 2017). Bt cotton showed 100% larval mortality of American bollworm at 30 DAS, while 60% to 80% larval mortality at 90 DAS (Bakhsh et al., 2015). The maximum feeding of larvae was observed on non-Bt cotton (Control) genotype as reported earlier (Zhang et al., 2017).

### Table 1: Insect bioassay of both armyworm and American bollworm

<table>
<thead>
<tr>
<th>G</th>
<th></th>
<th>Armyworm</th>
<th>American bollworm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IW (mg)</td>
<td>FW (mg)</td>
<td>WG(+)/WL(-)</td>
</tr>
<tr>
<td>G1</td>
<td>1.54±0.09*</td>
<td>6.5±1.03*</td>
<td>+4.96±0.21*</td>
</tr>
<tr>
<td>G2</td>
<td>1.63±0.04*</td>
<td>7.5±1.08*</td>
<td>+5.87±1.04*</td>
</tr>
<tr>
<td>G3</td>
<td>1.35±0.08*</td>
<td>2.5±1.09*</td>
<td>+1.15±0.07**</td>
</tr>
<tr>
<td>G4</td>
<td>1.39±0.03*</td>
<td>1.0±0.04*</td>
<td>-0.39±0.09*</td>
</tr>
<tr>
<td>G5</td>
<td>1.70±0.06*</td>
<td>4.5±1.01*</td>
<td>+2.80±0.08</td>
</tr>
<tr>
<td>G6</td>
<td>1.74±0.05*</td>
<td>4.0±0.09*</td>
<td>+2.26±0.06</td>
</tr>
<tr>
<td>G7</td>
<td>1.64±0.07*</td>
<td>8.3±1.07*</td>
<td>-6.66±1.19*</td>
</tr>
</tbody>
</table>

* = Correlation significant at 0.05 level; ** = Correlation highly significant at 0.01 level

**Legends**
- G: Genotypes; IW, Initial Weight; FW, Final Weight; WG/WL, Weight Gain/Weight Loss; Mort. Mortality

### Correlation analysis

The correlation analysis was used to find out the intensity, direction and magnitude of relationship and impact of one trait on the other one. Cry1Ac had non-significant relation with Cry2A in IW, FW and WG/WL, but all other remaining traits had significant correlations with each other in the insect bioassay of armyworms (Table 2). The traits of the insect bioassay of American worm showed similar results like those of armyworm, except IW, because initial weight showed non-significant results of all parameters (Table 3). In both experiments, the concentration of Cry1Ac was not correlated with Cry2A suggesting that there was no effect of Cry1Ac on the concentration of Cry2A, while it was positively and significantly correlated with larval mortality showing that if the concentration of Cry1Ac would increase the larval mortality would also increase. The larval weight was declined or increased very minutely due to the presence of Cry1Ac and Cry2A.

Cry2A had a negative and significant correlation with final weight, and weight loss/weight gain showing that if the concentration of Cry2A was high, the weight gain was very low and vice versa as observed earlier (Wan et al., 2017). It shows that there was no effect of initial weight on the final weight and weight gain/weight loss as reported by Ojha et al. (2014).

### Table 2. Correlation between Bt toxin and traits of insect bioassay (Armyworm)

<table>
<thead>
<tr>
<th></th>
<th>Cry1Ac</th>
<th>Cry2A</th>
<th>Cry1Ac + Cry2A</th>
<th>Mort. %</th>
<th>IW</th>
<th>FW</th>
<th>WG/WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac</td>
<td>1.0000</td>
<td>0.3870*</td>
<td>0.4872*</td>
<td>0.6234*</td>
<td>0.6689*</td>
<td>-0.5913*</td>
<td>-0.2696*</td>
</tr>
<tr>
<td>Cry2A</td>
<td>1.0000</td>
<td>0.9938*</td>
<td>0.9444*</td>
<td>0.7297*</td>
<td>-0.4120*</td>
<td>-0.2718*</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac + Cry2A</td>
<td>1.0000</td>
<td>0.9696**</td>
<td>0.7718*</td>
<td>-0.4616*</td>
<td>-0.3209**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mort.</td>
<td>1.0000</td>
<td>0.8321*</td>
<td>-0.5241*</td>
<td>-0.3804*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IW</td>
<td>1.0000</td>
<td>0.8714*</td>
<td>-0.7723*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FW</td>
<td>1.0000</td>
<td>0.9843**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WG/WL</td>
<td>1.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = Correlation significant at 0.05 level; ** = Correlation highly significant at 0.01 level; ns= Non-significant

**Legends**
- IW, Initial Weight; FW, Final Weight; WG/WL, Weight Gain/Weight Loss; Mort. Mortality

### Table 3. Correlation between Bt toxin and traits of insect bioassay (American bollworm)

<table>
<thead>
<tr>
<th></th>
<th>Cry1Ac</th>
<th>Cry2A</th>
<th>Cry1Ac + Cry2A</th>
<th>Mort. %</th>
<th>IW</th>
<th>FW</th>
<th>WG/WL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac</td>
<td>1.0000</td>
<td>0.0904*</td>
<td>0.0997*</td>
<td>0.3486*</td>
<td>0.0908*</td>
<td>-0.4181*</td>
<td>-0.4143*</td>
</tr>
<tr>
<td>Cry2A</td>
<td>1.0000</td>
<td>0.9819*</td>
<td>0.7844*</td>
<td>0.1758*</td>
<td>-0.7578*</td>
<td>-0.7617*</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac + Cry2A</td>
<td>1.0000</td>
<td>0.8500**</td>
<td>0.1584*</td>
<td>-0.8366*</td>
<td>-0.8398**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mort.</td>
<td>1.0000</td>
<td>0.0252*</td>
<td>-0.9098*</td>
<td>-0.9071**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IW</td>
<td>1.0000</td>
<td>0.0537*</td>
<td>0.0851*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FW</td>
<td>1.0000</td>
<td>0.9995**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WG/WL</td>
<td>1.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = Correlation significant at 0.05 level; ** = Correlation is highly significant at 0.01 level; ns= Non-significant

**Legends**
- IW, Initial Weight; FW, Final Weight; WG/WL, Weight Gain/Weight Loss; Mort. Mortality
Estimation of lethal concentrations

Lethal concentrations of double gene Cry1Ac+Cry2A for both insects (armyworm and American bollworm) were estimated through probit analysis (Table 4). The estimate of lethal concentrations, i.e., LC50, LC90, LC95, and LC99 for the local population of armyworm was 0.337 µg g⁻¹, 1.886 µg g⁻¹, 3.074 µg g⁻¹, and 7.597 µg g⁻¹, respectively. The lethal concentrations, i.e., LC50, LC90, LC95, and LC99 for the local population of the American bollworm were 0.310 µg g⁻¹, 1.898 µg g⁻¹, 3.170 µg g⁻¹, and 7.684 µg g⁻¹, respectively.

<table>
<thead>
<tr>
<th>LC levels</th>
<th>Armyworm</th>
<th></th>
<th>American bollworm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC (µg g⁻¹) ± S.E.</td>
<td>Lower limit</td>
<td>Upper limit</td>
</tr>
<tr>
<td>LC50</td>
<td>0.337 ± 0.0211</td>
<td>0.282</td>
<td>0.403</td>
</tr>
<tr>
<td>LC90</td>
<td>1.886 ± 0.0710</td>
<td>1.777</td>
<td>2.031</td>
</tr>
<tr>
<td>LC95</td>
<td>3.074 ± 0.0987</td>
<td>2.939</td>
<td>3.243</td>
</tr>
<tr>
<td>LC99</td>
<td>7.597 ± 0.1370</td>
<td>7.513</td>
<td>7.912</td>
</tr>
</tbody>
</table>

Legends: LCx = (50, 90, 95 and 99): Lethal concentration of double gene needed to kill x (50%, 90%, 95% and 99%) population of both insects.

Conclusion

Transgenic cotton is being cultivated on a large scale in many countries of the world including Pakistan. Bt cotton was developed against lepidopteran insects. The expression of Bt toxin in Bt cotton is not consistent and less efficient against target pests in Pakistan. The concentrations of Cry1Ac were lower than those of Cry2A in all genotypes. In both insect bioassays, weight loss or minute weight gain of larvae was observed on a double gene (Cry1Ac+Cry2A). Less weight gain was observed on genotypes with Cry2A and high weight gain in genotypes with Cry1Ac. Maximum weight gain was observed in non-Bt cotton. Armyworm was more affected by Bt toxin as compared to American bollworm. Armyworm lost weight on genotype 3, but American bollworm gained the maximum or little weight in all genotypes. Double gene showed the highest mortality percentage followed by that individually in Cry2A and Cry1Ac. Double gene Bt cotton genotypes had resistance against target pests, while single-gene Bt cotton genotypes tended to lose their efficacy against target pests.

Declaration of Author(s), Editor(s) and Publisher

Supplementary material
No supplementary material is included with this manuscript.

Conflict of interest
The authors declare no conflict of interest.

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Contribution of authors
Conceptualization and designing the study: MA. Review of initial draft: HMNC. Revisions and corrections: JNA. Conduction of experiment: KN. Moderation of laboratory activities: HAA. Preparation of initial draft: HA, ZA.

Ethical approval
This study does not involve human/animal subjects, and thus no ethical approval is needed.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discarded to minimize any types of bio-contamination(s).
Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors’ consent
All authors contributed in designing and writing the entire review article. All contributors have critically read this manuscript and agreed for publishing in IJAaEB.

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