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# Relationship Between Damage by Cotton Bollworm *Helicoverpa armigera* (Hübner) and Different Plant Characteristics of *Bt* and Non-*Bt* Cotton Varieties in Pakistan

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**MAR and SR planned the research idea and protocol. MF, ST and AMF executed the laboratory and field trials. MF, ST and IAN prepared the results. MAR and MZM performed statistical analyses. MF and MAR prepared the first draft. MZM and SR proofread the manuscript. MAR supervised the research and provided technical support in experimentation.**

**Key words**
***Bt* cotton, Bollworm resistance, Chlorophyll contents, *Cry*1Ac toxin, Gossypol glands, *Helicoverpa armigera*, Photosynthesis rate, Plant traits, Stomatal conductance**



## ABSTRACT

*Helicoverpa armigera* (Hübner) is one of the notorious lepidopterous pests of cotton (*Gossypium hirsutum* L.). Transgenic cotton varieties expressing *Bacillus thuringiensis* (*Bt*) induced insecticidal toxin (*Cry*1Ac) have been a key tool combating infestations of *H. armigera* and other cotton bollworms. However, the development of resistance in *H. armigera* to *Bt* transgenic cotton varieties is of major concern. This study assessed the *H. armigera* resistance to *Cry*1Ac expressing cotton varieties and association of plant biochemical, physico-morphic and physiological traits with boll damage by *H. armigera*. ELISA results revealed significantly high toxin (*Cry*1Ac) contents in *Bt* varieties particularly in PB-38, CRS-456 and PB-896 at 30-day post-germination. Moreover, toxin level in leaves decreased after 30 days of germination in most varieties except CRS-456 and PB-896. *Bt* varieties PB-896, CRS-456, PB-38 and VH-57 had the highest yield with low to moderate bollworm damage and exhibited a high and stable leaf *Cry*1Ac toxin level. The net photosynthesis rate, stomatal conductance, $CO_2$ emission and absorption were positively, while *Bt* toxin level, gossypol glands density and yield were negatively and significantly correlated with the boll damage by *H. armigera*. All *Bt* cotton varieties exhibited a differential level of *Cry*1Ac toxin and none of them was found bollworm free, suggesting that *H. armigera* is able to infest most of the *Bt* cotton varieties though the level of infestation varied from variety to variety. Overall study findings recommend the cultivation of three *Bt* (VH-57, CRS-456 and PB-38) and one non-*Bt* (L.A Fragobract) cotton varieties by indigenous cotton growers to diminish bollworm infestations.

## INTRODUCTION

Cotton plays a pivotal role in the economy of Pakistan and is a major source of fiber and edible oil. Currently its share in country's national GDP is 10% (Arshad *et al.*, 2022). Factors affecting cotton yield include the infestation by different insect pests and diseases. About 1300 species of insects and other arthropods have been reported feeding and damaging cotton plants. Among insects, lepidopterous species such as bollworms are the most damaging pests of cotton. *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), also known as American bollworm, is one of

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**Abbreviations**
GDP, gross domestic product; *Bt*, *Bacillus thuringiensis*; *Cry*1Ac, pesticidal crystal protein 1Ac; ANOVA, analysis of variance; RCBD, randomized complete block design; GGE, genotype and genotype-environment interaction; ELISA, enzyme linked immunosorbent assay.

the most serious pests of cotton in Pakistan. It damages all fruiting bodies of cotton plants including buds, squares, bolls and flowers (Bhatti *et al*., 2007; Hussain *et al*., 2015; Ali *et al*., 2020).

Synthetic chemicals are the prime tool most farmers rely on in combatting agricultural insect pests. About 75% of the synthetic insecticides being sprayed in cotton in the Indo-Pak regions are intended against *H. armigera* (Hussain *et al*., 2015; Nadeem *et al*., 2022). Based on field surveys, cotton is sprayed at least seven times during its growing season in Pakistan and such practice is done by almost half of the farming communities in Pakistan (Ahmad *et al*., 2019a). This trend of excessive and recurrent use of synthetic chemicals has led to different ecological consequences including environmental pollution and human health hazards. Another major concern regarding the excessive use of synthetic insecticides is the development of resistance in cotton insect pests including *H. armigera* (Hussain *et al*., 2015; Ahmad *et al*., 2019a; Wang *et al*., 2021).

Plant physico-morphic characters play an important role in insect pest management. Gossypol glands for instance are found on the roots, seeds, stems, leaves and reproductive tissues of the cotton plant and contain certain terpenoid compounds. Gossypol disrupts the digestive system of bollworm larvae while feeding on the cotton plant (Ali *et al*., 2020). Breeding cotton plants for natural resistance has been an effective pest control strategy being practiced since 1980s however, till now no cotton germplasm has been released exhibiting higher level of resistance and halted the use of insecticides in cotton crop (Majeed *et al*., 2016; Arora *et al*., 2017).

Growing transgenic cotton expressing *Bacillus thuringiensis* (*Bt*) induced insecticidal toxin *Cry* is another strategy to mitigate the insect pest pressure on cotton crop (Downes *et al*., 2017; Zafar *et al*., 2022). Lepidopterous insects mostly attack on cotton and maize and transgenic plants have the ability to decrease the infestations of these pests (Abro *et al*., 2004; Álvarez-Alfageme *et al*., 2021). The advantages of genetically modified crops include increased production and reduced insecticidal use (Jamil *et al*., 2021). The transgenic plants expressing *Cry*1Ac toxic proteins can effectively manage different lepidopterous pests particularly *H. armigera* in cotton and other crops (Sanyal *et al*., 2005; Downes *et al*., 2017).

*Bt* varieties of cotton have been effectively used against *H. armigera* infestations. However, *H. armigera* is exhibiting field-evolved resistance to these *Bt* varieties all over the world including Pakistan (Li *et al*., 2007; Tabashnik and Wu, 2012; Yang *et al*., 2013; Downes *et al*., 2017; Ahmad *et al*., 2019b; Del Pozo-Valdivia *et al*., 2021; Singh *et al*., 2021). This research work was aimed to make a comparative analysis of *H. armigera* resistance to different *Bt* cotton varieties being commonly cultivated in Pakistan and to determine the potential relationship between some biochemical, physico-morphic and physiological characteristics of cotton plants and boll damage by *H. armigera*.

## MATERIALS AND METHODS

*Sowing of cotton (*G. hirsutum*) varieties*

The study was conducted during the cotton seasons of 2019 and 2020 in the College of Agriculture, University of Sargodha, Punjab, Pakistan. The seeds of eight *Bt* varieties (CIM-600, CRS-456, MNH-586, PB-38, Gumbo Okra, VH-57, PB-52-NC63 and PB-896) and two non-*Bt* varieties (Coker 100A/2 and L.A Fragobract) of cotton were procured from the Central Cotton Research Institute (Multan, Pakistan). These seeds were sown in the farm area of the College of Agriculture, University of Sargodha (72°41'40.3" E and 32°07'49.8" N). Plants were sown on raised beds with 20, 30 and 60 cm plant to plant, row to row and bed to bed distance, respectively. Plot size was 2.0×1.2 $m^2$ and the experimental layout was randomized complete block (RCBD) with three replications for each treatment. Standard agronomic practices recommended by the Department of Agriculture; Government of Punjab (Pakistan) were adopted for the trial. Seeds were delinted with sulfuric acid and subsequently washed with water and treated with Confidor® 70 WSC (imidacloprid) @ 5g/Kg before sowing. A pre-emergence weedicide Dual Gold® 960 EC (S-metolachlor) was applied immediately after seed sowing.

H. armigera *damage assessment on cotton varieties*

Trial was conducted under unsprayed conditions to estimate the incidence and infestation by *H. armigera* larvae on *Bt* and non-*Bt* cotton varieties. Pest (larval) population level and damage assessment were determined by performing pest scouting at 30-, 60- and 90-days post-germination. Five plants were randomly selected from each plot and each plant was examined for the bollworm infestation. Number of infested or damaged bolls and the total number of bolls per plant were counted to determine the infestation percentage.

*Determination of biochemical, physiological and physico-morphic traits of cotton plants*

Total chlorophyll contents of cotton plants were determined with the help of a field-portable chlorophyll meter (Model CL-01; SPAD-Hansatech, Norfolk, England) by taking three leaves from three randomly selected plants

of each variety at 30-, 60- and 90-day post-germination. Photosynthetic activities were determined in maximum sunlight in the absence of dew drops between 10:00 to 11:00 am. The parameters such as the amount of $CO_2$ at the inlet and outlet of leaf chamber, temperature of the ambient air in the leaf chamber, atmospheric pressure, net photosynthesis rate (Pn), temperature of leaf measured by the infrared temperature sensor, leaf stomatal conductance (SC), transpiration rate (E) and the relative humidity at the inlet and outlet of the leaf chamber were assessed on randomly selected three canopy-top leaves on three randomly selected plants with a hand-held photosynthesis system (Model: CI-340; CID Bio-Science, Inc., Camas, WA, USA).

Number of gossypol glands was calculated using a disc of 1.0 $cm^2$ of the leaf of each cotton variety with three replications with the help of a biological microscope (Model: MCX100, Micros, Austria). Height of three randomly selected plants of each variety was recorded at above mentioned intervals. The yield of seed cotton was determined from each plot of cotton variety and was converted to per acre.

*Enzyme linked immunosorbent assay (ELISA)*

Enzyme linked immunosorbent assay was performed at the Center of Excellence in Microbiology, University of the Punjab (Lahore, Pakistan) to detect the presence and level of *Cry*1Ac toxin protein in the leaves of different *Bt* cotton varieties collected at different time intervals. Three leaves of each variety were collected from the research field and were stored in the refrigerator at 4°C before performing ELISA. The analysis was performed using Envirologix Qualiplate Kits for *Cry*1Ac/*Cry*1Ab proteins (Bio-Tek, Winooski, VT, USA) according to the manufacturer's instructions. In brief, the samples for ELISA were prepared by adding 10 $mm^2$ disc of cotton leaf to 250 µL of extraction buffer in 500 µL tubes. Leaf tissues were disrupted and mixed thoroughly. Then, 50 µL of the extracted samples and 50 µL of *Cry*1Ac/*Cry*1Ab enzyme conjugate were added to each well of the strip previously coated with antibodies. Only extraction buffer (50 µL) was added in the blank wells. The contents were thoroughly mixed by moving the well plate on bench top shaker in a circular motion for 20–30 sec. Then, the wells were covered with tape and were incubated for 1–2 h at ambient temperature (27°C). After the incubation period, the contents of wells were thoroughly shaken in a container. The wells were washed with wash buffer three times. Plates were placed on a paper towel to remove the excessive water and then 100 µL of substrate was added to each well. The contents of well were mixed thoroughly and incubated for 15–30 min and 100 µL of stop solution (1.0 N HCl) was added to each of the wells and was mixed thoroughly. Subsequently, the contents of wells were turned yellow due to the addition of stop solution. The plate was read by using an absorbance microplate reader (Model: ELx800; Bio-Tek, Winooski, VT, USA) at 450 nm after 30 min. Analytical software Gen5 Data was used for microplate reader data analysis. The standard curve of *Cry*1Ac was prepared by adding 4, 12 and 24 ng/g of toxin in the wells.

*Statistical analysis*

Means of all plant biochemical, physico-morphic and physiological parameters including *Cry*1Ac toxin concentration, number of gossypol glands, chlorophyll contents, stomatal conductance rate, $CO_2$ emission and absorption, transpiration, photosynthesis rates, yield and damaged bolls percentage were compared using analysis of variance (ANOVA) followed by the least significant difference (LSD) post-hoc test at standard level of significance (at $p = 0.05$). Association among the studied plant parameters and boll damage by *H. armigera* was determined by calculating Spearman's correlation coefficients. GGE biplot was constructed by plotting the PC1 (Principal component) scores of environments (plant parameters determined) and genotypes (PC2). Cluster analysis based upon all parameters of *Bt* and non-*Bt* varieties of cotton was performed by using R software version 3.2.1 (http://www.r-project.org/). Statistical criteria such as akaike information criterion (AIC) and bayesian information criterion (BIC) were used to determine the variables contributing to boll damage followed by regression analysis using R software with package lme4, matrix and nlme.

## RESULTS

*Biochemical and physico-morphic parameters of cotton plants*

Means of *Cry*1Ac toxin concentration, number of gossypol glands and chlorophyll contents are presented in Table I. The concentration of *Cry*1Ac was significantly different among most of cotton varieties after 60 days of germination. At 30-day post-germination, *Bt* varieties CRS-456 and PB-38 exhibited significantly higher toxin level (*i.e.* 39.6 and 38.8 ng/mg, respectively) followed by PB-896 (38.1 ng/mg) and VH-57 (37.6 ng/mg). Toxin contents were highest in CRS-456 (39.8 ng/mg) and PB-896 (39.6 ng/mg) at 60-day post-germination. Toxin contents of varieties PB-38, CRS-456 and PB-896 were non-significantly different from each other but were significantly different from MNH-586, CIM-600, Gumbo Okra and PB-52-NC-63 at 90-day post-germination.

**Table I. Mean concentration of *Cry*1Ac protein (ng/mg), gossypol glands (numbers/cm²), chlorophyll contents (mg/cm²) of transgenic *Bt* and non-*Bt* cotton varieties determined at different time intervals.**

| Cotton varieties | Cry1Ac concentration (ng/mg) | | | Gossypol glands (numbers/cm²) | | | Chlorophyll contents (mg/cm²) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 30 DAG | 60 DAG | 90 DAG | 30 DAG | 60 DAG | 90 DAG | 30 DAG | 60 DAG | 90 DAG |
| CIM-600 | 37.2±0.49 B(a) | 26.5±0.28 C(c) | 31.4±0.44 B(b) | 26.6±2.4F (c) | 191.7±5.6A (a) | 200±4.6AB (a) | 13.4±1.4A (a) | 15.7±1.9A (a) | 14.0±2.4A (a) |
| CRS-456 | 39.6±0.39 A(a) | 39.8±0.38 A(a) | 38.5 ±0.4 A (b) | 55.6±3CDE (c) | 241.6±6.1A (a) | 200±5.1AB (b) | 10.3±1.6 ABC(a) | 7.7±1.5C (a) | 11±2.2AB (a) |
| MNH-586 | 31.5±0.29 C(a) | 28±0.25 C(b) | 29.8±0.34 C(b) | 65±2.5BCD (c) | 275±3.5A (a) | 225±4.6AB (b) | 7.0±1.63 CD(b) | 13.0±2.1AB(a) | 13.6±2.1A (a) |
| PB-38 | 38.8±0.35 A(a) | 37.3±0.27 B(b) | 39.02±0.35 A(a) | 72.3±3.1BC (c) | 266.7±4.2A (a) | 137±3.9B (b) | 5.5±1.2D (c) | 8.7±0.9C (b) | 13.8±1.8A (a) |
| Gumbo Okra | 29±0.31 DE(a) | 23.8±0.4 D(b) | 10.89 ±0.34 D(c) | 85.6±3.2 B (c) | 200±3.9A (a) | 183.4±4.1AB (b) | 7.7±1.1 BCD(a) | 7.0±0.8C (a) | 7.5±1.9B (a) |
| VH-57 | 37.6±0.28 B(a) | 37.2±0.37 B(a) | 33.88 ±0.53 B(b) | 47±4.2 DEF (c) | 183.4±5.9A (a) | 136.7±3.5B (b) | 7.5±1.6 BCD(a) | 8.7±1.5C (a) | 7.2±2B (a) |
| PB-52-NC-63 | 19.9±0.36 D(b) | 10.1±0.23 E(c) | 28.1±0.4 C(a) | 41.6±3.2 EF (c) | 283.4±6.2A (a) | 216.7±3.1AB (b) | 10.9±2.1 AB(a) | 6.6±1.4C (a) | 10.3±2.3AB(a) |
| PB-896 | 38.1±0.59AB(a) | 39.6±0.52 A(a) | 38.54±0.61 A (a) | 51±4.4 CDE (c) | 183±5.6A (a) | 137±5.1B (b) | 12.0±1.3 A(a) | 9.2±1.8BC (a) | 9.8±1.4AB (a) |
| Coker 100 A/2 | 0.00 | 0.0 | 0.00 | 58±2.4 CDE (c) | 233±4.7A (a) | 133±3.9B (b) | 12.5±1.1A (a) | 6.1±0.9C (b) | 8.0±1.7B (b) |
| L.A Fragobract | 0.0 | 0.0 | 0.0 | 126±6.9A (c) | 191.6±5.6A (b) | 258.5±3.5A (a) | 6.1±1.3D (a) | 7.2±1.2C (a) | 10.7±1.4AB(a) |

*Means sharing different letters are significantly different at $p \leq 0.05$. Horizontally, small letters represent comparison among different time intervals' data of the same variety. Vertically, capital letters represent the mean comparison of varieties for each parameter. DAG, days after germination.

**Table II. Means stomatal conductance (SC, mmol/m²/s) and the emission and absorption of $CO_2$ (ppm) of different *Bt* and non-*Bt* cotton varieties determined at different time intervals.**

| Cotton varieties | SC (µmol/m²/s) | | | $CO_2$ out (ppm) | | | $CO_2$ in (ppm) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 30 DAG | 60 DAG | 90 DAG | 30 DAG | 60 DAG | 90 DAG | 30 DAG | 60 DAG | 90 DAG |
| CIM-600 | 79.5±6D (b) | 279.8±9.5C(a) | 72.5±17.2D(b) | 97.6±6.6 CD(c) | 154.9±11.2C(b) | 173.2±6B(a) | 134.6 ±7.1A(c) | 176.9±8.1BC(b) | 221.9 ±11.5A(a) |
| CRS-456 | 42.1±8.09 E(c) | 616.3±11.2A(a) | 78.7±12.5CD(b) | 92.63±6.1 D(c) | 145.2±10.2C(b) | 164±6.5BCD(a) | 109.8±6.3BC(b) | 160.8±8.4CD(a) | 186.8±11.9BC(a) |
| MNH-586 | 33.5±7.9 E(c) | 206±8.5E(a) | 79 ±11.5CD(b) | 96±5.2 D (c) | 148.6±11.3C(a) | 160.±6.1BCD(b) | 87.5 ±5.2D(b) | 151.5 ±8.6D(a) | 156.8 ±11.2D(a) |
| PB-38 | 47.6±6.2 E(c) | 211.6±9.2E(a) | 183.1±9.6B(b) | 121.3±7.1 A | 251.7 ±11.2A(a) | 154.4±5.2D(b) | 124.6±5.1AB(b) | 174.1±7.2BC(a) | 160.9 ±12D(a) |
| Gumbo Okra | 35.5±5.9 E(b) | 136.4±10.2F(a) | 180.9±10.5B(a) | 110±6.3 ABC(c) | 213.2 ±9.2B(a) | 15.60±7.1CD(b) | 119.4±5.2BC(b) | 185.1±7.1AB(a) | 171.1±9.5CD(a) |
| VH-57 | 72.4±6.5 D(b) | 135.3±8.5F(a) | 115±12.2C(ab) | 104±6.4 BCD(c) | 152.4 ±9.5C(b) | 169.8 ±5.2B(a) | 105.3 ±4.2C(c) | 168.8±8.9BC(b) | 197.4 ±9.2B(a) |
| PB-52-NC-63 | 10.3±6.5 F(c) | 114.3±6.5F(b) | 159.8±10.2B(a) | 104.7±6.6 BCD(b) | 160.3 ±9.2C(a) | 168.9 ±4.2BC(a) | 118.2±4.5BC(b) | 177 ±8.11BC(a) | 158.7 ±11.9D(a) |
| PB-896 | 296.2±8.7 A(b) | 111.37±7.8F(c) | 506.4±13.2A(a) | 120.1±6.9 A(c) | 153.80 ±11.5C(b) | 194±5.1A(a) | 119.9±5.3ABC(c) | 194.9±8.94A(a) | 161.1 ±10.9D(b) |
| Coker 100 A/2 | 172.8±6.3 B(b) | 311.8±8.2 B(a) | 183.5±14.2B(b) | 113.8±4.5 AB(b) | 161.03 ±8.5C(a) | 162±3.4BCD(a) | 113.8±5.5BC(b) | 163.6±8.4CD(a) | 188.2±11.2BC(a) |
| L.A Fragobract | 142.3±5.4 C(b) | 246.5±7.2 D(a) | 171.5±13.2B(b) | 103±65.5 BCD(b) | 173.1 ±7.9C(b) | 154.7±4.1D(a) | 123±6.2AB(b) | 180 ±8.1AB(a) | 158.4±10.8D(a) |

*Means sharing different letters are significantly different at $p \leq 0.05$. Horizontally small letters represent comparison among three intervals of the same variety. Vertically capital letters represent the mean comparison among cotton varieties for each parameter. DAG, days after germination.

The highest average number of gossypol glands was observed in PB-52-NC-63 (283) at 60 days of germination, while the lowest number of gossypol glands was recorded in case of CIM-600 (26) at 30 days of germination. There was highest number of glands after 60 days of germination in almost all cotton varieties. The non-*Bt* variety L.A Fragobract was significantly different from all other *Bt* varieties and non-*Bt* variety Coker 100A/2 at 30-day post-germination.

Similarly, significantly higher chlorophyll contents were recorded in CIM-600 (15.7 mg/cm$^2$), MNH-586 (13.0) followed by PB-896 (9.2), PB-38 (8.78), VH-57 (8.7), CRS-456 (7.7), L.A. Fragobract (7.29), Gumbo Okra (7), PH-52-NC-63 (6.6), Coker 100 A/2 (6.1) at 60-day post-germination stage, while the lowest chlorophyll contents were observed in PB-38 (5.5) at 30 days. Total chlorophyll contents were not significantly different in *Bt* and non-*Bt* varieties except CIM-600 and MNH-586 at 60-day post-germination stage. The total chlorophyll contents increased significantly with time in MNH-586 and PB-38 varieties.

*Physiological parameters of cotton plants*

Mean values of stomatal conductance (SC) rate (mmol/m$^2$/s) and $CO_2$ emission and absorption (ppm) are given in Table II. Variety CRS-456 showed the highest SC (616.31 mmol/m$^2$/s) after 60 days of germination, while the lowest SC was recorded in L.A Fragobract (17.50) after 90 days of germination. Non-*Bt* varieties exhibited significantly higher SC than *Bt* varieties at 30-, 60- and 90-day post-germination.

The highest emission of $CO_2$ was observed in PB-38 (251) and Gumbo Okra (213) varieties after 60 days of germination. All *Bt* varieties had no significant difference of $CO_2$ emission from non-*Bt* varieties except PB-38 (251) and Gumbo Okra (213) after 60 days and PB-896 after 90 days of germination. The highest absorption of $CO_2$ was found in CIM-600 (221.90) after 90 days and was significantly different from all other varieties. *Bt* and non-*Bt* varieties were not significantly different from each other. Absorption of $CO_2$ increased significantly with the post-germination time (Table II).

Regarding mean transpiration rates (Table III), cotton varieties PB-38 and MNH-586 showed the highest transpiration rates (3.2 and 3.16 µmol/m$^2$/s, respectively) at 30-day post-germination. Non-*Bt* variety L.A Fragobract was non-significantly different from *Bt* varieties CRS-456, CIM-600, Gumbo Okra, VH-57, PB-520NC-63 and PB-896 but was significantly different from MNH-586 and PB-38 at 30-day post-germination. Non-*Bt* varieties were non-significantly different from *Bt* varieties after 60 and 90 days of germination. Transpiration rate increased significantly after 30 days in Gumbo Okra and Coker 100A/2 and decreased significantly in MNH-586, PB-38 and PB-896 varieties. It remained unchanged in CIM-600, CRS-456 and PB-52-NC-63 (Table III).

Similarly, regarding net photosynthetic rates, non-*Bt* variety L.A Fragobract was non-significantly different from CRS-456, MNH-586, GUMBO OKRA, PB-52-NC-63 and PB-896 and significantly different from CIM-600, PB-38 and VH-57 after 30 days of germination. Non-*Bt* varieties L.A Fragobract and Coker 100A/2 were significantly different from Gumbo Okra, PB-38, PB-52-NC-63 and PB-896 and were non-significantly different



**Table III. Mean transpiration rate and net photosynthesis rate (µmol/m$^2$/s) of different *Bt* and non-*Bt* cotton varieties determined at different time intervals.**

| Cotton varieties | Transpiration rate (µmol/m$^2$/s) | | | Net photosynthesis (µmol/m$^2$/s) | | |
|---|---|---|---|---|---|---|
| | 30 DAG | 60 DAG | 90 DAG | 30 DAG | 60 DAG | 90 DAG |
| CIM-600 | 2.4±0.7AB(a) | 1.92±0.34AB(a) | 0.52±0.17DE(a) | 14.2±2.4A(a) | 10.4±3.5F(a) | 5.2±0.6D(b) |
| CRS-456 | 0.6±0.78CD(a) | 1.19±0.41ABCD(a) | 0.65±0.19CD(a) | 8.8±2.3B(a) | 8.1±3.1F(a) | 3.8±0.4E(b) |
| MNH-586 | 3.16±0.8A(a) | 1.02±0.39BCD(b) | 0.62±0.20CD(b) | 8.7±2.5B(b) | 2.21±0.8H(c) | 13.4±1.6B(a) |
| PB-38 | 3.2±0.81A(a) | 0.96±0.34CD(b) | 1.02±0.18B(b) | 2.6±2.7D(b) | 48.2±3.4C(a) | 3.7±0.4E(b) |
| Gumbo Okra | 0.15±0.82D(b) | 1.32±0.40ABCD(a) | 0.90±0.19BC(ab) | 9.1±2.6AB(c) | 76.43±3.1A(a) | 25.6±1.6A(b) |
| VH-57 | 0.66±0.83CD(b) | 1.96±0.49AB(a) | 0.19±0.21E(b) | 3.5±2.2D(c) | 20.50±3.2D(a) | 6.83±0.7C(b) |
| PB-52-NC-63 | 1.13±0.82BCD(a) | 1.50±0.45ABC(a) | 1.04±0.20B(a) | 11±2.5A(b) | 50.05±3.9C(a) | 3.44±0.5E(c) |
| PB-896 | 1.7±0.85ABC(a) | 0.65±0.43CD(b) | 1.06±0.17B(b) | 5.1±2.5BC(a) | 62.81±3.5B(a) | 22.54±1.5A(b) |
| Coker 100 A/2 | 0.6±0.2CD(b) | 1.04±0.42ABCD(b) | 1.83±0.18A(a) | 6.2±2.4BC(b) | 10.61±2.5F(a) | 6.63±0.8C(b) |
| L.A Fragobract | 1.06±0.8BCD(a) | 0.38±0.21D(b) | 0.76±0.20BCD(a) | 9.1±2.3AB(b) | 15.43±1.5E(a) | 4.73±0.5D(c) |

*Means sharing different letters are significantly different at $p \geq 0.05$. Horizontally, small letters represent comparison among three intervals of the same variety. Vertically, capital letters represent the mean comparison of varieties for each parameter. DAG, days after germination.

from CIM-600, CRS-456, MNH-586 and VH-57 after 60 days of germination. At 90-day post-germination, L.A Fragobract and Coker 100A/2 were significantly different from CIM-600, MNH-586 but were non-significantly different from rest of the varieties (Table III).

*Bolls damage, bollworm larvae and yield*

Mean number of damaged bolls were the highest in MNH-586 (13.3%) after 90 days of germination, whereas the lowest damaged bolls were found in Gumbo Okra (2.0%) at 90 days of germination. Non-*Bt* varieties had significantly higher number of damaged bolls as compared to *Bt* varieties at 60- and 90-days post-germination except a *Bt* variety MNH-586 (13.3%). The number of bollworm larvae were the highest on non-*Bt* varieties, Coker 100 A/2 followed by L.A Fragobract, while, the lowest was on *Bt*-variety Gumbo Okra followed by CRS-456, VH-57, PB-38, CIM-600, PB-896, PB-52-NC-63 and MNH-586. The number of bollworm larvae were significantly increased after 60 days of germination in two non-*Bt*- and three *Bt* varieties while, other varieties did not show significant different of larvae after 60 and 90 days of germination (Table IV). The highest yield was recorded for *Bt* variety PB-896 (664.7 kg/acre) and non-*Bt* variety L.A Fragobract (661.3 kg/acre) followed by CRS-456 and VH-57 (Table IV). Gumbo Okra was having significantly lowest yield. Other non-*Bt* variety coker 100A/2 was significantly different from CRS-456, PB-38, VH-57 and PB-896 and non-significantly different from CIM-600, MNH-586, PB-52-NC-63 and Gumbo Okra.

*Correlation of plant parameters with boll damage and bollworm larvae*

Damaged bolls percentage in cotton plants was significantly and negatively correlated with *Cry*1Ac toxin contents (-0.49), gossypol glands (-0.46) and yield (-0.26), and positively and significantly correlated with Pn (0.22), SC (0.29), $CO_2$ absorption (0.51) and emission (0.53) and number of bollworm larvae (0.75). Similarly, number of bollworm larvae was significantly and negatively correlated with *Cry*1Ac toxin contents (-0.48), gossypol contents (-0.41) and yield (-0.27) and positively and significantly correlated with net photosynthesis rate (0.29) (Table V).

*Determination of variables contributing to boll damage by using different criteria and regression models*

The variables contributing to boll damage after 60 and 90 days are presented in Table VI. The regression analysis was performed to determine the dependence of damaged boll (DB) on other variables. The result of regression analysis showed that three variables such as number of bollworm larvae, *Bt* toxin and SC had impact on DB after 60 days. The coefficients of models 3 showed that the *Bt* toxin contributed negatively while two variables such as BW and SC contributed positively to DB. Regression analysis performed on these variables depicted that the values of R-squared and Adjusted R-squared increased by adding variables to the models. The model 3 (DB after 60 days) has maximum value of R-squared (0.89) and Adjusted R-squared (0.88). All of the three models have significant P-value. Akaike information criterion (AIC) and Bayesian information criterion (BIC) of the regression models were evaluated for their quality. AIC and BIC decreased by adding the contributing variable. Model 3 (DB after 60 days) was having the lowest AIC (1.63) and BIC (95.7) values indicating the good quality among the models.



**Table IV. Mean yield (Kg/acre), number of bollworm larvae and bolls (%) damaged by *Helicoverpa armigera* in different transgenic *Bt* and non-*Bt* cotton varieties.**

| Cotton varieties | Yield (Kg/acre) | No of bollworm larvae | | | Damaged bolls (%) | | |
|---|---|---|---|---|---|---|---|
| | | 60 DAG | 90 DAG | Cumulative average | 60 DAG | 90 DAG | Cumulative average |
| CIM-600 | 425 ±10.2D | 3.3±0.5DE | 3.7±1.52DE | 2.3DE | 5.33±2.2CD | 3±1.9EF | 4.16EF |
| CRS-456 | 660.7±11.2A | 2±0.9E | 2.7±0.57DE | 1.5E | 2.33±1.9F | 3.33±1.1EF | 2.83F |
| MNH-586 | 431.7±12.2F | 1.3±1.1E | 14±2.64AB | 5.1BC | 3.00±1.4EF | 13.33±1.3A | 8.16ABC |
| PB-38 | 632.0±9.1B | 3±1.1DE | 3.3±1.52DE | 2.1DE | 3.00±1.7EF | 4.33±1.2DEF | 5.16DEF |
| Gumbo Okra | 411.7±7.4D | 1.7±1.15E | 1.6±0.57E | 1.1E | 4.66±1.6DE | 2±1.5F | 3.33F |
| VH-57 | 611.7±10.1C | 2.3±0.57DE | 3±1.1DE | 1.7E | 4.00±1.5DE | 6.33±1.1CD | 6.66BCDE |
| PB-52-NC-63 | 554.7±11.1CD | 2.6±1.15DE | 9.3±1.2C | 4CD | 6.33±1.2C | 4.66±0.9DE | 5.5CDEF |
| PB-896 | 664.7±9.6A | 3.3±1.5DE | 8.7±1.5C | 4CD | 5.00±1.8CD | 7.6±0.8BC | 7.33ABCD |
| Coker 100 A/2 | 431.7±8.2D | 9±1.9C | 18±1.6A | 9A | 10.66±2.1A | 9±1.3B | 9.83A |
| L.A Fragobract | 661.3±7.3A | 6.3±0.57CD | 13.7±1.5B | 6.6B | 9.1±1.6AB | 9±1.9B | 9AB |

*Within each column, means sharing different letters are significantly different at $p \leq 0.05$. DAG, days after germination.

**Table V. Correlation among the biochemical, physiological and physico-morphic characters of cotton varieties and boll damage by *Helicoverpa armigera.***

| | Cry1Ac toxin | Pn | CP | SC | E | GG | Yield | $CO_2$in | $CO_2$out | BW |
|---|---|---|---|---|---|---|---|---|---|---|
| Pn | 0.0901§ | | | | | | | | | |
| P-value | 0.4938 | | | | | | | | | |
| CP | 0.2913 | -0.2606 | | | | | | | | |
| P-value | * | * | | | | | | | | |
| SC | 0.0748 | -0.2767 | -0.2384 | | | | | | | |
| P-value | 0.5698 | * | 0.0666 | | | | | | | |
| E | -0.0481 | -0.0588 | 0.1128 | 0.1019 | | | | | | |
| P-value | 0.715 | 0.6555 | 0.3909 | 0.4385 | | | | | | |
| GG | -0.1599 | 0.1321 | -0.0156 | -0.0063 | 0.0393 | | | | | |
| P-value | 0.2222 | 0.3144 | 0.9056 | 0.962 | 0.7653 | | | | | |
| Yield | 0.1016 | -0.3494 | 0.0875 | -0.1952 | -0.392 | 0.4701 | | | | |
| P-value | 0.4397 | ** | 0.5064 | 0.135 | ** | *** | | | | |
| $CO_2$in | 0.0488 | 0.428 | -0.0993 | -0.3698 | -0.1845 | -0.2545 | 0.0934 | | | |
| P-value | 0.7114 | *** | 0.4501 | ** | 0.1583 | * | 0.4777 | | | |
| $CO_2$out | 0.1497 | 0.44 | -0.1647 | -0.0129 | -0.0964 | 0.0293 | -0.0281 | 0.1893 | | |
| P-value | 0.2535 | *** | 0.2086 | 0.9218 | 0.4636 | 0.824 | 0.8313 | 0.1475 | | |
| BW | -0.4882 | 0.2938 | 0.0145 | 0.0016 | 0.0313 | -0.4141 | -0.2702 | -0.1807 | -0.0704 | |
| P-value | *** | * | 0.9123 | 0.9901 | 0.8126 | * | * | 0.1671 | 0.5928 | |
| DB | -0.4941 | 0.2245 | -0.0116 | 0.2901 | -0.0677 | -0.4673 | -0.26181 | 0.5121 | 0.5312 | 0.754 |
| P-value | *** | * | 0.9302 | * | 0.6072 | * | * | ** | ** | *** |

§, Spearman's correlation coefficient; Pn, net photosynthesis; CP, chlorophyll contents; SC, stomatal conductance; E, transpiration rate; GG, gossypol glands; CO2in, CO2 absorption; CO2out, CO2 emission; BW, Bollworm larvae; DB, damaged bolls; Significance levels: *, $p \leq 0.05$; **, $p \leq 0.01$ and ***, $p \leq 0.001$.

**Table VI. Selection of regression model of different variables of cotton varieties contributing to boll damage using different statistical criterion.**

| S. | Models | $R^2$ | Adj $R^2$ | F-value | P-value | AIC | BIC |
|---|---|---|---|---|---|---|---|
| | **Regression model of DB at 60 DAG** | | | | | | |
| 1 | DB ~ 1.88 + 0.98 BW | 0.77 | 0.76 | 93.7 | *** | 20.5 | 111.9 |
| 2 | DB ~ 5.03 +0.65 BW – 0.081 *Bt* Toxin | 0.87 | 0.86 | 94.6 | *** | 4.23 | 96.9 |
| 3 | DB ~ 5.29 + 0.69 BW – 0.073 *Bt* Toxin + 0.002 SC | 0.89 | 0.88 | 72.3 | *** | 1.63 | 95.7 |
| | **Regression model of DB at 90 DAG** | | | | | | |
| 1 | DB ~ 2.46 + 0.48 BW | 0.61 | 0.59 | 44.1 | *** | 51.9 | 143.2 |
| 2 | DB ~ 4 + 0.59 BW -2.73 E | 0.70 | 0.68 | 31.8 | *** | 45.9 | 138.7 |
| 3 | DB ~ 1.65 + 0.66 BW – 2.33 E + 0.05 *Bt* Toxin | 0.74 | 0.71 | 24.7 | *** | 43.7 | 137.9 |
| 4 | DB ~ 6.53 + 0.63 BW – 2.57 E + 0.05 *Bt* Toxin + 0.02 CO2in | 0.76 | 0.72 | 20.3 | *** | 42.8 | 136.3 |
| 5 | DB ~ 9.49 + 0.59 BW – 1.59 E + 0.04 *Bt* Toxin + 0.05 CO2in + 0.12 Pn | 0.79 | 0.75 | 19 | *** | 40.1 | 134.1 |
| 6 | DB ~ 13.25 + 0.62 BW – 2.48 E + 0.03 *Bt* Toxin + 0.05 CO2in + 0.11 Pn – 0.01 GG | 0.81 | 0.76 | 16.6 | *** | 40 | 132.3 |
| 7 | DB ~ 18.78 + 0.65 BW - 2.39 E + 0.04 *Bt* Toxin + 0.05 CO2in + 0.12 Pn – 0.01 GG +0.03 $CO_2$out | 0.82 | 0.77 | 15 | *** | 39.7 | 130.4 |

Unlike after 60 days, among ten variables, seven variables such as BW, E, *Bt* Toxin, $CO_2$in, Pn, GG and $CO_2$ out were found to contribute in DB after 90 days. The data of R-squared and Adjusted R-squared depicted that their values increased by adding variables to the models. Model 7 showed the highest value of R-squared (0.82) and Adjusted R-squared (0.77) and the lowest AIC value (39.7) and BIC value (130.4). The coefficients of regression models 7 showed that five variables such as BW, *Bt* Toxin, $CO_2$into, Pn and $CO_2$out contributed positively, while, two variables (E and GG) contributed negatively to DB.

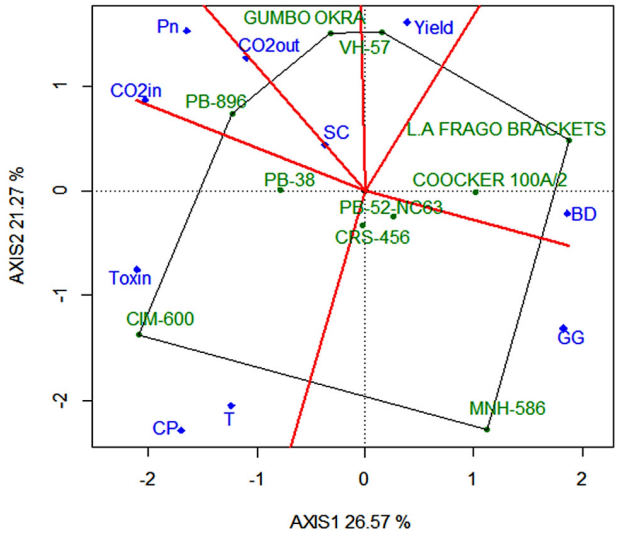


Fig. 1. GGE biplot of different *Bt* and non-*Bt* cotton varieties with their traits. Pn, net photosynthesis; CP, chlorophyll contents; SC, stomatal conductance; T, transpiration rate; GG, gossypol glands; $CO_2$in, $CO_2$ absorption; $CO_2$out, $CO_2$ emission; DB, damaged boll.

*Association of plant traits with cotton genotypes*

A genotype and genotype-environment interaction (GGE) biplot was constructed by plotting the first PC1 (Principal component) scores of environment and genotypes against the second PC2. The "which-won-where" of GGE is an effective tool and consists of irregular polygon and a set of lines drawn from the biplot origin (Yan *et al*., 2007). The 10 plant traits (environment) determined in this study were divided into 6 sectors with different winning varieties. According to the biplot (Fig. 1), the polygon had six corners with six corner genotypes VH-57, Gumbo Okra, PB-896, CIM-600, MNH-586 and L.A Fragobract that were the most responsive ones. The first sector represents yield with VH-57 cotton variety as the most favorable. The second sector represents boll damage with non-*Bt* cotton varieties (Coker 100 A/2 and L.A Fragobract) as the most favorable. The third sector represents gossypol glands density with genotype MNH-586, CRS-456 and PB-52-NC-63 as the most favorable. The fourth sector represents transpiration rate, total chlorophyll contents and *Cry*1Ac toxin with two *Bt*-varieties (CIM-600 and PB-38). The fifth sector represents net photosynthesis rate and $CO_2$in with one *Bt*-variety PB-896. The last sector represents stomatal conductance and $CO_2$out with one *Bt*-variety Gumbo Okra.

Cluster analysis, based upon all parameters of *Bt* and non-*Bt* varieties of cotton, produced a dendrogram (Fig. 2) with three clusters. Cluster-I had only one *Bt* variety VH-57. Cluster-2 was comprised of one non-*Bt* (Coker 100A/2) and two *Bt* varieties (PB-896 and CRS-456), while five *Bt* (PB-38, CIM-600, Gumbo Okra, MNH-586 and PB-52-NC-63) and one non-*Bt* (L.A. Fragobract) variety were present in Cluster-3 (Fig. 2).

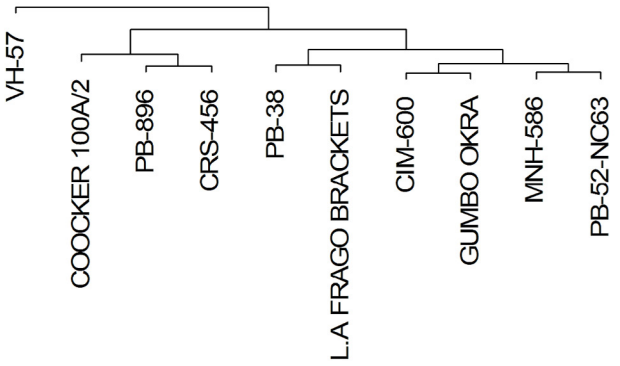


Fig. 2. Cluster analysis of different *Bt* and non-*Bt* cotton varieties.

## DISCUSSION

In this study, we assessed the susceptibility of different cotton varieties to *H. armigera* infestation by correlating the percent boll damage caused by bollworms to different plant biochemical and physico-morphic parameters including concentration of *Cry*1Ac toxin level, gossypol glands' density, total chlorophyll contents, plant height and yield, and to different physiological parameters including photosynthesis and transpiration rates and $CO_2$ emission and absorption rates determined for eight *Bt* and two non-*Bt* cotton varieties at 30, 60 and 90 days post-germination.

Our results showed that *Cry*1Ac toxin contents were significantly higher in *Bt* varieties particularly in CRS-456 and PB-896. The concentration of *Cry*1Ac in leaves decreased after 30 days of germination in most of the *Bt*

varieties, whereas the level of toxin increased with the passage of time in varieties MNH-586, CRS-456 and PB-52-NC-63, hence considered good for the control of bollworm. It is likely that the concentration of toxin is growth and time dependent. Its concentration decreases as the plant matures. Wan *et al*. (2005) also reported that the amount of *Bt* toxin decreased with the passage of time especially in the middle and last stages of the crop.

Total chlorophyll contents, $CO_2$ emission and absorption, transpiration rate and net photosynthesis rates did not differ significantly among *Bt* and non-*Bt* varieties. However, the stomatal conductance of *Bt* varieties was significantly lower than non-*Bt* varieties. These results are in association with those of Guo *et al*. (2016) showing that *Bt* cotton varieties had lower stomatal conductance and net transpiration rates than the non-*Bt* ones.

The number of bollworm larvae increased with the passage of time in non-*Bt* varieties and three *Bt* varieties (MNH-586, PB-52-NC-63 ad PB-896). Overall, there was no significant change in the number of bollworm larvae on *Bt* varieties after 60 and 90 days of germination. Similarly, non-*Bt* varieties had significantly higher number of damaged bolls as compared to *Bt* varieties except MNH-586 and PB-896. *Bt* varieties CIM-600, CRS-456, PB-38, Gumbo Okra and PB-52-NC-63 exhibited the lowest damage by *H. armigera*. These results are in line with Mann *et al*. (2010) who showed that the damage by *H. armigera* and *Earias* spp. were the lowest till harvest in Bollgard® and Bollgard-II® cotton varieties as compared to severe damage incurred in the conventional non-*Bt* cotton varieties. Sharma and Pampapathy (2006) and Jamil *et al*. (2021) also demonstrated that *Bt* cotton hybrids exhibited significantly less bolls damage than non-*Bt* ones.

Boll damage was significantly related to number of bollworm larvae and negatively correlated to *Cry*1Ac toxin contents, gossypol glands and yield. Although, boll damage was not significantly related to total chlorophyll contents and transpiration rates, the net photosynthesis rate, stomatal conductance, $CO_2$ absorption and emission were positively and significantly correlated with boll damage by *H. armigera*. It would be probably due to the fact that the adults of bollworms are attracted for oviposition towards the cotton plants displaying good growth irrespective of *Bt* and non-*Bt* nature of the plant. Gossypol glands are important physico-morphic character of cotton and other solanaceous plants and play a significant role in conferring plant resistance to insect pests (Ali *et al*., 2020).

We observed that boll damage was negatively correlated with *Cry*1Ac toxin contents, gossypol glands' density and yield, while positively correlated with the number of *H. armigera* larvae. Regression analysis confirmed that *Cry1Ac* toxin contents contributed negatively, however, number of bollworm larvae and stomatal conductance contributed positively after 60 days of germination. Conversely, the *Cry1Ac* toxin contents contributed positively after 90 days of germination. It is likely that the level of *Cry1Ac* toxin contents in *Bt* varieties is not stable over the life span of plants. These findings are consistent with many previous studies (Wan *et al*., 2005: Sharma and Pampapathy, 2006; Mann *et al*., 2010).

## CONCLUSION AND RECOMMENDATIONS

This study concluded that the boll damage by *H. armigera* depends on biochemical, physico-morphic and physiological characteristics of cotton plants. Plant characteristics conferring plant resistance to insect pests could enhance the plant protection as narrated by Sharma and Pampapathy (2006) and Ali *et al*. (2020). Indeed, none of the *Bt* varieties was found bollworm free indicating increased survival of *H. armigera* on *Bt* varieties expressing *Cry*1Ac toxin. However, the larval survival may vary from variety to variety as demonstrated by Mann *et al*. (2010).

Based from the results, it is recommended that farmers can use the three *Bt* (VH-57, CRS-456 and PB-38) and one non-*Bt* (L.A Fragobract) cotton varieties for planting which showed resistance to bollworm. For instance, *Bt* varieties of cotton with high gossypol gland could be effective tool to manage the population of *H. armigera*. Non-*Bt* variety L.A Fargobract has the highest number of gossypol glands as compared to *Bt* varieties. The higher number of gossypol glands help the plant to survive better against early instar of *H. armigera* larvae. It is likely that this variety has good survival and yield potential and have traits that make the variety resistant or tolerant. The highest yield of non-Bt variety could be indirectly due to higher number of gossypol glands. Females of bollworms lay eggs on leaf surface. The neonates feeding on leaves with higher gossypol glands likely to have less ability to survive. Hence, it provided better protection against larvae.

*H. armigera* is a voracious feeder of cotton. According to one study, the boll consumption rate of sixth instar larva is about 23 squares or 8 bolls (Mohapatra and Sahu, 2005). Therefore, the data of insect count (*H. armigera*) is less informative as compared to damage which vary with plant characteristics. There have not been any breeding efforts to incorporate plant resistant traits in *Bt* varieties. We have suggested that the relying on transgenic cotton could not decrease the damage of *H. armigera*. Use of *Bt* cotton varieties in combination with the plant characteristics conferring plant resistance to insect pests will be helpful for the management of *H. armigera*. The leaf characters are important to adults for oviposition. Females lay eggs on leaves and the neonates feed on leaves first, the later

instar move to bolls, so that's why it is important to focus on the leaf and boll characters while studying bollworm infestation.

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*IRB approval*

Authors declare that this study did not require ethical committee's approval or any other ethical considerations.

*Ethical statement*

Not applicable.

*Statement of conflict of interest*

The authors have declared no conflict of interest.

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